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54	GHRELIN-CARRIER CONJUGATES
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The figure of the drawing to which the abstract refers is attached.

ABSTRACT

The present invention is related to the fields of molecular biology, virology, immunology and medicine. The invention provides a composition comprising an ordered and repetitive antigen or antigenic determinant array, and in particular a ghrelin or ghrelin-derived peptide-array. More specifically, the invention provides a composition comprising a virus-like particle and at least one ghrelin or a ghrelin-derived peptide bound thereto. The invention also provides a process for producing the conjugates and the ordered and repetitive arrays, respectively. The compositions of the invention are useful in the production of vaccines for the treatment of obesity and other disease associated with increased food-uptake or increased body weight and to efficiently induce immune responses, in particular antibody responses. Furthermore, the compositions of the invention are particularly useful to efficiently induce self-specific immune responses within the indicated context.

## GHRELIN-CARRIER CONJUGATES

## BACKGROUND OF THE INVENTION

## Field of the Invention

5           The present invention is related to the fields of molecular biology, virology, immunology and medicine. The invention provides a composition comprising an ordered and repetitive antigen or antigenic determinant array, and in particular a ghrelin or ghrelin-derived peptide-array. More specifically, the invention provides a composition comprising a virus-like particle and at least one ghrelin or a ghrelin-  
10           derived peptide bound thereto.

          The invention also provides a process for producing the conjugates and the ordered and repetitive arrays, respectively. The compositions of the invention are useful in the production of vaccines for the treatment of obesity and other disease associated with increased food-uptake or increased body weight and to efficiently  
15           induce immune responses, in particular antibody responses. Furthermore, the compositions of the invention are particularly useful to efficiently induce self-specific immune responses within the indicated context.

## Related Art

          Obesity is a disease afflicting millions of people world-wide. Although the  
20           underlying causes for obesity may be manifold, a common reason for almost all forms of obesity is increased food-uptake. Many factors regulate hunger and feeding behaviour, including leptin, growth-hormone (GH), neuropeptide Y (NPY), agouti-related protein (AGRP) and others. A recently identified key regulator of feeding behaviour is ghrelin, an acylated peptide produced in the stomach and also some  
25           parts of the brain (hypothalamus) (Kojima et al. *Nature* 402: 656-660 (1999)). Ghrelin is derived by enzymatic cleavage from a prepro form encompassing 117 amino acids resulting in a 28 amino acid long peptide with a n-octanoylation at serine 3. Biologically active ghrelin needs to be n-octanylated at this position. A second, 27 aa isoform of ghrelin (Ghrelin-desQ14), lacking a glutamine (Q) at

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position 14, has been identified, however, this isoform represents only a minor component of circulating ghrelin. Like full length ghrelin, the biological activity of Ghrelin-des-Q14 is dependant on the n-octanoyl group on serine 3. Nevertheless, most functional activity is derived from the 28 aa ghrelin isoform (Hosoda et al. Biochem. Biophys. Res. Commun. 279(3): 909-913 (2000)). Ghrelin is highly conserved, since human and rat ghrelin differ by only 2 amino-acids (GSSFLSPEHQRVQQRKESKKPPAKLQPR (SEQ ID NO: 31) versus GSSFLSPEHQKAQQ-RKESKKPPAKLQPR (SEQ ID NO: 32)) (Kojima et al. *Nature* 402: 656-660 (1999)).

Receptors for ghrelin (GHS-R) are expressed in various regions of the brain, including the arcuate nucleus (Arc) and ventromedial nucleus of the hypothalamus and in the pituitary gland (Howard et al. *Science* 273:974-977 (1996)); McKee et al. *Mol Endokrin.* 11:415-423 (1997); Guan et al., *Mol brain research* 48:23-29 (1997)), indicating that ghrelin primarily acts in the brain. In addition to stimulating release of GH from the pituitary gland (Kojima et al. *Nature* 402: 656-660 (1999)), ghrelin has more recently been identified as a key central regulator of feeding (Nakazato et al., *Nature* 409: 194-198 (2001)). Specifically, upon intracerebroventricular application, ghrelin was shown to stimulate feeding. Moreover, intracerebroventricular application of anti-ghrelin antibodies inhibited feeding. Ghrelin injection induced upregulated release of NPY and anti-NPY antibodies together with AGRP antagonists blocked ghrelin induced feeding, suggesting that ghrelin modulates feeding via enhancing expression of NPY and AGRP (Nakazato et al., *Nature* 409: 194-198 (2001)). Moreover, peripheral daily administration of ghrelin induced body weight gain in mice and rats and serum ghrelin concentrations were increased in fasting rats and reduced by feeding, further suggestin that ghrelin plays a key role in regulating feeding (Tschop et al, *Nature* 407:908-912). Transgenic rats expressing anti-sense GHS-R RNA in the Arc exhibited lower body weight and less adipose tissue, supporting the notion that ghrelin regulates body weight (Shuto et al., *JCI* 109:14291436 (2002)). There is also evidence for a key role for ghrelin in human feeding behaviour. Peripheral administration of ghrelin in humans enhanced appetite and increased food uptake in humans (Wren et al., *J Clin Endocrinol Metab* 86:

5992-5998 (2001)). Humans with Prader-Willi syndrome, the most common form of human syndromic obesity, exhibit highly increased ghrelin levels (Cummings et al. Nat Med 8:643-644 (2002)). In addition, plasma ghrelin levels in humans are strongly increased after diet-induced weight loss, correlating with rapid regain of weight when people stop the diet. In contrast, in patients with gastric bypass surgery, ghrelin levels remained low during and after diet and patients do not usually regain their weight under these conditions ((Cummings et al., N Engl J Med 21: 1623-1630 (2002)). Hence, ghrelin appears to be a key regulator of food uptake and body weight in humans.

10 Since peripheral administration of ghrelin was able to increase food uptake leading to increased body weight (Tschop et al, Nature 407:908-912), it is likely that ghrelin produced in the stomach reaches the brain through the blood stream and triggers feeding. Thus, it may be possible to block migration of ghrelin from the blood to the brain to stop food uptake in animals and humans. As it has been shown  
15 that specific antibodies can block ghrelin action in the brain (Nakazato et al., Nature 409: 194-198 (2001)) it is likely that peripheral antibodies will also be able to block the action of peripheral ghrelin. In addition, since antibodies inefficiently penetrate the blood brain barrier, ghrelin-specific antibodies would probably be able to seclude ghrelin from the brain but would not act on ghrelin within the brain. This would be a particularly attractive possibility, since ghrelin is also produced in the brain where it probably exerts functions different from regulating food uptake (Nakazato et al., Nature 409: 194-198 (2001)). Therefore, a potential therapy for obesity would be to induce ghrelin-specific antibodies in the host, leading to the long-term blockage  
20 obstruction of ghrelin resulting in reduced food-uptake, similarly to that observed in gastric bypass patients.  
25

WO98/42840 discloses the influence of ghrelin and ghrelin-derived fragments on the gastrointestinal tract and hereby in particular their effect on gastric motility and gastric emptying. Moreover, US 6'420'521 discloses the use of short ghrelin peptides for effects on gastric function, including gastric emptying, gastric contractility and glucose absorption.  
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However, it is usually difficult to induce antibody responses against peptides, in particular against self-peptides. One way to improve the efficiency of vaccination is to increase the degree of repetitiveness of the antigen applied. Unlike isolated proteins, viruses induce prompt and efficient immune responses in the absence of any adjuvants both with and without T-cell help (Bachmann and Zinkernagel, *Ann. Rev. Immunol.* 15:235-270 (1991)). Although viruses often consist of few proteins, they are able to trigger much stronger immune responses than their isolated components. For B-cell responses, it is known that one crucial factor for the immunogenicity of viruses is the repetitiveness and order of surface epitopes. Many viruses exhibit a quasi-crystalline surface that displays a regular array of epitopes which efficiently crosslinks epitope-specific immunoglobulins on B cells (Bachmann and Zinkernagel, *Immunol. Today* 17:553-558 (1996)). This crosslinking of surface immunoglobulins on B cells is a strong activation signal that directly induces cell-cycle progression and the production of IgM antibodies. Further, such triggered B cells are able to activate T helper cells, which in turn induce a switch from IgM to IgG antibody production in B cells and the generation of long-lived B cell memory -- the goal of any vaccination (Bachmann and Zinkernagel, *Ann. Rev. Immunol.* 15:235-270 (1997)). Viral structure is even linked to the generation of anti-antibodies in autoimmune disease and as a part of the natural response to pathogens (see Fehr, T., *et al.*, *J Exp. Med.* 185:1785-1792 (1997)). Thus, antibodies presented by a highly organized viral surface are able to induce strong anti-antibody responses.

As indicated, however, the immune system usually fails to produce antibodies against self-derived structures. For soluble antigens present at low concentrations, this is due to tolerance at the Th cell level. Under these conditions, coupling the self-antigen to a carrier that can deliver T help may break tolerance. For soluble proteins present at high concentrations or membrane proteins at low concentration, B and Th cells may be tolerant. However, B cell tolerance may be reversible (anergy) and can be broken by administration of the antigen in a highly organized fashion coupled to a foreign carrier (Bachmann and Zinkernagel, *Ann. Rev. Immunol.* 15:235-270 (1997)).

## BRIEF SUMMARY OF THE INVENTION

We have found that ghrelin or ghrelin peptides, which are bound to a core particle having a structure with an inherent repetitive organization, and hereby in particular to virus-like-particles (VLPs) and subunits of VLPs, respectively, leading to highly ordered and repetitive conjugates represent potent immunogens for the induction of specific antibodies. The present invention provides a prophylactic and therapeutic mean for the treatment of obesity and related diseases, which is based on an ordered and repetitive ghrelin or ghrelin-derived peptide core particle array and in particular on a VLP-ghrelin/ghrelin peptide-conjugate and -array, respectively. This prophylactic and therapeutic composition is able to induce high titers of anti-ghrelin antibodies in a vaccinated animal or human. Therefore, the present invention focuses on ghrelin and its brain-related properties. The present invention, moreover, focuses on the central effects of ghrelin in the brain, more importantly the regulation of appetite, growth hormone secretion and energy homeostasis. Hence, it is preferred that the antibodies induced by our vaccination strategy are able to bind the *n*-octanoylated form(s) of ghrelin. As indicated, ghrelin or shorter ghrelin peptide fragments could be used, when coupled to a core particle, and alternatively or preferably administered in adjuvant, to induce ghrelin-specific antibodies in humans and in animals.

Therefore, short peptide fragments of ghrelin, particularly the shorter peptides of residues 24-33, 42-51, 31-41 and 28-37, coupled either C- or N-terminally to a core particle or a virus-like particle, respectively, are capable of inducing highly specific anti-ghrelin antibodies being capable of neutralizing peripheral circulating ghrelin before it entered the CNS and exerted an effect on growth hormone and hence, food intake.

In a preferred embodiment of the present invention, thus, the antigen or antigenic determinant is selected from the group of ghrelin peptides corresponding to residues of 24-33, 42-51, 31-41 and 28-37 of any of the sequences set forth in SEQ ID NO: 144 to 146, wherein said preferred ghrelin peptide fragments are selected

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from the group consisting of human ghrelin; (b) bovine ghrelin; (c) sheep ghrelin; (d) dog ghrelin; (e) cat ghrelin; (f) mouse ghrelin; (g) pig ghrelin; and (h) horse ghrelin.

More specifically, in the present invention, we were able to induce high levels of antibodies that recognize, surprisingly, the *n*-octanoylated form of ghrelin as shown herein, and in particular in example 17. Furthermore, generated antibodies also recognized the alternative isorform, Ghrelin-desQ14. As a result, antibodies generated from vaccination with C- or N-terminally linked ghrelin and ghrelin peptide, respectively, to a core particle or, preferably to a VLP, were able to block the entry of *n*-octanoylated ghrelin into the brain and modulated food intake in mice. Therefore, the present invention focuses on vaccination strategies against active ghrelin as a treatment for obesity and other related diseases.

As shown herein, and in particular in example 18, vaccination with C- or N-terminally linked ghrelin and ghrelin peptide, respectively, to a core particle or, preferably to a VLP, leads to reduced food uptake in mice, suggesting that ghrelin and ghrelin peptide, respectively, is a key regulator of food intake and antibodies that target ghrelin and ghrelin peptide, respectively, is supposed to be a potential therapy for obesity and other related diseases.

The present invention, thus, provides for a composition comprising: (a) a core particle with at least one first attachment site; and (b) at least one antigen or antigenic determinant with at least one second attachment site, wherein said antigen or antigenic determinant is ghrelin or a ghrelin-derived peptide, and wherein said second attachment site being selected from the group consisting of (i) an attachment site not naturally occurring with said antigen or antigenic determinant; and (ii) an attachment site naturally occurring with said antigen or antigenic determinant, wherein said second attachment site is capable of association to said first attachment site; and wherein said antigen or antigenic determinant and said core particle interact through said association to form an ordered and repetitive antigen array. Preferred embodiments of core particles suitable for use in the present invention are a virus, a virus-like particle, a bacteriophage, a virus-like particle of a RNA-phage, a bacterial pilus or flagella or any other core particle having an inherent repetitive structure



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capable of forming an ordered and repetitive antigen array in accordance with the present invention.

More specifically, the invention provides a composition comprising an ordered and repetitive antigen or antigenic determinant array, and hereby in particular ghrelin or ghrelin-derived peptide VLP conjugates. More specifically, the invention provides  
5 a composition comprising a virus-like particle and at least one ghrelin or ghrelin-derived peptide bound thereto. The invention also provides a process for producing the conjugates and the ordered and repetitive arrays, respectively. The compositions of the invention are useful in the production of vaccines for the treatment of obesity  
10 and related diseases and as a pharmaccine to prevent or cure obesity and related diseases and to efficiently induce immune responses, in particular antibody responses. Furthermore, the compositions of the invention are particularly useful to efficiently induce self-specific immune responses within the indicated context.

In the present invention, ghrelin or a ghrelin-derived peptide is bound to a core  
15 particle and VLP, respectively, typically in an oriented manner, yielding an ordered and repetitive ghrelin or ghrelin-derived peptide antigen array. Furthermore, the highly repetitive and organized structure of the core particles and VLPs, respectively, mediates the display of the ghrelin or ghrelin-derived peptide in a highly ordered and repetitive fashion leading to a highly organized and repetitive antigen array.  
20 Furthermore, binding of the ghrelin or ghrelin-derived peptide to the core particle and VLP, respectively, provides T helper cell epitopes, since the core particle and VLP is foreign to the host immunized with the core particle-ghrelin or ghrelin-derived peptide array and VLP-ghrelin or ghrelin-derived peptide array, respectively. Those arrays differ from prior art conjugates, in particular, in their highly organized  
25 structure, dimensions, and in the repetitiveness of the antigen on the surface of the array.

In one aspect of the invention, the ghrelin or ghrelin-derived peptide is expressed in a suitable expression host, or synthesized, while the core particle and the VLP, respectively, is expressed and purified from an expression host suitable  
30 for the folding and assembly of the core particle and the VLP, respectively. Ghrelin or ghrelin-derived peptides may be chemically synthesized. Since biologically active

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ghrelin contains a n-octanyolated serine at position three, chemical synthesis will be the preferred way of producing ghrelin for a vaccine formulation containing biologically active forms of ghrelin. The ghrelin or ghrelin-derived peptide array is then assembled by binding the ghrelin or ghrelin-derived peptide to the core particle and the VLP, respectively.

In another aspect, the present invention provides for a composition comprising (a) a virus-like particle, and (b) at least one antigen or antigenic determinant, wherein said antigen or said antigenic determinant is ghrelin or a ghrelin-derived peptide, and wherein said at least one antigen or antigenic determinant is bound to said virus-like particle.

In a further aspect, the present invention provides for a pharmaceutical composition comprising (a) the inventive composition, and (b) an acceptable pharmaceutical carrier.

In still a further aspect, the present invention provides for a vaccine composition comprising a composition comprising: (a) a core particle with at least one first attachment site; and (b) at least one antigen or antigenic determinant with at least one second attachment site, wherein said antigen or antigenic determinant is ghrelin or a ghrelin-derived peptide, and wherein said second attachment site being selected from the group consisting of (i) an attachment site not naturally occurring with said antigen or antigenic determinant; and (ii) an attachment site naturally occurring with said antigen or antigenic determinant, wherein said second attachment site is capable of association to said first attachment site; and wherein said antigen or antigenic determinant and said core particle interact through said association to form an ordered and repetitive antigen array.

In a further aspect, the present invention provides for a vaccine composition comprising a composition, wherein said composition comprising (a) a virus-like particle; and (b) at least one antigen or antigenic determinant, wherein said antigen or said antigenic determinant is ghrelin or a ghrelin-derived peptide; and wherein said at least one antigen or antigenic determinant is bound to said virus-like particle.

In still a further aspect, the present invention provides for a process for producing a composition of claim 1 comprising (a) providing a virus-like particle;

and (b) providing at least one antigen or antigenic determinant, wherein said antigen or said antigenic determinant is ghrelin or a ghrelin-derived peptide; (c) combining said virus-like particle and said at least one antigen or antigenic determinant so that said at least one said antigen or antigenic determinant is bound to said virus-like  
5 particle.

Analogously, the present invention provides a process for producing an inventive composition comprising: (a) providing a core particle with at least one first attachment site; (b) providing at least one antigen or antigenic determinant with at least one second attachment site, wherein said antigen or antigenic determinant is  
10 ghrelin or a ghrelin-derived peptide, and wherein said second attachment site being selected from the group consisting of (i) an attachment site not naturally occurring with said antigen or antigenic determinant; and (ii) an attachment site naturally occurring with said antigen or antigenic determinant; and wherein said second attachment site is capable of association to said first attachment site; and (c)  
15 combining said core particle and said at least one antigen or antigenic determinant, wherein said antigen or antigenic determinant and said core particle interact through said association to form an ordered and repetitive antigen array.

In another aspect, the present invention provides for a method of immunization comprising administering the composition of claim 1 to an animal or human.

20 In a further aspect, the present invention provides for a use of a composition of claim 1 for the manufacture of a medicament for treatment of obesity or a related disease.

In a still further aspect, the present invention provides for a use of a composition of claim 1 for the preparation of a medicament for the therapeutic or  
25 prophylactic treatment of obesity or a related disease. Furthermore, in a still further aspect, the present invention provides for a use of a composition of claim 1, either in isolation or in combination with other agents, for the manufacture of a composition, vaccine, drug or medicament for therapy or prophylaxis of obesity or a related disease, and/or for stimulating the mammalian immune system.

30 Therefore, the invention provides, in particular, vaccine compositions which are suitable for preventing and/or reducing or curing obesity or conditions related

thereto. The invention further provides immunization and vaccination methods, respectively, for preventing and/or reducing or curing obesity or conditions related thereto, in animals, and in particular in pets such as cats or dogs as well as in humans. The inventive compositions may be used prophylactically or therapeutically.

5 In specific embodiments, the invention provides methods for preventing, curing and/or attenuating obesity or conditions related thereto which are caused or exacerbated by "self" gene products, i.e. "self antigens" as used herein. In related embodiments, the invention provides methods for inducing immunological responses in animals and individuals, respectively, which lead to the production of antibodies  
10 that prevent, cure and/or attenuate obesity or conditions related thereto, which are caused or exacerbated by "self" gene products.

As would be understood by one of ordinary skill in the art, when compositions of the invention are administered to an animal or a human, they may be in a composition which contains salts, buffers, adjuvants, or other substances which are  
15 desirable for improving the efficacy of the composition. Examples of materials suitable for use in preparing pharmaceutical compositions are provided in numerous sources including *Remington's Pharmaceutical Sciences* (Osol, A, ed., Mack Publishing Co. (1990)).

Compositions of the invention are said to be "pharmacologically acceptable" if  
20 their administration can be tolerated by a recipient individual. Further, the compositions of the invention will be administered in a "therapeutically effective amount" (*i.e.*, an amount that produces a desired physiological effect).

The compositions of the present invention may be administered by various methods known in the art, but will normally be administered by injection, infusion,  
25 inhalation, oral administration or other suitable physical methods. The compositions may alternatively be administered intramuscularly, intravenously, or subcutaneously. Components of compositions for administration include sterile aqueous (*e.g.*, physiological saline) or non-aqueous solutions and suspensions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as  
30 olive oil, and injectable organic esters such as ethyl oleate. Carriers or occlusive dressings can be used to increase skin permeability and enhance antigen absorption.

Other embodiments of the present invention will be apparent to one of ordinary skill in light of what is known in the art, the following description of the invention, and the claims.

#### BRIEF DESCRIPTION OF THE DRAWINGS/FIGURES

5 Figure 1 shows the coupling products from the reaction of murine C-Ghrelin (SEQ ID: No. 77) or murine Ghrelin-GC (SEQ ID: No. 105) to Q $\beta$  capsid protein. Lane M is the marker, lane 1 shows derivatized Q $\beta$  VLP, lane 2 shows Q $\beta$ -C-Ghrelin in soluble fraction, lane 3 shows Q $\beta$ -C-Ghrelin in insoluble fraction, lane 4 shows Ghrelin-GC-Q $\beta$  in soluble fraction, and lane 5 shows Ghrelin-GC-Q $\beta$  in insoluble  
10 fraction. Very little product is in the insoluble fraction.

Figure 2 shows the average titers of ghrelin-specific IgG antibodies that were detected in pooled sera from mice, which had been immunized with murine Q $\beta$ -C-Ghrelin (Qb-cGhr), murine Ghrelin-GC-Q $\beta$  (Qb-GhrC) or PBS on day 0, 14, 21 and  
15 42. ELISA titers are expressed as serum dilutions which lead to half maximal OD in the ELISA assay. ELISA plates were coated with serine-octanylated, murine ghrelin (Bachem, Product No. H-4862) at a concentration of 20 $\mu$ g/ml. The plates were blocked and then incubated with serially diluted mouse sera from day 14, 21 and 42. Bound antibodies were detected with enzymatically labeled anti-mouse IgG  
20 antibody. As a control, pre-immune serum of the same mice was also tested.

Figure 3 shows the cumulative food consumption of mice, which had been immunized with murine Q $\beta$ -C-Ghrelin (Qb-cGhr), murine Ghrelin-GC-Q $\beta$  (Qb-GhrC) or PBS. As a control, mice were immunized with PBS. All mice were placed on a normal diet between days 0 and 14. Subsequently, all mice were given a high fat (45%) diet to facilitate the development of diet-induced obesity. Food and water was  
25 administered *ad libitum*. Individual mice were monitored for body weight changes and food and water consumption per cage (i.e. group) was also monitored at regular intervals after immunization (at day 5, 11, 14, 21, 28, 35, 40, 49, and 55 after immunization).

## DETAILED DESCRIPTION OF THE INVENTION

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or  
5 equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are hereinafter described.

## 1. Definitions:

Adjuvant: The term "adjuvant" as used herein refers to non-specific stimulators  
10 of the immune response or substances that allow generation of a depot in the host which when combined with the vaccine and pharmaceutical composition, respectively, of the present invention may provide for an even more enhanced immune response. A variety of adjuvants can be used. Examples include complete and incomplete Freund's adjuvant, aluminum hydroxide and modified  
15 muramyl dipeptide. Further adjuvants are mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Such adjuvants are also well known in the art. Further adjuvants that can  
20 be administered with the compositions of the invention include, but are not limited to, Monophosphoryl lipid immunomodulator, AdjuVax 100a, QS-21, QS-18, CRL1005, Aluminum salts (Alum), MF-59, OM-174, OM-197, OM-294, and Virosomal adjuvant technology. The adjuvants can also comprise a mixture of these substances.

25 Immunologically active saponin fractions having adjuvant activity derived from the bark of the South American tree *Quillaja Saponaria* Molina are known in the art. For example QS21, also known as QA21, is an Hplc purified fraction from the *Quillaja Saponaria* Molina tree and its method of its production is disclosed (as QA21) in U.S. Pat. No. 5,057,540. *Quillaja* saponin has also been disclosed as an

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adjuvant by Scott et al, Int. Archs. Allergy Appl. Immun., 1985, 77, 409. Monosphoryl lipid A and derivatives thereof are known in the art. A preferred derivative is 3 de-o-acylated monophosphoryl lipid A, and is known from British Patent No. 2220211. Further preferred adjuvants are described in WO00/00462, the disclosure of which is herein incorporated by reference.

However, an advantageous feature of the present invention is the high immunogenicity of the inventive compositions. As already outlined herein or will become apparent as this specification proceeds, vaccines and pharmaceutical compositions devoid of adjuvants are provided, in further alternative or preferred embodiments, leading to vaccines and pharmaceutical compositions for treating AD being devoid of adjuvants and, thus, having a superior safety profile since adjuvants may cause side-effects. The term "devoid" as used herein in the context of vaccines and pharmaceutical compositions for treating AD refers to vaccines and pharmaceutical compositions that are used without adjuvants.

Amino acid linker: An "amino acid linker", or also just termed "linker" within this specification, as used herein, either associates the antigen or antigenic determinant with the second attachment site, or more preferably, already comprises or contains the second attachment site, typically - but not necessarily - as one amino acid residue, preferably as a cysteine residue. The term "amino acid linker" as used herein, however, does not intend to imply that such an amino acid linker consists exclusively of amino acid residues, even if an amino acid linker consisting of amino acid residues is a preferred embodiment of the present invention. The amino acid residues of the amino acid linker are, preferably, composed of naturally occurring amino acids or unnatural amino acids known in the art, all-L or all-D or mixtures thereof. However, an amino acid linker comprising a molecule with a sulfhydryl group or cysteine residue is also encompassed within the invention. Such a molecule comprise preferably a C1-C6 alkyl-, cycloalkyl (C5, C6), aryl or heteroaryl moiety. However, in addition to an amino acid linker, a linker comprising preferably a C1-C6 alkyl-, cycloalkyl- (C5, C6), aryl- or heteroaryl- moiety and devoid of any amino acid(s) shall also be encompassed within the scope of the invention. Association between the antigen or antigenic determinant or optionally the second attachment site

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and the amino acid linker is preferably by way of at least one covalent bond, more preferably by way of at least one peptide bond.

Animal: As used herein, the term "animal" is meant to include, for example, humans, sheep, elks, deer, mule deer, minks, mammals, monkeys, horses, cattle, pigs, goats, dogs, cats, rats, mice, birds, chicken, reptiles, fish, insects and arachnids.

Antibody: As used herein, the term "antibody" refers to molecules which are capable of binding an epitope or antigenic determinant. The term is meant to include whole antibodies and antigen-binding fragments thereof, including single-chain antibodies. Most preferably the antibodies are human antigen binding antibody fragments and include, but are not limited to, Fab, Fab' and F(ab')<sub>2</sub>, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv) and fragments comprising either a V<sub>L</sub> or V<sub>H</sub> domain. The antibodies can be from any animal origin including birds and mammals. Preferably, the antibodies are human, murine, rabbit, goat, guinea pig, camel, horse or chicken. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries or from animals transgenic for one or more human immunoglobulins and that do not express endogenous immunoglobulins, as described, for example, in U.S. Patent No. 5,939,598 by Kucherlapati *et al.*

Antigen: As used herein, the term "antigen" refers to a molecule capable of being bound by an antibody or a T cell receptor (TCR) if presented by MHC molecules. The term "antigen", as used herein, also encompasses T-cell epitopes. An antigen is additionally capable of being recognized by the immune system and/or being capable of inducing a humoral immune response and/or cellular immune response leading to the activation of B- and/or T-lymphocytes. This may, however, require that, at least in certain cases, the antigen contains or is linked to a Th cell epitope and is given in adjuvant. An antigen can have one or more epitopes (B- and T- epitopes). The specific reaction referred to above is meant to indicate that the antigen will preferably react, typically in a highly selective manner, with its corresponding antibody or TCR and not with the multitude of other antibodies or



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TCRs which may be evoked by other antigens. Antigens as used herein may also be mixtures of several individual antigens.

Antigenic determinant: As used herein, the term "antigenic determinant" is meant to refer to that portion of an antigen that is specifically recognized by either B-  
5 or T-lymphocytes. B-lymphocytes responding to antigenic determinants produce antibodies, whereas T-lymphocytes respond to antigenic determinants by proliferation and establishment of effector functions critical for the mediation of cellular and/or humoral immunity.

Association: As used herein, the term "association" as it applies to the first and  
10 second attachment sites, refers to the binding of the first and second attachment sites that is preferably by way of at least one non-peptide bond. The nature of the association may be covalent, ionic, hydrophobic, polar or any combination thereof, preferably the nature of the association is covalent.

Attachment Site, First: As used herein, the phrase "first attachment site" refers  
15 to an element of non-natural or natural origin, to which the second attachment site located on the antigen or antigenic determinant may associate. The first attachment site may be a protein, a polypeptide, an amino acid, a peptide, a sugar, a polynucleotide, a natural or synthetic polymer, a secondary metabolite or compound (biotin, fluorescein, retinol, digoxigenin, metal ions, phenylmethylsulfonylfluoride),  
20 or a combination thereof, or a chemically reactive group thereof. The first attachment site is located, typically and preferably on the surface, of the core particle such as, preferably the virus-like particle. Multiple first attachment sites are present on the surface of the core and virus-like particle, respectively, typically in a repetitive configuration.

25 Attachment Site, Second: As used herein, the phrase "second attachment site" refers to an element associated with the antigen or antigenic determinant to which the first attachment site located on the surface of the core particle and virus-like particle, respectively, may associate. The second attachment site of the antigen or antigenic determinant may be a protein, a polypeptide, a peptide, a sugar, a polynucleotide, a  
30 natural or synthetic polymer, a secondary metabolite or compound (biotin, fluorescein, retinol, digoxigenin, metal ions, phenylmethylsulfonylfluoride), or a

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combination thereof, or a chemically reactive group thereof. At least one second attachment site is present on the antigen or antigenic determinant. The term "antigen or antigenic determinant with at least one second attachment site" refers, therefore, to an antigen or antigenic construct comprising at least the antigen or antigenic determinant and the second attachment site. However, in particular for a second attachment site, which is of non-natural origin, i.e. not naturally occurring within the antigen or antigenic determinant, these antigen or antigenic constructs comprise an "amino acid linker".

Bound: As used herein, the term "bound" refers to binding or attachment that may be covalent, *e.g.*, by chemically coupling, or non-covalent, *e.g.*, ionic interactions, hydrophobic interactions, hydrogen bonds, etc. Covalent bonds can be, for example, ester, ether, phosphoester, amide, peptide, imide, carbon-sulfur bonds, carbon-phosphorus bonds, and the like. The term "bound" is broader than and includes terms such as "coupled," "fused" and "attached".

Coat protein(s): As used herein, the term "coat protein(s)" refers to the protein(s) of a bacteriophage or a RNA-phage capable of being incorporated within the capsid assembly of the bacteriophage or the RNA-phage. However, when referring to the specific gene product of the coat protein gene of RNA-phages the term "CP" is used. For example, the specific gene product of the coat protein gene of RNA-phage Q $\beta$  is referred to as "Q $\beta$  CP", whereas the "coat proteins" of bacteriophage Q $\beta$  comprise the "Q $\beta$  CP" as well as the A1 protein. The capsid of Bacteriophage Q $\beta$  is composed mainly of the Q $\beta$  CP, with a minor content of the A1 protein. Likewise, the VLP Q $\beta$  coat protein contains mainly Q $\beta$  CP, with a minor content of A1 protein.

Core particle: As used herein, the term "core particle" refers to a rigid structure with an inherent repetitive organization. A core particle as used herein may be the product of a synthetic process or the product of a biological process.

Coupled: The term "coupled", as used herein, refers to attachment by covalent bonds or by strong non-covalent interactions, typically and preferably to attachment by covalent bonds. Any method normally used by those skilled in the art for the coupling of biologically active materials can be used in the present invention.

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Effective Amount: As used herein, the term "effective amount" refers to an amount necessary or sufficient to realize a desired biologic effect. An effective amount of the composition would be the amount that achieves this selected result, and such an amount could be determined as a matter of routine by a person skilled in the art. For example, an effective amount for treating an immune system deficiency could be that amount necessary to cause activation of the immune system, resulting in the development of an antigen specific immune response upon exposure to antigen. The term is also synonymous with "sufficient amount."

The effective amount for any particular application can vary depending on such factors as the disease or condition being treated, the particular composition being administered, the size of the subject, and/or the severity of the disease or condition. One of ordinary skill in the art can empirically determine the effective amount of a particular composition of the present invention without necessitating undue experimentation.

Epitope: As used herein, the term "epitope" refers to continuous or discontinuous portions of a polypeptide having antigenic or immunogenic activity in an animal, preferably a mammal, and most preferably in a human. An epitope is recognized by an antibody or a T cell through its T cell receptor in the context of an MHC molecule. An "immunogenic epitope," as used herein, is defined as a portion of a polypeptide that elicits an antibody response or induces a T-cell response in an animal, as determined by any method known in the art. (*See, for example, Geysen et al., Proc. Natl. Acad. Sci. USA 81:3998-4002 (1983)*). The term "antigenic epitope," as used herein, is defined as a portion of a protein to which an antibody can immunospecifically bind its antigen as determined by any method well known in the art. Immunospecific binding excludes non-specific binding but does not necessarily exclude cross-reactivity with other antigens. Antigenic epitopes need not necessarily be immunogenic. Antigenic epitopes can also be T-cell epitopes, in which case they can be bound immunospecifically by a T-cell receptor within the context of an MHC molecule.

An epitope can comprise 3 amino acids in a spatial conformation which is unique to the epitope. Generally, an epitope consists of at least about 5 such amino

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acids, and more usually, consists of at least about 8-10 such amino acids. If the epitope is an organic molecule, it may be as small as Nitrophenyl.

Fusion: As used herein, the term "fusion" refers to the combination of amino acid sequences of different origin in one polypeptide chain by in-frame combination of their coding nucleotide sequences. The term "fusion" explicitly encompasses internal fusions, *i.e.*, insertion of sequences of different origin within a polypeptide chain, in addition to fusion to one of its termini.

Ghrelin: The term "ghrelin" as used herein refers to a peptide derived from a protein encoded by a ghrelin gene. As used herein ghrelin includes all forms of ghrelins known in humans, cats, dogs and all domesticated animals as well as of other animals. Ghrelin, as used herein, includes ghrelin with or without a n-octanoyl-modification. Moreover, ghrelin also includes all splice variants that exist of ghrelin. In addition, due to high sequence homology between ghrelins of different species (only 2 aa exchanged between rat and human ghrelin (Kojima et al. *Nature* 402: 656-660 (1999)), all natural variants of ghrelin with more than 80% identity, preferably more than 90%, more preferably more than 95%, and even more preferably more than 99% with human ghrelin are referred to as "ghrelin" herein.

As used herein, the term "ghrelin-derived peptide" or "ghrelin-peptides" are broadly defined as any peptide which represents a fraction of ghrelin and containing at least two, preferably at least three, more preferably at least four, more preferably at least five, or at least six, and even more preferably at least eight or nine or even more preferably at least ten consecutive amino acids of the original ghrelin peptide. Typically, "ghrelin peptide" and "ghrelin-fragment" are used interchangeably. Moreover, the terms "ghrelin peptide and fragments thereof" as used herein, shall encompass beside the ghrelin peptide, any fraction of said ghrelin peptide, wherein said fraction may be, preferably, derived by deletion of one or more amino acids at the N and/or C terminus. The ghrelin peptide can be obtained by recombinant expression in eukaryotic or prokaryotic expression systems as ghrelin peptide alone or as a fusion with other amino acids or proteins, e.g. to facilitate folding, expression or solubility of the ghrelin peptide or to facilitate purification of the ghrelin peptide. To facilitate or enable correct folding of fusion proteins between ghrelin peptides and

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subunit proteins of VLPs or capsids, one or more amino acids may be added N- or C-terminally to ghrelin peptides. To enable coupling of ghrelin peptides to subunit proteins of VLPs or capsids or core particles, at least one second attachment site may be added to the ghrelin peptide. Alternatively ghrelin peptides may be synthesized  
5 using methods known to the art. Such peptides may even contain amino acids which are not present in the corresponding ghrelin protein. The peptides may be modified by n-octanoylation.

Residue: As used herein, the term "residue" is meant to mean a specific amino acid in a polypeptide backbone or side chain.

10 Immune response: As used herein, the term "immune response" refers to a humoral immune response and/or cellular immune response leading to the activation or proliferation of B- and/or T-lymphocytes and/or and antigen presenting cells. In some instances, however, the immune responses may be of low intensity and become detectable only when using at least one substance in accordance with the invention.  
15 "Immunogenic" refers to an agent used to stimulate the immune system of a living organism, so that one or more functions of the immune system are increased and directed towards the immunogenic agent. An "immunogenic polypeptide" is a polypeptide that elicits a cellular and/or humoral immune response, whether alone or linked to a carrier in the presence or absence of an adjuvant. Preferably, antigen  
20 presenting cell may be activated.

A substance which "enhances" an immune response refers to a substance in which an immune response is observed that is greater or intensified or deviated in any way with the addition of the substance when compared to the same immune response measured without the addition of the substance. For example, the lytic  
25 activity of cytotoxic T cells can be measured, *e.g.* using a <sup>51</sup>Cr release assay, in samples obtained with and without the use of the substance during immunization. The amount of the substance at which the CTL lytic activity is enhanced as compared to the CTL lytic activity without the substance is said to be an amount sufficient to enhance the immune response of the animal to the antigen. In a  
30 preferred embodiment, the immune response is enhanced by a factor of at least about 2, more preferably by a factor of about 3 or more. The amount or type of cytokines

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secreted may also be altered. Alternatively, the amount of antibodies induced or their subclasses may be altered.

Immunization: As used herein, the terms "immunize" or "immunization" or related terms refer to conferring the ability to mount a substantial immune response (comprising antibodies and/or cellular immunity such as effector CTL) against a target antigen or epitope. These terms do not require that complete immunity be created, but rather that an immune response be produced which is substantially greater than baseline. For example, a mammal may be considered to be immunized against a target antigen if the cellular and/or humoral immune response to the target antigen occurs following the application of methods of the invention.

Natural origin: As used herein, the term "natural origin" means that the whole or parts thereof are not synthetic and exist or are produced in nature.

Non-natural: As used herein, the term generally means not from nature, more specifically, the term means from the hand of man.

Non-natural origin: As used herein, the term "non-natural origin" generally means synthetic or not from nature; more specifically, the term means from the hand of man.

Ordered and repetitive antigen or antigenic determinant array: As used herein, the term "ordered and repetitive antigen or antigenic determinant array" generally refers to a repeating pattern of antigen or antigenic determinant, characterized by a typically and preferably uniform spacial arrangement of the antigens or antigenic determinants with respect to the core particle and virus-like particle, respectively. In one embodiment of the invention, the repeating pattern may be a geometric pattern. Typical and preferred examples of suitable ordered and repetitive antigen or antigenic determinant arrays are those which possess strictly repetitive paracrystalline orders of antigens or antigenic determinants, preferably with spacings of 1 to 30 nanometers, preferably 5 to 15 nanometers.

Pili: As used herein, the term "pili" (singular being "pilus") refers to extracellular structures of bacterial cells composed of protein monomers (*e.g.*, pilin monomers) which are organized into ordered and repetitive patterns. Further, pili are structures which are involved in processes such as the attachment of bacterial cells to

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host cell surface receptors, inter-cellular genetic exchanges, and cell-cell recognition. Examples of pili include Type-1 pili, P-pili, F1C pili, S-pili, and 987P-pili. Additional examples of pili are set out below.

Pilus-like structure: As used herein, the phrase "pilus-like structure" refers to structures having characteristics similar to that of pili and composed of protein monomers. One example of a "pilus-like structure" is a structure formed by a bacterial cell which expresses modified pilin proteins that do not form ordered and repetitive arrays that are identical to those of natural pili.

Polypeptide: As used herein, the term "polypeptide" refers to a molecule composed of monomers (amino acids) linearly linked by amide bonds (also known as peptide bonds). It indicates a molecular chain of amino acids and does not refer to a specific length of the product. Thus, peptides, dipeptides, tripeptides, oligopeptides and proteins are included within the definition of polypeptide. This term is also intended to refer to post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations, and the like. A recombinant or derived polypeptide is not necessarily translated from a designated nucleic acid sequence. It may also be generated in any manner, including chemical synthesis.

Self antigen: As used herein, the term "self antigen" refers to proteins encoded by the host's DNA and products generated by proteins or RNA encoded by the host's DNA are defined as self. In addition, proteins that result from a combination of two or several self-molecules or that represent a fraction of a self-molecule and proteins that have a high homology two self-molecules as defined above (>95%, preferably >97%, more preferably >99%) may also be considered self.

Treatment: As used herein, the terms "treatment", "treat", "treated" or "treating" refer to prophylaxis and/or therapy. When used with respect to an infectious disease, for example, the term refers to a prophylactic treatment which increases the resistance of a subject to infection with a pathogen or, in other words, decreases the likelihood that the subject will become infected with the pathogen or will show signs of illness attributable to the infection, as well as a treatment after the subject has become infected in order to fight the infection, *e.g.*, reduce or eliminate the infection or prevent it from becoming worse. When used with respect to obesity

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or related diseases, the term "treatment" refers to a prophylactic or therapeutic treatment which increases the resistance of a subject against, and/or which reverts obesity.

Vaccine: As used herein, the term "vaccine" refers to a formulation which contains the composition of the present invention and which is in a form that is capable of being administered to an animal. Typically, the vaccine comprises a conventional saline or buffered aqueous solution medium in which the composition of the present invention is suspended or dissolved. In this form, the composition of the present invention can be used conveniently to prevent, ameliorate, or otherwise treat a condition. Upon introduction into a host, the vaccine is able to provoke an immune response including, but not limited to, the production of antibodies and/or cytokines and/or the activation of cytotoxic T cells, antigen presenting cells, helper T cells, dendritic cells and/or other cellular responses.

Optionally, the vaccine of the present invention additionally includes an adjuvant which can be present in either a minor or major proportion relative to the compound of the present invention.

Virus-like particle (VLP): As used herein, the term "virus-like particle" refers to a structure resembling a virus particle. Moreover, a virus-like particle in accordance with the invention is non-replicative and noninfectious since it lacks all or part of the viral genome, in particular the replicative and infectious components of the viral genome. A virus-like particle in accordance with the invention may contain nucleic acid distinct from their genome. A typical and preferred embodiment of a virus-like particle in accordance with the present invention is a viral capsid such as the viral capsid of the corresponding virus, bacteriophage, or RNA-phage. The terms "viral capsid" or "capsid", as interchangeably used herein, refer to a macromolecular assembly composed of viral protein subunits. Typically and preferably, the viral protein subunits assemble into a viral capsid and capsid, respectively, having a structure with an inherent repetitive organization, wherein said structure is, typically, spherical or tubular. For example, the capsids of RNA-phages or HBcAgs have a spherical form of icosahedral symmetry. The term "capsid-like structure" as used herein, refers to a macromolecular assembly composed of viral protein subunits



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resembling the capsid morphology in the above defined sense but deviating from the typical symmetrical assembly while maintaining a sufficient degree of order and repetitiveness.

Virus-like particle of a bacteriophage: As used herein, the term "virus-like particle of a bacteriophage" refers to a virus-like particle resembling the structure of a bacteriophage, being non replicative and noninfectious, and lacking at least the gene or genes encoding for the replication machinery of the bacteriophage, and typically also lacking the gene or genes encoding the protein or proteins responsible for viral attachment to or entry into the host. This definition should, however, also encompass virus-like particles of bacteriophages, in which the aforementioned gene or genes are still present but inactive, and, therefore, also leading to non-replicative and noninfectious virus-like particles of a bacteriophage.

VLP of RNA phage coat protein: The capsid structure formed from the self-assembly of 180 subunits of RNA phage coat protein and optionally containing host RNA is referred to as a "VLP of RNA phage coat protein." A specific example is the VLP of Q $\beta$  coat protein. In this particular case, the VLP of Q $\beta$  coat protein may either be assembled exclusively from Q $\beta$  CP subunits (generated by expression of a Q $\beta$  CP gene containing, for example, a TAA stop codon precluding any expression of the longer A1 protein through suppression, see Kozlovska, T.M., *et al.*, *Intervirology* 39: 9-15 (1996)), or additionally contain A1 protein subunits in the capsid assembly.

Virus particle: The term "virus particle" as used herein refers to the morphological form of a virus. In some virus types it comprises a genome surrounded by a protein capsid; others have additional structures (*e.g.*, envelopes, tails, etc.).

One, a, or an: When the terms "one," "a," or "an" are used in this disclosure, they mean "at least one" or "one or more," unless otherwise indicated.

As will be clear to those skilled in the art, certain embodiments of the invention involve the use of recombinant nucleic acid technologies such as cloning, polymerase chain reaction, the purification of DNA and RNA, the expression of recombinant proteins in prokaryotic and eukaryotic cells, etc. Such methodologies

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are well known to those skilled in the art and can be conveniently found in published laboratory methods manuals (e.g., Sambrook, J. *et al.*, eds., *Molecular Cloning, A Laboratory Manual*, 2nd. edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); Ausubel, F. *et al.*, eds., *Current Protocols in Molecular Biology*, John H. Wiley & Sons, Inc. (1997)).  
5 Fundamental laboratory techniques for working with tissue culture cell lines (Celis, J., ed., *Cell Biology*, Academic Press, 2<sup>nd</sup> edition, (1998)) and antibody-based technologies (Harlow, E. and Lane, D., *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1988); Deutscher, M.P., "Guide to Protein Purification," *Meth.*  
10 *Enzymol.* 128, Academic Press San Diego (1990); Scopes, R.K., *Protein Purification Principles and Practice*, 3rd ed., Springer-Verlag, New York (1994)) are also adequately described in the literature, all of which are incorporated herein by reference.

15           2.           Compositions and Methods for Enhancing an Immune Response

The disclosed invention provides compositions and methods for enhancing an immune response against ghrelin or a ghrelin peptide in an animal. Compositions of the invention comprise, or alternatively consist of (a) a core particle with at least one first attachment site; and (b) at least one antigen or antigenic determinant with at  
20 least one second attachment site, wherein said antigen or antigenic determinant is ghrelin or a ghrelin peptide, and wherein said second attachment site being selected from the group consisting of (i) an attachment site not naturally occurring with said antigen or antigenic determinant; and (ii) an attachment site naturally occurring with said antigen or antigenic determinant, wherein said second attachment site is capable  
25 of association to said first attachment site; and wherein said antigen or antigenic determinant and said core particle interact through said association to form an ordered and repetitive antigen array. More specifically, compositions of the invention comprise, or alternatively consist of, a virus-like particle and at least one antigen or antigenic determinant, wherein the antigen or antigenic determinant is ghrelin or a  
30 ghrelin peptide and wherein the at least one antigen or antigenic determinant is

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bound to the virus-like particle so as to form an ordered and repetitive antigen-VLP-array. Furthermore, the invention conveniently enables the practitioner to construct such a composition, inter alia, for treatment and/or prophylactic prevention of obesity.

5           In one embodiment, the core particle comprises, or is selected from a group consisting of, a virus, a bacterial pilus, a structure formed from bacterial pilin, a bacteriophage, a virus-like particle, a virus-like particle of a RNA phage, a viral capsid particle or a recombinant form thereof. Any virus known in the art having an ordered and repetitive coat and/or core protein structure may be selected as a core  
10 particle of the invention; examples of suitable viruses include sindbis and other alphaviruses, rhabdoviruses (*e.g.* vesicular stomatitis virus), picornaviruses (*e.g.*, human rhino virus, Aichi virus), togaviruses (*e.g.*, rubella virus), orthomyxoviruses (*e.g.*, Thogoto virus, Batken virus, fowl plague virus), polyomaviruses (*e.g.*, polyomavirus BK, polyomavirus JC, avian polyomavirus BFDV), parvoviruses,  
15 rotaviruses, Norwalk virus, foot and mouth disease virus, a retrovirus, Hepatitis B virus, Tobacco mosaic virus, Flock House Virus, and human Papillomavirus, and preferably a RNA phage, bacteriophage Q $\beta$ , bacteriophage R17, bacteriophage M11, bacteriophage MX1, bacteriophage NL95, bacteriophage fr, bacteriophage GA, bacteriophage SP, bacteriophage MS2, bacteriophage f2, bacteriophage PP7 (for  
20 example, *see* Table 1 in Bachmann, M.F. and Zinkernagel, R.M., *Immunol. Today* 17:553-558 (1996)).

In a further embodiment, the invention utilizes genetic engineering of a virus to create a fusion between an ordered and repetitive viral envelope protein and a first attachment site being comprised by, or alternatively or preferably being a  
25 heterologous protein, peptide, antigenic determinant or a reactive amino acid residue of choice. Other genetic manipulations known to those in the art may be included in the construction of the inventive compositions; for example, it may be desirable to restrict the replication ability of the recombinant virus through genetic mutation. Furthermore, the virus used for the present invention is replication incompetent due  
30 to chemical or physical inactivation or, as indicated, due to lack of a replication competent genome. The viral protein selected for fusion to the first attachment site

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should have an organized and repetitive structure. Such an organized and repetitive structure includes paracrystalline organizations with a spacing of 5-30 nm, preferably 5-15 nm, on the surface of the virus. The creation of this type of fusion protein will result in multiple, ordered and repetitive first attachment sites on the surface of the virus and reflect the normal organization of the native viral protein. As will be understood by those in the art, the first attachment site may be or be a part of any suitable protein, polypeptide, sugar, polynucleotide, peptide (amino acid), natural or synthetic polymer, a secondary metabolite or combination thereof that may serve to specifically attach the antigen or antigenic determinant leading an ordered and repetitive antigen array.

In another embodiment of the invention, the core particle is a recombinant alphavirus, and more specifically, a recombinant Sindbis virus. Alphaviruses are positive stranded RNA viruses that replicate their genomic RNA entirely in the cytoplasm of the infected cell and without a DNA intermediate (Strauss, J. and Strauss, E., *Microbiol. Rev.* 58:491-562 (1994)). Several members of the alphavirus family, Sindbis (Xiong, C. *et al.*, *Science* 243:1188-1191 (1989); Schlesinger, S., *Trends Biotechnol.* 11:18-22 (1993)), Semliki Forest Virus (SFV) (Liljeström, P. & Garoff, H., *Bio/Technology* 9:1356-1361 (1991)) and others (Davis, N.L. *et al.*, *Virology* 171:189-204 (1989)), have received considerable attention for use as virus-based expression vectors for a variety of different proteins (Lundstrom, K., *Curr. Opin. Biotechnol.* 8:578-582 (1997); Liljeström, P., *Curr. Opin. Biotechnol.* 5:495-500 (1994)) and as candidates for vaccine development. Recently, a number of patents have issued directed to the use of alphaviruses for the expression of heterologous proteins and the development of vaccines (*see* U.S. Patent Nos. 5,766,602; 5,792,462; 5,739,026; 5,789,245 and 5,814,482). The construction of the alphaviral core particles of the invention may be done by means generally known in the art of recombinant DNA technology, as described by the aforementioned articles, which are incorporated herein by reference.

A variety of different recombinant host cells can be utilized to produce a viral-based core particle for antigen or antigenic determinant attachment. For example, alphaviruses are known to have a wide host range; Sindbis virus infects cultured

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mammalian, reptilian, and amphibian cells, as well as some insect cells (Clark, H., *J. Natl. Cancer Inst.* 51:645 (1973); Leake, C., *J. Gen. Virol.* 35:335 (1977); Stollar, V. in *THE TOGAVIRUSES*, R.W. Schlesinger, Ed., Academic Press, (1980), pp.583-621). Thus, numerous recombinant host cells can be used in the practice of the invention.

5 BHK, COS, Vero, HeLa and CHO cells are particularly suitable for the production of heterologous proteins because they have the potential to glycosylate heterologous proteins in a manner similar to human cells (Watson, E. *et al.*, *Glycobiology* 4:227, (1994)) and can be selected (Zang, M. *et al.*, *Bio/Technology* 13:389 (1995)) or genetically engineered (Renner W. *et al.*, *Biotech. Bioeng.* 4:476 (1995); Lee K. *et al.* *Biotech. Bioeng.* 50:336 (1996)) to grow in serum-free medium, as well as in

10 suspension.

Introduction of the polynucleotide vectors into host cells can be effected by methods described in standard laboratory manuals (*see, e.g.*, Sambrook, J. *et al.*, eds., *MOLECULAR CLONING, A LABORATORY MANUAL*, 2nd. edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989), Chapter 9; Ausubel, F. *et al.*, eds., *CURRENT PROTOCOLS IN MOLECULAR BIOLOGY*, John H. Wiley & Sons, Inc. (1997), Chapter 16), including methods such as electroporation, DEAE-dextran mediated transfection, transfection, microinjection, cationic lipid-mediated transfection, transduction, scrape loading, ballistic introduction, and infection.

15 Methods for the introduction of exogenous DNA sequences into host cells are discussed in Felgner, P. *et al.*, U.S. Patent No. 5,580,859.

Packaged RNA sequences can also be used to infect host cells. These packaged RNA sequences can be introduced to host cells by adding them to the culture medium. For example, the preparation of non-infective alphaviral particles is

25 described in a number of sources, including "Sindbis Expression System", Version C (*Invitrogen* Catalog No. K750-1).

When mammalian cells are used as recombinant host cells for the production of viral-based core particles, these cells will generally be grown in tissue culture. Methods for growing cells in culture are well known in the art (*see, e.g.*, Celis, J., ed., *CELL BIOLOGY*, Academic Press, 2<sup>nd</sup> edition, (1998); Sambrook, J. *et al.*, eds., *MOLECULAR CLONING, A LABORATORY MANUAL*, 2nd. edition, Cold Spring Harbor

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Laboratory Press, Cold Spring Harbor, N.Y. (1989); Ausubel, F. *et al.*, eds., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John H. Wiley & Sons, Inc. (1997); Freshney, R., CULTURE OF ANIMAL CELLS, Alan R. Liss, Inc. (1983)).

Further examples of RNA viruses suitable for use as core particle in the present invention include, but are not limited to, the following: members of the family Reoviridae, including the genus Orthoreovirus (multiple serotypes of both mammalian and avian retroviruses), the genus Orbivirus (Bluetongue virus, Eugenangee virus, Kemerovo virus, African horse sickness virus, and Colorado Tick Fever virus), the genus Rotavirus (human rotavirus, Nebraska calf diarrhea virus, murine rotavirus, simian rotavirus, bovine or ovine rotavirus, avian rotavirus); the family Picomaviridae, including the genus Enterovirus (poliovirus, Coxsackie virus A and B, enteric cytopathic human orphan (ECHO) viruses, hepatitis A, C, D, E and G viruses, Simian enteroviruses, Murine encephalomyelitis (ME) viruses, Poliovirus muris, Bovine enteroviruses, Porcine enteroviruses, the genus Cardiovirus (Encephalomyocarditis virus (EMC), Mengovirus), the genus Rhinovirus (Human rhinoviruses including at least 113 subtypes; other rhinoviruses), the genus Aphovirus (Foot and Mouth disease (FMDV); the family Calciviridae, including Vesicular exanthema of swine virus, San Miguel sea lion virus, Feline picornavirus and Norwalk virus; the family Togaviridae, including the genus Alphavirus (Eastern equine encephalitis virus, Semliki forest virus, Sindbis virus, Chikungunya virus, O'Nyong-Nyong virus, Ross river virus, Venezuelan equine encephalitis virus, Western equine encephalitis virus), the genus Flavivirus (Mosquito borne yellow fever virus, Dengue virus, Japanese encephalitis virus, St. Louis encephalitis virus, Murray Valley encephalitis virus, West Nile virus, Kunjin virus, Central European tick borne virus, Far Eastern tick borne virus, Kyasanur forest virus, Louping III virus, Powassan virus, Omsk hemorrhagic fever virus), the genus Rubivirus (Rubella virus), the genus Pestivirus (Mucosal disease virus, Hog cholera virus, Border disease virus); the family Bunyaviridae, including the genus Bunyavirus (Bunyamwera and related viruses, California encephalitis group viruses), the genus Phlebovirus (Sandfly fever Sicilian virus, Rift Valley fever virus), the genus Nairovirus (Crimean-Congo hemorrhagic fever virus, Nairobi sheep disease virus),

and the genus Uukuvirus (Uukuniemi and related viruses); the family Orthomyxoviridae, including the genus Influenza virus (Influenza virus type A, many human subtypes); Swine influenza virus, and Avian and Equine Influenza viruses; influenza type B (many human subtypes), and influenza type C (possible  
5 separate genus); the family paramyxoviridae, including the genus Paramyxovirus (Parainfluenza virus type 1, Sendai virus, Hemadsorption virus, Parainfluenza viruses types 2 to 5, Newcastle Disease Virus, Mumps virus), the genus Morbillivirus (Measles virus, subacute sclerosing panencephalitis virus, distemper virus, Rinderpest *virus*), the genus Pneumovirus (respiratory syncytial virus (RSV), Bovine  
10 respiratory syncytial virus and Pneumonia virus of mice); forest virus, Sindbis virus, Chikungunya virus, O'Nyong-Nyong virus, Ross river virus, Venezuelan equine encephalitis virus, Western equine encephalitis virus), the genus Flavivirus (Mosquito borne yellow fever virus, Dengue virus, Japanese encephalitis virus, St. Louis encephalitis virus, Murray Valley encephalitis virus, West Nile virus, Kunjin virus,  
15 Central European tick borne virus, Far Eastern tick borne virus, Kyasanur forest virus, Louping III virus, Powassan virus, Omsk hemorrhagic fever virus), the genus Rubivirus (Rubella virus), the genus Pestivirus (Mucosal disease virus, Hog cholera virus, Border disease virus); the family Bunyaviridae, including the genus Bunyavirus (Bunyamwera and related viruses, California encephalitis group viruses), the genus  
20 Phlebovirus (Sandfly fever Sicilian virus, Rift Valley fever virus), the genus Nairovirus (Crimean-Congo hemorrhagic fever virus, Nairobi sheep disease virus), and the genus Uukuvirus (Uukuniemi and related viruses); the family Orthomyxoviridae, including the genus Influenza virus (Influenza virus type A, many human subtypes); Swine influenza virus, and Avian and Equine Influenza  
25 viruses; influenza type B (many human subtypes), and influenza type C (possible separate genus); the family paramyxoviridae, including the genus Paramyxovirus (Parainfluenza virus type 1, Sendai virus, Hemadsorption virus, Parainfluenza viruses types 2 to 5, Newcastle Disease Virus, Mumps virus), the genus Morbillivirus (Measles virus, subacute sclerosing panencephalitis virus, distemper virus,  
30 Rinderpest virus), the genus Pneumovirus (respiratory syncytial virus (RSV), Bovine respiratory syncytial *virus* and Pneumonia virus of mice); the family Rhabdoviridae,

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including the genus Vesiculovirus (VSV), Chandipura virus, Flanders-Hart Park virus), the genus Lyssavirus (Rabies virus), fish Rhabdoviruses and, filoviruses (Marburg virus and Ebola virus); the family Arenaviridae, including Lymphocytic choriomeningitis virus (LCM), Tacaribe virus complex, and Lassa virus; the family  
5 Coronaviridae, including Infectious Bronchitis Virus (IBV), Mouse Hepatitis virus, Human enteric corona virus, and Feline infectious peritonitis (Feline coronavirus).

Illustrative DNA viruses that may be used as core particles include, but are not limited to: the family Poxviridae, including the genus Orthopoxvirus (Variola major, Variola minor, Monkey pox Vaccinia, Cowpox, Buffalopox, Rabbitpox, Ectromelia),  
10 the genus Leporipoxvirus (Myxoma, Fibroma), the genus Avipoxvirus (Fowlpox, other avian poxvirus), the genus Capripoxvirus (sheeppox, goatpox), the genus Suipoxvirus (Swinepox), the genus Parapoxvirus (contagious postular dermatitis virus, pseudocowpox, bovine papular stomatitis virus); the family Iridoviridae (African swine fever virus, Frog viruses 2 and 3, Lymphocystis virus of fish); the  
15 family Herpesviridae, including the alpha-Herpesviruses (Herpes Simplex Types 1 and 2, Varicella-Zoster, Equine abortion virus, Equine herpes virus 2 and 3, pseudorabies virus, infectious bovine keratoconjunctivitis virus, infectious bovine rhinotracheitis virus, feline rhinotracheitis virus, infectious laryngotracheitis virus) the Beta-herpesviruses (Human cytomegalovirus and cytomegaloviruses of swine,  
20 monkeys and rodents); the gamma-herpesviruses (Epstein-Barr virus (EBV), Marek's disease virus, Herpes saimiri, Herpesvirus ateles, Herpesvirus sylvilagus, guinea pig herpes virus, Lucke tumor virus); the family Adenoviridae, including the genus Mastadenovirus (Human subgroups A, B, C, D and E and ungrouped; simian adenoviruses (at least 23 serotypes), infectious canine hepatitis, and adenoviruses of  
25 cattle, pigs, sheep, frogs and many other species, the genus Aviadenovirus (Avian adenoviruses); and non-cultivable adenoviruses; the family Papoviridae, including the genus Papillomavirus (Human papilloma viruses, bovine papilloma viruses, Shope rabbit papilloma virus, and various pathogenic papilloma viruses of other species), the genus Polyomavirus (polyomavirus, Simian vacuolating agent (SV-40),  
30 Rabbit vacuolating agent (RKV), K virus, BK virus, JC virus, and other primate polyoma viruses such as Lymphotropic papilloma virus); the family Parvoviridae



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including the genus Adeno-associated viruses, the genus Parvovirus (Feline panleukopenia virus, bovine parvovirus, canine parvovirus, Aleutian mink disease virus, etc.). Finally, DNA viruses may include viruses such as chronic infectious neuropathic agents (CHINA virus).

5 In other embodiments, a bacterial pilin, a subportion of a bacterial pilin, or a fusion protein which contains either a bacterial pilin or subportion thereof is used to prepare compositions and vaccine compositions, respectively, of the invention. Examples of pilin proteins include pilins produced by *Escherichia coli*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Caulobacter crescentus*,  
10 *Pseudomonas stutzeri*, and *Pseudomonas aeruginosa*. The amino acid sequences of pilin proteins suitable for use with the present invention include those set out in GenBank reports AJ000636, AJ132364, AF229646, AF051814, AF051815), and X00981, the entire disclosures of which are incorporated herein by reference.

Bacterial pilin proteins are generally processed to remove N-terminal leader  
15 sequences prior to export of the proteins into the bacterial periplasm. Further, as one skilled in the art would recognize, bacterial pilin proteins used to prepare compositions and vaccine compositions, respectively, of the invention will generally not have the naturally present leader sequence.

One specific example of a pilin protein suitable for use in the present invention  
20 is the P-pilin of *E. coli* (GenBank report AF237482 (SEQ ID NO:1)). An example of a Type-1 *E. coli* pilin suitable for use with the invention is a pilin having the amino acid sequence set out in GenBank report P04128 (SEQ ID NO:2), which is encoded by nucleic acid having the nucleotide sequence set out in GenBank report M27603 (SEQ ID NO:3). The entire disclosures of these GenBank reports are incorporated  
25 herein by reference. Again, the mature form of the above referenced protein would generally be used to prepare compositions and vaccine compositions, respectively, of the invention.

Bacterial pilins or pilin subportions suitable for use in the practice of the  
present invention will generally be able to associate to form ordered and repetitive  
30 antigen arrays.

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Methods for preparing pili and pilus-like structures *in vitro* are known in the art. Bullitt *et al.*, *Proc. Natl. Acad. Sci. USA* 93:12890-12895 (1996), for example, describe the *in vitro* reconstitution of *E. coli* P-pili subunits. Furthermore, Eshdat *et al.*, *J. Bacteriol.* 148:308-314 (1981) describe methods suitable for dissociating  
5 Type-1 pili of *E. coli* and the reconstitution of pili. In brief, these methods are as follows: pili are dissociated by incubation at 37°C in saturated guanidine hydrochloride. Pilin proteins are then purified by chromatography, after which pilin dimers are formed by dialysis against 5 mM tris(hydroxymethyl) aminomethane hydrochloride (pH 8.0). Eshdat *et al.* also found that pilin dimers reassemble to form  
10 pili upon dialysis against the 5 mM tris(hydroxymethyl) aminomethane (pH 8.0) containing 5 mM MgCl<sub>2</sub>.

Further, using, for example, conventional genetic engineering and protein modification methods, pilin proteins may be modified to contain a first attachment site to which an antigen or antigenic determinant is linked through a second  
15 attachment site. Alternatively, antigens or antigenic determinants can be directly linked through a second attachment site to amino acid residues which are naturally resident in these proteins. These modified pilin proteins may then be used in vaccine compositions of the invention.

Bacterial pilin proteins used to prepare compositions and vaccine  
20 compositions, respectively, of the invention may be modified in a manner similar to that described herein for HBcAg. For example, cysteine and lysine residues may be either deleted or substituted with other amino acid residues and first attachment sites may be added to these proteins. Further, pilin proteins may either be expressed in modified form or may be chemically modified after expression. Similarly, intact pili  
25 may be harvested from bacteria and then modified chemically.

In another embodiment, pili or pilus-like structures are harvested from bacteria (*e.g.*, *E. coli*) and used to form compositions and vaccine compositions of the invention. One example of pili suitable for preparing compositions and vaccine compositions is the Type-1 pilus of *E. coli*, which is formed from pilin monomers  
30 having the amino acid sequence set out in SEQ ID NO:2.

A number of methods for harvesting bacterial pili are known in the art. Bullitt and Makowski (*Biophys. J.* 74:623-632 (1998)), for example, describe a pilus purification method for harvesting P-pili from *E. coli*. According to this method, pili are sheared from hyperpiliated *E. coli* containing a P-pilus plasmid and purified by  
5 cycles of solubilization and MgCl<sub>2</sub> (1.0 M) precipitation.

Once harvested, pili or pilus-like structures may be modified in a variety of ways. For example, a first attachment site can be added to the pili to which antigens or antigen determinants may be attached through a second attachment site. In other words, bacterial pili or pilus-like structures can be harvested and modified to lead to  
10 ordered and repetitive antigen arrays.

Antigens or antigenic determinants could be linked to naturally occurring cysteine residues or lysine residues present in Pili or pilus-like structures. In such instances, the high order and repetitiveness of a naturally occurring amino acid residue would guide the coupling of the antigens or antigenic determinants to the pili  
15 or pilus-like structures. For example, the pili or pilus-like structures could be linked to the second attachment sites of the antigens or antigenic determinants using a heterobifunctional cross-linking agent.

When structures which are naturally synthesized by organisms (*e.g.*, pili) are used to prepare compositions and vaccine compositions of the invention, it will often  
20 be advantageous to genetically engineer these organisms so that they produce structures having desirable characteristics. For example, when Type-1 pili of *E. coli* are used, the *E. coli* from which these pili are harvested may be modified so as to produce structures with specific characteristics. Examples of possible modifications of pilin proteins include the insertion of one or more lysine residues, the deletion or  
25 substitution of one or more of the naturally resident lysine residues, and the deletion or substitution of one or more naturally resident cysteine residues (*e.g.*, the cysteine residues at positions 44 and 84 in SEQ ID NO:2).

Further, additional modifications can be made to pilin genes which result in the expression products containing a first attachment site other than a lysine residue  
30 (*e.g.*, a *FOS* or *JUN* domain). Of course, suitable first attachment sites will generally

be limited to those which do not prevent pilin proteins from forming pili or pilus-like structures suitable for use in vaccine compositions of the invention.

Pilin genes which naturally reside in bacterial cells can be modified *in vivo* (e.g., by homologous recombination) or pilin genes with particular characteristics can be inserted into these cells. For examples, pilin genes could be introduced into  
5 bacterial cells as a component of either a replicable cloning vector or a vector which inserts into the bacterial chromosome. The inserted pilin genes may also be linked to expression regulatory control sequences (e.g., a *lac* operator).

In most instances, the pili or pilus-like structures used in compositions and  
10 vaccine compositions, respectively, of the invention will be composed of single type of a pilin subunit. Pili or pilus-like structures composed of identical subunits will generally be used because they are expected to form structures which present highly ordered and repetitive antigen arrays.

However, the compositions of the invention also include compositions and  
15 vaccines comprising pili or pilus-like structures formed from heterogenous pilin subunits. The pilin subunits which form these pili or pilus-like structures can be expressed from genes naturally resident in the bacterial cell or may be introduced into the cells. When a naturally resident pilin gene and an introduced gene are both expressed in a cell which forms pili or pilus-like structures, the result will generally  
20 be structures formed from a mixture of these pilin proteins. Further, when two or more pilin genes are expressed in a bacterial cell, the relative expression of each pilin gene will typically be the factor which determines the ratio of the different pilin subunits in the pili or pilus-like structures.

When pili or pilus-like structures having a particular composition of mixed  
25 pilin subunits is desired, the expression of at least one of the pilin genes can be regulated by a heterologous, inducible promoter. Such promoters, as well as other genetic elements, can be used to regulate the relative amounts of different pilin subunits produced in the bacterial cell and, hence, the composition of the pili or pilus-like structures.

30 In additional, the antigen or antigenic determinant can be linked to bacterial pili or pilus-like structures by a bond which is not a peptide bond, bacterial cells

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which produce pili or pilus-like structures used in the compositions of the invention can be genetically engineered to generate pilin proteins which are fused to an antigen or antigenic determinant. Such fusion proteins which form pili or pilus-like structures are suitable for use in vaccine compositions of the invention.

5 Virus-like particles in the context of the present application refer to structures resembling a virus particle but which are not pathogenic. In general, virus-like particles lack the viral genome and, therefore, are noninfectious. Also, virus-like particles can be produced in large quantities by heterologous expression and can be easily purified.

10 In a preferred embodiment, the core particle is a virus-like particle, wherein the virus-like particle is a recombinant virus-like particle. The skilled artisan can produce VLPs using recombinant DNA technology and virus coding sequences which are readily available to the public. For example, the coding sequence of a virus envelope or core protein can be engineered for expression in a baculovirus  
15 expression vector using a commercially available baculovirus vector, under the regulatory control of a virus promoter, with appropriate modifications of the sequence to allow functional linkage of the coding sequence to the regulatory sequence. The coding sequence of a virus envelope or core protein can also be engineered for expression in a bacterial expression vector, for example.

20 Examples of VLPs include, but are not limited to, the capsid proteins of Hepatitis B virus (Ulrich, *et al.*, *Virus Res.* 50:141-182 (1998)), measles virus (Warnes, *et al.*, *Gene* 160:173-178 (1995)), Sindbis virus, rotavirus (US 5,071,651 and US 5,374,426), foot-and-mouth-disease virus (Twomey, *et al.*, *Vaccine* 13:1603-1610, (1995)), Norwalk virus (Jiang, X., *et al.*, *Science* 250:1580-1583  
25 (1990); Matsui, S.M., *et al.*, *J. Clin. Invest.* 87:1456-1461 (1991)), the retroviral GAG protein (WO 96/30523), the retrotransposon Ty protein p1, the surface protein of Hepatitis B virus (WO 92/11291), human papilloma virus (WO 98/15631), RNA phages, Ty, fr-phage, GA-phage, AP205-phage and Q $\beta$ -phage.

30 As will be readily apparent to those skilled in the art, the VLP of the invention is not limited to any specific form. The particle can be synthesized chemically or through a biological process, which can be natural or non-natural. By way of

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example, this type of embodiment includes a virus-like particle or a recombinant form thereof.

In a more specific embodiment, the VLP can comprise, or alternatively essentially consist of, or alternatively consist of recombinant polypeptides, or fragments thereof, being selected from recombinant polypeptides of Rotavirus, 5 recombinant polypeptides of Norwalk virus, recombinant polypeptides of Alphavirus, recombinant polypeptides of Foot and Mouth Disease virus, recombinant polypeptides of measles virus, recombinant polypeptides of Sindbis virus, recombinant polypeptides of Polyoma virus, recombinant polypeptides of Retrovirus, 10 recombinant polypeptides of Hepatitis B virus (*e.g.*, a HBcAg), recombinant polypeptides of Tobacco mosaic virus, recombinant polypeptides of Flock House Virus, recombinant polypeptides of human Papillomavirus, recombinant polypeptides of bacteriophages, recombinant polypeptides of RNA phages, recombinant polypeptides of Ty, recombinant polypeptides of fr-phage, recombinant 15 polypeptides of GA-phage and recombinant polypeptides of Q $\beta$ -phage. The virus-like particle can further comprise, or alternatively essentially consist of, or alternatively consist of, one or more fragments of such polypeptides, as well as variants of such polypeptides. Variants of polypeptides can share, for example, at least 80%, 85%, 90%, 95%, 97%, or 99% identity at the amino acid level with their 20 wild-type counterparts.

In a preferred embodiment, the virus-like particle comprises, consists essentially of, or alternatively consists of recombinant proteins, or fragments thereof, of a RNA-phage. Preferably, the RNA-phage is selected from the group consisting of a) bacteriophage Q $\beta$ ; b) bacteriophage R17; c) bacteriophage fr; d) bacteriophage 25 GA; e) bacteriophage SP; f) bacteriophage MS2; g) bacteriophage M11; h) bacteriophage MX1; i) bacteriophage NL95; k) bacteriophage f2; l) bacteriophage PP7, and m) bacteriophage AP205.

In another preferred embodiment of the present invention, the virus-like particle comprises, or alternatively consists essentially of, or alternatively consists of 30 recombinant proteins, or fragments thereof, of the RNA-bacteriophage Q $\beta$  or of the RNA-bacteriophage fr, or of the RNA-bacteriophage AP205.

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In a further preferred embodiment of the present invention, the recombinant proteins comprise, or alternatively consist essentially of, or alternatively consist of coat proteins of RNA phages.

RNA-phage coat proteins forming capsids or VLPs, or fragments of the bacteriophage coat proteins compatible with self-assembly into a capsid or a VLP, are, therefore, further preferred embodiments of the present invention. Bacteriophage Q $\beta$  coat proteins, for example, can be expressed recombinantly in *E. coli*. Further, upon such expression these proteins spontaneously form capsids. Additionally, these capsids form a structure with an inherent repetitive organization.

Specific preferred examples of bacteriophage coat proteins which can be used to prepare compositions of the invention include the coat proteins of RNA bacteriophages such as bacteriophage Q $\beta$  (SEQ ID NO:4; PIR Database, Accession No. VCBPQ $\beta$  referring to Q $\beta$  CP and SEQ ID NO: 5; Accession No. AAA16663 referring to Q $\beta$  A1 protein), bacteriophage R17 (SEQ ID NO:6; PIR Accession No. VCBPR7), bacteriophage fr (SEQ ID NO:7; PIR Accession No. VCBPFR), bacteriophage GA (SEQ ID NO:8; GenBank Accession No. NP-040754), bacteriophage SP (SEQ ID NO:9; GenBank Accession No. CAA30374 referring to SP CP and SEQ ID NO: 10; Accession No. NP 695026 referring to SP A1 protein), bacteriophage MS2 (SEQ ID NO:11; PIR Accession No. VCBPM2), bacteriophage M11 (SEQ ID NO:12; GenBank Accession No. AAC06250), bacteriophage MX1 (SEQ ID NO:13; GenBank Accession No. AAC14699), bacteriophage NL95 (SEQ ID NO:14; GenBank Accession No. AAC14704), bacteriophage f2 (SEQ ID NO: 15; GenBank Accession No. P03611), bacteriophage PP7 (SEQ ID NO: 16), and bacteriophage AP205 (SEQ ID NO: 28). Furthermore, the A1 protein of bacteriophage Q $\beta$  (SEQ ID NO: 5) or C-terminal truncated forms missing as much as 100, 150 or 180 amino acids from its C-terminus may be incorporated in a capsid assembly of Q $\beta$  coat proteins. Generally, the percentage of Q $\beta$  A1 protein relative to Q $\beta$  CP in the capsid assembly will be limited, in order to ensure capsid formation.

Q $\beta$  coat protein has also been found to self-assemble into capsids when expressed in *E. coli* (Kozlovska TM. *et al.*, *GENE* 137: 133-137 (1993)). The

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obtained capsids or virus-like particles showed an icosahedral phage-like capsid structure with a diameter of 25 nm and T=3 quasi symmetry. Further, the crystal structure of phage Q $\beta$  has been solved. The capsid contains 180 copies of the coat protein, which are linked in covalent pentamers and hexamers by disulfide bridges (Golmohammadi, R. *et al.*, *Structure 4*: 543-5554 (1996)) leading to a remarkable stability of the capsid of Q $\beta$  coat protein. Capsids or VLPs made from recombinant Q $\beta$  coat protein may contain, however, subunits not linked via disulfide links to other subunits within the capsid, or incompletely linked. However, typically more than about 80% of the subunits are linked via disulfide bridges to each other within the VLP. Thus, upon loading recombinant Q $\beta$  capsid on non-reducing SDS-PAGE, bands corresponding to monomeric Q $\beta$  coat protein as well as bands corresponding to the hexamer or pentamer of Q $\beta$  coat protein are visible. Incompletely disulfide-linked subunits could appear as dimer, trimer or even tetramer band in non-reducing SDS-PAGE. Q $\beta$  capsid protein also shows unusual resistance to organic solvents and denaturing agents. Surprisingly, we have observed that DMSO and acetonitrile concentrations as high as 30%, and Guanidinium concentrations as high as 1 M do not affect the stability of the capsid. The high stability of the capsid of Q $\beta$  coat protein is an advantageous feature, in particular, for its use in immunization and vaccination of mammals and humans in accordance of the present invention.

Upon expression in *E. coli*, the N-terminal methionine of Q $\beta$  coat protein is usually removed, as we observed by N-terminal Edman sequencing as described in Stoll, E. *et al.* *J. Biol. Chem.* 252:990-993 (1977). VLP composed from Q $\beta$  coat proteins where the N-terminal methionine has not been removed, or VLPs comprising a mixture of Q $\beta$  coat proteins where the N-terminal methionine is either cleaved or present are also within the scope of the present invention.

Further preferred virus-like particles of RNA-phages, in particular of Q $\beta$ , in accordance of this invention are disclosed in WO 02/056905, the disclosure of which is herewith incorporated by reference in its entirety.

Further RNA phage coat proteins have also been shown to self-assemble upon expression in a bacterial host (Kastelcin, RA. *et al.*, *Gene 23*: 245-254 (1983),



Kozlovskaya, TM. *et al.*, *Dokl. Akad. Nauk SSSR* 287: 452-455 (1986), Adhin, MR. *et al.*, *Virology* 170: 238-242 (1989), Ni, CZ., *et al.*, *Protein Sci.* 5: 2485-2493 (1996), Priano, C. *et al.*, *J. Mol. Biol.* 249: 283-297 (1995)). The Q $\beta$  phage capsid contains, in addition to the coat protein, the so called read-through protein A1 and the maturation protein A2. A1 is generated by suppression at the UGA stop codon and has a length of 329 aa. The capsid of phage Q $\beta$  recombinant coat protein used in the invention is devoid of the A2 lysis protein, and contains RNA from the host. The coat protein of RNA phages is an RNA binding protein, and interacts with the stem loop of the ribosomal binding site of the replicase gene acting as a translational repressor during the life cycle of the virus. The sequence and structural elements of the interaction are known (Witherell, GW. & Uhlenbeck, OC. *Biochemistry* 28: 71-76 (1989); Lim F. *et al.*, *J. Biol. Chem.* 271: 31839-31845 (1996)). The stem loop and RNA in general are known to be involved in the virus assembly (Golmohammadi, R. *et al.*, *Structure* 4: 543-5554 (1996)).

In a further preferred embodiment of the present invention, the virus-like particle comprises, or alternatively consists essentially of, or alternatively consists of recombinant proteins, or fragments thereof, of a RNA-phage, wherein the recombinant proteins comprise, alternatively consist essentially of or alternatively consist of mutant coat proteins of a RNA phage, preferably of mutant coat proteins of the RNA phages mentioned above. In another preferred embodiment, the mutant coat proteins of the RNA phage have been modified by removal of at least one, or alternatively at least two, lysine residue by way of substitution, or by addition of at least one lysine residue by way of substitution; alternatively, the mutant coat proteins of the RNA phage have been modified by deletion of at least one, or alternatively at least two, lysine residue, or by addition of at least one lysine residue by way of insertion. The deletion, substitution or addition of at least one lysine residue allows varying the degree of coupling, i.e. the amount of A $\beta$ 1-6 peptides per subunits of the VLP of the RNA-phages, in particular, to match and tailor the requirements of the vaccine.

In another preferred embodiment, the virus-like particle comprises, or alternatively consists essentially of, or alternatively consists of recombinant proteins,

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or fragments thereof, of the RNA-bacteriophage Q $\beta$ , wherein the recombinant proteins comprise, or alternatively consist essentially of, or alternatively consist of coat proteins having an amino acid sequence of SEQ ID NO:4, or a mixture of coat proteins having amino acid sequences of SEQ ID NO:4 and of SEQ ID NO: 5 or mutants of SEQ ID NO: 5 and wherein the N-terminal methionine is preferably cleaved.

In a further preferred embodiment of the present invention, the virus-like particle comprises, consists essentially of or alternatively consists of recombinant proteins of Q $\beta$ , or fragments thereof, wherein the recombinant proteins comprise, or alternatively consist essentially of, or alternatively consist of mutant Q $\beta$  coat proteins. In another preferred embodiment, these mutant coat proteins have been modified by removal of at least one lysine residue by way of substitution, or by addition of at least one lysine residue by way of substitution. Alternatively, these mutant coat proteins have been modified by deletion of at least one lysine residue, or by addition of at least one lysine residue by way of insertion.

Four lysine residues are exposed on the surface of the capsid of Q $\beta$  coat protein. Q $\beta$  mutants, for which exposed lysine residues are replaced by arginines can also be used for the present invention. The following Q $\beta$  coat protein mutants and mutant Q $\beta$  VLPs can, thus, be used in the practice of the invention: "Q $\beta$ -240" (Lys13-Arg; SEQ ID NO:17), "Q $\beta$ -243" (Asn 10-Lys; SEQ ID NO:18), "Q $\beta$ -250" (Lys 2-Arg, Lys13-Arg; SEQ ID NO:19), "Q $\beta$ -251" (SEQ ID NO:20) and "Q $\beta$ -259" (Lys 2-Arg, Lys16-Arg; SEQ ID NO:21). Thus, in further preferred embodiment of the present invention, the virus-like particle comprises, consists essentially of or alternatively consists of recombinant proteins of mutant Q $\beta$  coat proteins, which comprise proteins having an amino acid sequence selected from the group of a) the amino acid sequence of SEQ ID NO:17; b) the amino acid sequence of SEQ ID NO:18; c) the amino acid sequence of SEQ ID NO:19; d) the amino acid sequence of SEQ ID NO:20; and e) the amino acid sequence of SEQ ID NO:21. The construction, expression and purification of the above indicated Q $\beta$  coat proteins, mutant Q $\beta$  coat

protein VLPs and capsids, respectively, are described in WO 02/056905. In particular is hereby referred to Example 18 of above mentioned application.

In a further preferred embodiment of the present invention, the virus-like particle comprises, or alternatively consists essentially of, or alternatively consists of recombinant proteins of Q $\beta$ , or fragments thereof, wherein the recombinant proteins  
5 comprise, consist essentially of or alternatively consist of a mixture of either one of the foregoing Q $\beta$  mutants and the corresponding A1 protein.

In a further preferred embodiment of the present invention, the virus-like particle comprises, or alternatively essentially consists of, or alternatively consists of recombinant proteins, or fragments thereof, of RNA-phage AP205.  
10

The AP205 genome consists of a maturation protein, a coat protein, a replicase and two open reading frames not present in related phages; a lysis gene and an open reading frame playing a role in the translation of the maturation gene (Klovins, J., *et al.*, *J. Gen. Virol.* 83: 1523-33 (2002)). AP205 coat protein can be expressed from plasmid pAP283-58 (SEQ ID NO: 27), which is a derivative of pQb10 (Kozlovskaja, T. M., *et al.*, *Gene* 137:133-37 (1993)), and which contains an AP205 ribosomal binding site. Alternatively, AP205 coat protein may be cloned into pQb185, downstream of the ribosomal binding site present in the vector. Both approaches lead to expression of the protein and formation of capsids as described in the co-pending  
15 US provisional patent application with the title "Molecular Antigen Arrays" and having filed by the present assignee on July 17, 2002, which is incorporated by reference in its entirety. Vectors pQb10 and pQb185 are vectors derived from pGEM vector, and expression of the cloned genes in these vectors is controlled by the *trp* promoter (Kozlovskaja, T. M., *et al.*, *Gene* 137:133-37 (1993)). Plasmid pAP283-58  
20 (SEQ ID NO:27) comprises a putative AP205 ribosomal binding site in the following sequence, which is downstream of the XbaI site, and immediately upstream of the ATG start codon of the AP205 coat protein: *tctagaATTTTCTGCGCACCCAT CCCGGGTGGCGCCCAA-GTGAGGAAAATCACatg* (SEQ ID NO:57). The vector pQb185 comprises a Shine-Dalgarno sequence downstream from the XbaI site  
25 and upstream of the start codon

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(*tctaga*TTAACCCAACGCGTAGGAGTCAGGCC*catg* (SEQ ID NO:58), Shine-Dalgarno sequence underlined).

In a further preferred embodiment of the present invention, the virus-like particle comprises, or alternatively essentially consists of, or alternatively consists of recombinant coat proteins, or fragments thereof, of the RNA-phage AP205.

This preferred embodiment of the present invention, thus, comprises AP205 coat proteins that form capsids. Such proteins are recombinantly expressed or prepared from natural sources. AP205 coat proteins produced in bacteria spontaneously form capsids, as evidenced by Electron Microscopy (EM) and immunodiffusion. The structural properties of the capsid formed by the AP205 coat protein (SEQ ID NO: 28) and those formed by the coat protein of the AP205 RNA phage are nearly indistinguishable when seen in EM. AP205 VLPs are highly immunogenic, and can be linked with antigens and/or antigenic determinants to generate vaccine constructs displaying the antigens and/or antigenic determinants oriented in a repetitive manner. High titers are elicited against the so displayed antigens showing that bound antigens and/or antigenic determinants are accessible for interacting with antibody molecules and are immunogenic.

In a further preferred embodiment of the present invention, the virus-like particle comprises, or alternatively essentially consists of, or alternatively consists of recombinant mutant coat proteins, or fragments thereof, of the RNA-phage AP205.

Assembly-competent mutant forms of AP205 VLPs, including AP205 coat protein with the substitution of proline at amino acid 5 to threonine (SEQ ID NO: 29), may also be used in the practice of the invention and leads to a further preferred embodiment of the invention. These VLPs, AP205 VLPs derived from natural sources, or AP205 viral particles, may be bound to antigens to produce ordered repetitive arrays of the antigens in accordance with the present invention.

AP205 P5-T mutant coat protein can be expressed from plasmid pAP281-32 (SEQ ID No. 30), which is derived directly from pQb185, and which contains the mutant AP205 coat protein gene instead of the Q $\beta$  coat protein gene. Vectors for expression of the AP205 coat protein are transfected into *E. coli* for expression of the AP205 coat protein.

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Methods for expression of the coat protein and the mutant coat protein, respectively, leading to the self-assembly into VLPs are described in Examples 20 and 21. Suitable *E. coli* strains include, but are not limited to, *E. coli* K802, JM 109, RR1. Suitable vectors and strains and combinations thereof can be identified by  
5 testing expression of the coat protein and mutant coat protein, respectively, by SDS-PAGE and capsid formation and assembly by optionally first purifying the capsids by gel filtration and subsequently testing them in an immunodiffusion assay (Ouchterlony test) or Electron Microscopy (Kozłowska, T. M., *et al.*, *Gene* 137:133-37 (1993)).

10 AP205 coat proteins expressed from the vectors pAP283-58 and pAP281-32 may be devoid of the initial Methionine amino-acid, due to processing in the cytoplasm of *E. coli*. Cleaved, uncleaved forms of AP205 VLP or mixtures thereof are further preferred embodiments of the invention.

In a further preferred embodiment of the present invention, the virus-like  
15 particle comprises, or alternatively essentially consists of, or alternatively consists of a mixture of recombinant coat proteins, or fragments thereof, of the RNA-phage AP205 and of recombinant mutant coat proteins, or fragments thereof, of the RNA-phage AP205.

In a further preferred embodiment of the present invention, the virus-like  
20 particle comprises, or alternatively essentially consists of, or alternatively consists of fragments of recombinant coat proteins or recombinant mutant coat proteins of the RNA-phage AP205.

Recombinant AP205 coat protein fragments capable of assembling into a VLP and a capsid, respectively are also useful in the practice of the invention. These  
25 fragments may be generated by deletion, either internally or at the termini of the coat protein and mutant coat protein, respectively. Insertions in the coat protein and mutant coat protein sequence or fusions of antigen sequences to the coat protein and mutant coat protein sequence, and compatible with assembly into a VLP, are further  
30 embodiments of the invention and lead to chimeric AP205 coat proteins, and particles, respectively. The outcome of insertions, deletions and fusions to the coat

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protein sequence and whether it is compatible with assembly into a VLP can be determined by electron microscopy.

The particles formed by the AP205 coat protein, coat protein fragments and chimeric coat proteins described above, can be isolated in pure form by a combination of fractionation steps by precipitation and of purification steps by gel  
5 filtration using *e.g.* Sepharose CL-4B, Sepharose CL-2B, Sepharose CL-6B columns and combinations thereof. Other methods of isolating virus-like particles are known in the art, and may be used to isolate the virus-like particles (VLPs) of bacteriophage AP205. For example, the use of ultracentrifugation to isolate VLPs of the yeast  
10 retrotransposon Ty is described in U.S. Patent No. 4,918,166, which is incorporated by reference herein in its entirety.

The crystal structure of several RNA bacteriophages has been determined (Golmohammadi, R. *et al.*, *Structure* 4:543-554 (1996)). Using such information, surface exposed residues can be identified and, thus, RNA-phage coat proteins can  
15 be modified such that one or more reactive amino acid residues can be inserted by way of insertion or substitution. As a consequence, those modified forms of bacteriophage coat proteins can also be used for the present invention. Thus, variants of proteins which form capsids or capsid-like structures (*e.g.*, coat proteins of  
20 bacteriophage Q $\beta$ , bacteriophage R17, bacteriophage fr, bacteriophage GA, bacteriophage SP, bacteriophage AP205, and bacteriophage MS2) can also be used to prepare compositions of the present invention.

Although the sequence of the variants proteins discussed above will differ from their wild-type counterparts, these variant proteins will generally retain the ability to form capsids or capsid-like structures. Thus, the invention further includes  
25 compositions and vaccine compositions, respectively, which further includes variants of proteins which form capsids or capsid-like structures, as well as methods for preparing such compositions and vaccine compositions, respectively, individual protein subunits used to prepare such compositions, and nucleic acid molecules which encode these protein subunits. Thus, included within the scope of the  
30 invention are variant forms of wild-type proteins which form capsids or capsid-like

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structures and retain the ability to associate and form capsids or capsid-like structures.

As a result, the invention further includes compositions and vaccine compositions, respectively, comprising proteins, which comprise, or alternatively  
5 consist essentially of, or alternatively consist of amino acid sequences which are at least 80%, 85%, 90%, 95%, 97%, or 99% identical to wild-type proteins which form ordered arrays and having an inherent repetitive structure, respectively.

Further included within the scope of the invention are nucleic acid molecules which encode proteins used to prepare compositions of the present invention.

10 In other embodiments, the invention further includes compositions comprising proteins, which comprise, or alternatively consist essentially of, or alternatively consist of amino acid sequences which are at least 80%, 85%, 90%, 95%, 97%, or 99% identical to any of the amino acid sequences shown in SEQ ID NOs:4-21.

Proteins suitable for use in the present invention also include C-terminal  
15 truncation mutants of proteins which form capsids or capsid-like structures, or VLPs. Specific examples of such truncation mutants include proteins having an amino acid sequence shown in any of SEQ ID NOs:4-21 where 1, 2, 5, 7, 9, 10, 12, 14, 15, or 17 amino acids have been removed from the C-terminus. Typically, these C-terminal truncation mutants will retain the ability to form capsids or capsid-like structures.

20 Further proteins suitable for use in the present invention also include N-terminal truncation mutants of proteins which form capsids or capsid-like structures. Specific examples of such truncation mutants include proteins having an amino acid sequence shown in any of SEQ ID NOs:4-21 where 1, 2, 5, 7, 9, 10, 12, 14, 15, or 17 amino acids have been removed from the N-terminus. Typically, these  
25 N-terminal truncation mutants will retain the ability to form capsids or capsid-like structures.

Additional proteins suitable for use in the present invention include N- and C-terminal truncation mutants which form capsids or capsid-like structures. Suitable truncation mutants include proteins having an amino acid sequence shown in any of  
30 SEQ ID NOs:4-21 where 1, 2, 5, 7, 9, 10, 12, 14, 15, or 17 amino acids have been removed from the N-terminus and 1, 2, 5, 7, 9, 10, 12, 14, 15, or 17 amino acids have

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been removed from the C-terminus. Typically, these N-terminal and C-terminal truncation mutants will retain the ability to form capsids or capsid-like structures.

The invention further includes compositions comprising proteins which comprise, or alternatively consist essentially of, or alternatively consist of, amino acid sequences which are at least 80%, 85%, 90%, 95%, 97%, or 99% identical to the above described truncation mutants.

The invention thus includes compositions and vaccine compositions prepared from proteins which form capsids or VLPs, methods for preparing these compositions from individual protein subunits and VLPs or capsids, methods for preparing these individual protein subunits, nucleic acid molecules which encode these subunits, and methods for vaccinating and/or eliciting immunological responses in individuals using these compositions of the present invention.

In one embodiment, the invention provides a vaccine composition of the invention further comprising an adjuvant. In another embodiment, the vaccine composition of is devoid of an adjuvant. In a further embodiment of the invention, the vaccine composition comprises a core particle of the invention, wherein the core particle comprises, preferably is, a virus-like particle, wherein preferably said virus-like particle is a recombinant virus-like particle. Preferably, the virus-like particle comprises, or alternatively consist essentially of, or alternatively consists of, recombinant proteins, or fragments thereof, of a RNA-phage, preferably of coat proteins of RNA phages,. In a preferred embodiment, the coat protein of the RNA phages has an amino acid are selected from the group consisting of: (a) SEQ ID NO:4; (b) a mixture of SEQ ID NO:4 and SEQ ID NO:5; (c) SEQ ID NO:6; (d) SEQ ID NO:7; (e) SEQ ID NO:8; (f) SEQ ID NO:9; (g) a mixture of SEQ ID NO:9 and SEQ ID NO:10; (h) SEQ ID NO:11; (i) SEQ ID NO:12; (k) SEQ ID NO:13; (l) SEQ ID NO:14; (m) SEQ ID NO:15; (n) SEQ ID NO:16; and (o) SEQ ID NO:28. Alternatively, the recombinant proteins of the virus-like particle of the vaccine composition of the invention comprise, or alternatively consist essentially of, or alternatively consist of mutant coat proteins of RNA phages, wherein the RNA-phage is selected from the group consisting of: (a) bacteriophage Q $\beta$ ; (b) bacteriophage R17; (c)



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bacteriophage fr; (d) bacteriophage GA; (e) bacteriophage SP; (f) bacteriophage MS2; (g) bacteriophage M11; (h) bacteriophage MX1; (i) bacteriophage NL95; (k) bacteriophage f2; (l) bacteriophage PP7; and (m) bacteriophage AP205.

5 In a preferred embodiment, the mutant coat proteins of said RNA phage have been modified by removal, or by addition of at least one lysine residue by way of substitution. In another preferred embodiment, the mutant coat proteins of said RNA phage have been modified by deletion of at least one lysine residue or by addition of at least one lysine residue by way of insertion. In a preferred embodiment, the virus-  
10 like particle comprises recombinant proteins or fragments thereof, of RNA-phage Q $\beta$ , or alternatively of RNA-phage fr, or of RNA-phage AP205.

As previously stated, the invention includes virus-like particles or recombinant forms thereof. In one further preferred embodiment, the particles used in compositions of the invention are composed of a Hepatitis B core protein (HBcAg) or a fragment of a HBcAg. In a further embodiment, the particles used in  
15 compositions of the invention are composed of a Hepatitis B core protein (HBcAg) or a fragment of a HBcAg protein, which has been modified to either eliminate or reduce the number of free cysteine residues. Zhou *et al.* (*J. Virol.* 66:5393-5398 (1992)) demonstrated that HBcAgs which have been modified to remove the  
20 naturally resident cysteine residues retain the ability to associate and form capsids. Thus, VLPs suitable for use in compositions of the invention include those comprising modified HBcAgs, or fragments thereof, in which one or more of the naturally resident cysteine residues have been either deleted or substituted with another amino acid residue (*e.g.*, a serine residue).

25 The HBcAg is a protein generated by the processing of a Hepatitis B core antigen precursor protein. A number of isotypes of the HBcAg have been identified and their amino acids sequences are readily available to those skilled in the art. In most instances, compositions and vaccine compositions, respectively, of the invention will be prepared using the processed form of a HBcAg (*i.e.*, an HBcAg  
30 from which the N-terminal leader sequence of the Hepatitis B core antigen precursor protein has been removed).

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Further, when HBcAgs are produced under conditions where processing will not occur, the HBcAgs will generally be expressed in "processed" form. For example, when an *E. coli* expression system directing expression of the protein to the cytoplasm is used to produce HBcAgs of the invention, these proteins will generally  
5 be expressed such that the N-terminal leader sequence of the Hepatitis B core antigen precursor protein is not present.

The preparation of Hepatitis B virus-like particles, which can be used for the present invention, is disclosed, for example, in WO 00/32227, and hereby in particular in Examples 17 to 19 and 21 to 24, as well as in WO 01/85208, and hereby  
10 in particular in Examples 17 to 19, 21 to 24, 31 and 41, and in WO 02/056905. For the latter application, it is in particular referred to Example 23, 24, 31 and 51. All three documents are explicitly incorporated herein by reference.

The present invention also includes HBcAg variants which have been modified to delete or substitute one or more additional cysteine residues. It is known in the art  
15 that free cysteine residues can be involved in a number of chemical side reactions. These side reactions include disulfide exchanges, reaction with chemical substances or metabolites that are, for example, injected or formed in a combination therapy with other substances, or direct oxidation and reaction with nucleotides upon  
20 exposure to UV light. Toxic adducts could thus be generated, especially considering the fact that HBcAgs have a strong tendency to bind nucleic acids. The toxic adducts would thus be distributed between a multiplicity of species, which individually may each be present at low concentration, but reach toxic levels when together.

In view of the above, one advantage to the use of HBcAgs in vaccine compositions which have been modified to remove naturally resident cysteine  
25 residues is that sites to which toxic species can bind when antigens or antigenic determinants are attached would be reduced in number or eliminated altogether.

A number of naturally occurring HBcAg variants suitable for use in the practice of the present invention have been identified. Yuan *et al.*, (*J. Virol.* 73:10122-10128 (1999)), for example, describe variants in which the isoleucine  
30 residue at position corresponding to position 97 in SEQ ID NO:22 is replaced with either a leucine residue or a phenylalanine residue. The amino acid sequences of a

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number of HBcAg variants, as well as several Hepatitis B core antigen precursor variants, are disclosed in GenBank reports AAF121240, AF121239, X85297, X02496, X85305, X85303, AF151735, X85259, X85286, X85260, X85317, X85298, AF043593, M20706, X85295, X80925, X85284, X85275, X72702, X85291, X65258, X85302, M32138, X85293, X85315, U95551, X85256, X85316, X85296, AB033559, X59795, X85299, X85307, X65257, X85311, X85301 (SEQ ID NO:23), X85314, X85287, X85272, X85319, AB010289, X85285, AB010289, AF121242, M90520 (SEQ ID NO:24), P03153, AF110999, and M95589, the disclosures of each of which are incorporated herein by reference. The sequences of the hereinabove mentioned Hepatitis B core antigen precursor variants are further disclosed in WO 01/85208 in SEQ ID NOs: 89 – 138. These HBcAg variants differ in amino acid sequence at a number of positions, including amino acid residues which corresponds to the amino acid residues located at positions 12, 13, 21, 22, 24, 29, 32, 33, 35, 38, 40, 42, 44, 45, 49, 51, 57, 58, 59, 64, 66, 67, 69, 74, 77, 80, 81, 87, 92, 93, 97, 98, 100, 103, 105, 106, 109, 113, 116, 121, 126, 130, 133, 135, 141, 147, 149, 157, 176, 178, 182 and 183 in SEQ ID NO:25. Further HBcAg variants suitable for use in the compositions of the invention, and which may be further modified according to the disclosure of this specification are described in WO 00/198333, WO 00/177158 and WO 00/214478.

As noted above, generally processed HBcAgs (*i.e.*, those which lack leader sequences) will be used in the compositions and vaccine compositions, respectively, of the invention. The present invention includes vaccine compositions, as well as methods for using these compositions, which employ the above described variant HBcAgs.

Whether the amino acid sequence of a polypeptide has an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97% or 99% identical to one of the above wild-type amino acid sequences, or a subportion thereof, can be determined conventionally using known computer programs such the Bestfit program. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference amino acid sequence, the parameters are set such that the percentage of identity is calculated

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over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

The amino acid sequences of the hereinabove mentioned HBcAg variants and precursors are relatively similar to each other. Thus, reference to an amino acid residue of a HBcAg variant located at a position which corresponds to a particular position in SEQ ID NO:25, refers to the amino acid residue which is present at that position in the amino acid sequence shown in SEQ ID NO:25. The homology between these HBcAg variants is for the most part high enough among Hepatitis B viruses that infect mammals so that one skilled in the art would have little difficulty reviewing both the amino acid sequence shown in SEQ ID NO:25 and that of a particular HBcAg variant and identifying "corresponding" amino acid residues. Furthermore, the HBcAg amino acid sequence shown in SEQ ID NO:24, which shows the amino acid sequence of a HBcAg derived from a virus which infect woodchucks, has enough homology to the HBcAg having the amino acid sequence shown in SEQ ID NO:25 that it is readily apparent that a three amino acid residue insert is present in SEQ ID NO:23 between amino acid residues 155 and 156 of SEQ ID NO:25.

The invention also includes vaccine compositions which comprise HBcAg variants of Hepatitis B viruses which infect birds, as well as vaccine compositions which comprise fragments of these HBcAg variants. For these HBcAg variants one, two, three or more of the cysteine residues naturally present in these polypeptides could be either substituted with another amino acid residue or deleted prior to their inclusion in vaccine compositions of the invention.

As discussed above, the elimination of free cysteine residues reduces the number of sites where toxic components can bind to the HBcAg, and also eliminates sites where cross-linking of lysine and cysteine residues of the same or of neighboring HBcAg molecules can occur. Therefore, in another embodiment of the present invention, one or more cysteine residues of the Hepatitis B virus capsid protein have been either deleted or substituted with another amino acid residue.

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In other embodiments, compositions and vaccine compositions, respectively, of the invention will contain HBcAgs from which the C-terminal region (*e.g.*, amino acid residues 145-185 or 150-185 of SEQ ID NO:25) has been removed. Thus, additional modified HBcAgs suitable for use in the practice of the present invention include C-terminal truncation mutants. Suitable truncation mutants include HBcAgs where 1, 5, 10, 15, 20, 25, 30, 34, 35, amino acids have been removed from the C-terminus.

HBcAgs suitable for use in the practice of the present invention also include N-terminal truncation mutants. Suitable truncation mutants include modified HBcAgs where 1, 2, 5, 7, 9, 10, 12, 14, 15, or 17 amino acids have been removed from the N-terminus.

Further HBcAgs suitable for use in the practice of the present invention include N- and C-terminal truncation mutants. Suitable truncation mutants include HBcAgs where 1, 2, 5, 7, 9, 10, 12, 14, 15, and 17 amino acids have been removed from the N-terminus and 1, 5, 10, 15, 20, 25, 30, 34, 35 amino acids have been removed from the C-terminus.

The invention further includes compositions and vaccine compositions, respectively, comprising HBcAg polypeptides comprising, or alternatively essentially consisting of, or alternatively consisting of, amino acid sequences which are at least 80%, 85%, 90%, 95%, 97%, or 99% identical to the above described truncation mutants.

In certain embodiments of the invention, a lysine residue is introduced into a HBcAg polypeptide, to mediate the binding of ghrelin or ghrelin peptide to the VLP of HBcAg. In preferred embodiments, compositions of the invention are prepared using a HBcAg comprising, or alternatively consisting of, amino acids 1-144, or 1-149, 1-185 of SEQ ID NO:25, which is modified so that the amino acids corresponding to positions 79 and 80 are replaced with a peptide having the amino acid sequence of Gly-Gly-Lys-Gly-Gly (SEQ ID NO:33) resulting in the HBcAg polypeptide having the sequence shown in SEQ ID NO: 26). In further preferred embodiments, the cysteine residues at positions 48 and 107 of SEQ ID NO:25 are mutated to serine. The invention further includes compositions comprising the

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corresponding polypeptides having amino acid sequences shown in any of the hereinabove mentioned Hepatitis B core antigen precursor variants, which also have above noted amino acid alterations. Further included within the scope of the invention are additional HBcAg variants which are capable of associating to form a capsid or VLP and have the above noted amino acid alterations. Thus, the invention further includes compositions and vaccine compositions, respectively, comprising HBcAg polypeptides which comprise, or alternatively consist of, amino acid sequences which are at least 80%, 85%, 90%, 95%, 97% or 99% identical to any of the wild-type amino acid sequences, and forms of these proteins which have been processed, where appropriate, to remove the N-terminal leader sequence and modified with above noted alterations.

Compositions or vaccine compositions of the invention may comprise mixtures of different HBcAgs. Thus, these vaccine compositions may be composed of HBcAgs which differ in amino acid sequence. For example, vaccine compositions could be prepared comprising a "wild-type" HBcAg and a modified HBcAg in which one or more amino acid residues have been altered (*e.g.*, deleted, inserted or substituted). Further, preferred vaccine compositions of the invention are those which present highly ordered and repetitive antigen array, wherein the antigen is ghrelin or a ghrelin peptide.

In a further preferred embodiment of the present invention, the at least one ghrelin or ghrelin peptide is bound to said core particle and virus-like particle, respectively, by at least one covalent bond. Preferably, the least one ghrelin or ghrelin peptide is bound to the core particle and virus-like particle, respectively, by at least one covalent bond, said covalent bond being a non-peptide bond leading to a core particle-ghrelin ordered and repetitive array and a ghrelin-VLP-array or -conjugate, respectively. This ghrelin-VLP array and conjugate, respectively, has typically and preferably a repetitive and ordered structure since the at least one, but usually more than one, ghrelin or a ghrelin peptide is bound to the VLP and core particle, respectively, in an oriented manner. Preferably, more than, 120, more than 180, preferably more than 270, and preferably more than 360 ghrelin or ghrelin peptides are bound to the VLP. The formation of a repetitive and ordered ghrelin-

VLP and core particle, respectively, -array and conjugate, respectively, is ensured by an oriented and directed as well as defined binding and attachment, respectively, of the at least one ghrelin or ghrelin peptide to the VLP and core particle, respectively, as will become apparent in the following. Furthermore, the typical inherent highly  
5 repetitive and organized structure of the VLPs and core particles, respectively, advantageously contributes to the display of the ghrelin or ghrelin peptide in a highly ordered and repetitive fashion leading to a highly organized and repetitive ghrelin-VLP/core particle array and conjugate, respectively.

Therefore, the preferred inventive conjugates and arrays, respectively, differ  
10 from prior art conjugates in their highly organized structure, dimensions, and in the repetitiveness of the antigen on the surface of the array. The preferred embodiment of this invention, furthermore, allows expression of both the particle and the antigen in an expression host guaranteeing proper folding of the antigen, i.e. the at least one ghrelin or ghrelin peptide, and proper folding and assembly of the VLP.

15 The present invention discloses methods of binding of ghrelin or ghrelin peptide to core particles and VLPs, respectively. As indicated, in one aspect of the invention, the ghrelin or ghrelin peptide is bound to the core particle and VLP, respectively, by way of chemical cross-linking, typically and preferably by using a heterobifunctional cross-linker. Several hetero-bifunctional cross-linkers are known  
20 to the art. In preferred embodiments, the hetero-bifunctional cross-linker contains a functional group which can react with preferred first attachment sites, i.e. with the side-chain amino group of lysine residues of the core particle and the VLP or at least one VLP subunit, respectively, and a further functional group which can react with a preferred second attachment site, i.e. a cysteine residue naturally present, made  
25 available for reaction by reduction, or engineered on the ghrelin or ghrelin peptide, and optionally also made available for reaction by reduction. The first step of the procedure, typically called the derivatization, is the reaction of the core particle or the VLP with the cross-linker. The product of this reaction is an activated core particle or activated VLP, also called activated carrier. In the second step, unreacted  
30 cross-linker is removed using usual methods such as gel filtration or dialysis. In the third step, the ghrelin or ghrelin peptide is reacted with the activated carrier, and this

step is typically called the coupling step. Unreacted ghrelin or ghrelin peptide may be optionally removed in a fourth step, for example by dialysis. Several hetero-bifunctional cross-linkers are known to the art. These include the preferred cross-linkers SMPH (Pierce), Sulfo-MBS, Sulfo-EMCS, Sulfo-GMBS, Sulfo-SIAB, Sulfo-SMPB, Sulfo-SMCC, SVSB, SIA and other cross-linkers available for example  
5 from the Pierce Chemical Company (Rockford, IL, USA), and having one functional group reactive towards amino groups and one functional group reactive towards cysteine residues. The above mentioned cross-linkers all lead to formation of an amide bond after reaction with the amino group and a thioether linkage with the  
10 cysteine. Another class of cross-linkers suitable in the practice of the invention is characterized by the introduction of a disulfide linkage between the ghrelin or ghrelin peptide and the core particle or VLP upon coupling. Preferred cross-linkers belonging to this class include for example SPDP and Sulfo-LC-SPDP (Pierce). The extent of derivatization of the core particle and VLP, respectively, with cross-linker  
15 can be influenced by varying experimental conditions such as the concentration of each of the reaction partners, the excess of one reagent over the other, the pH, the temperature and the ionic strength. The degree of coupling, i.e. the amount of ghrelin or ghrelin peptides per subunits of the core particle and VLP, respectively, can be adjusted by varying the experimental conditions described above to match the  
20 requirements of the vaccine. Solubility of the ghrelin or ghrelin peptide may impose a limitation on the amount of ghrelin or ghrelin peptide that can be coupled on each subunit and in those cases where the obtained vaccine would be insoluble reducing the amount of ghrelin or ghrelin peptide per subunit is beneficial.

A particularly favored method of binding of ghrelin or ghrelin peptide to the  
25 core particle and the VLP, respectively, is the linking of a lysine residue on the surface of the core particle and the VLP, respectively, with a cysteine residue on the ghrelin or ghrelin peptide. Thus, in a preferred embodiment of the present invention, the first attachment site is a lysine residue and the second attachment site is a cysteine residue. In some embodiments, engineering of an amino acid linker  
30 containing a cysteine residue, as a second attachment site or as a part thereof, to the ghrelin or ghrelin peptide for coupling to the core particle and VLP, respectively,



may be required. Alternatively, a cysteine may be introduced either by insertion or mutation within the ghrelin or ghrelin peptide. Alternatively, the cysteine residue may be introduced by chemical coupling.

The selection of the amino acid linker will be dependent on the nature of the antigen and self-antigen, respectively, i.e. on the nature of the ghrelin or ghrelin peptide, on its biochemical properties, such as pI, charge distribution and glycosylation. In general, flexible amino acid linkers are favored. Preferred embodiments of the amino acid linker are selected from the group consisting of: (a) CGG; (b) N-terminal gamma 1-linker; (c) N-terminal gamma 3-linker; (d) Ig hinge regions; (e) N-terminal glycine linkers; (f)  $(G)_kC(G)_n$  with  $n=0-12$  and  $k=0-5$  (SEQ ID NO: 34); (g) N-terminal glycine-serine linkers; (h)  $(G)_kC(G)_m(S)_l(GGGGS)_n$  with  $n=0-3$ ,  $k=0-5$ ,  $m=0-10$ ,  $l=0-2$  (SEQ ID NO: 35); (i) GGC; (k) GGC-NH<sub>2</sub>; (l) C-terminal gamma 1-linker; (m) C-terminal gamma 3-linker; (n) C-terminal glycine linkers; (o)  $(G)_nC(G)_k$  with  $n=0-12$  and  $k=0-5$  (SEQ ID NO: 36); (p) C-terminal glycine-serine linkers; (q)  $(G)_m(S)_l(GGGGS)_n(G)_oC(G)_k$  with  $n=0-3$ ,  $k=0-5$ ,  $m=0-10$ ,  $l=0-2$ , and  $o=0-8$  (SEQ ID NO: 37).

Further preferred examples of amino acid linkers are the hinge region of Immunoglobulins, glycine serine linkers  $(GGGGS)_n$  (SEQ ID NO: 38), and glycine linkers  $(G)_n$  all further containing a cysteine residue as second attachment site and optionally further glycine residues. Typically preferred examples of said amino acid linkers are N-terminal gamma 1: CGDKTHTSPP (SEQ ID NO: 39); C-terminal gamma 1: DKTHTSPPCG (SEQ ID NO: 40); N-terminal gamma 3: CGGPKPSTPPGSSGGAP (SEQ ID NO: 41); C-terminal gamma 3: PKPSTPPGSSGGAPGGCG (SEQ ID NO: 42); N-terminal glycine linker: GCGGGG (SEQ ID NO: 43); C-terminal glycine linker: GGGGCG (SEQ ID NO: 44); C-terminal glycine-lysine linker: GGKKGC (SEQ ID NO: 45); N-terminal glycine-lysine linker: CGKKGG (SEQ ID NO: 46).

In a further preferred embodiment of the present invention, and in particular if the antigen is a ghrelin peptide, GGCG (SEQ ID NO: 47), GGC or GGC-NH<sub>2</sub> ("NH<sub>2</sub>" stands for amidation) linkers at the C-terminus of the peptide or CGG at its N-terminus are preferred as amino acid linkers. In general, glycine residues will be

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inserted between bulky amino acids and the cysteine to be used as second attachment site, to avoid potential steric hindrance of the bulkier amino acid in the coupling reaction.

5 The cysteine residue present on the ghrelin or ghrelin peptide has to be in its reduced state to react with the hetero-bifunctional cross-linker on the activated VLP, that is a free cysteine or a cysteine residue with a free sulfhydryl group has to be available. In the instance where the cysteine residue to function as binding site is in an oxidized form, for example if it is forming a disulfide bridge, reduction of this disulfide bridge with *e.g.* DTT, TCEP or  $\beta$ -mercaptoethanol is required.

10 Binding of the ghrelin or ghrelin peptide to the core particle and VLP, respectively, by using a hetero-bifunctional cross-linker according to the preferred methods described above, allows coupling of the ghrelin or ghrelin peptide to the core particle and the VLP, respectively, in an oriented fashion. Other methods of binding the ghrelin or ghrelin peptide to the core particle and the VLP, respectively,  
15 include methods wherein the ghrelin or ghrelin peptide is cross-linked to the core particle and the VLP, respectively, using the carbodiimide EDC, and NHS. The ghrelin or ghrelin peptide may also be first thiolated through reaction, for example with SATA, SATP or iminothiolane. The ghrelin or ghrelin peptide, after deprotection if required, may then be coupled to the core particle and the VLP,  
20 respectively, as follows. After separation of the excess thiolation reagent, the ghrelin or ghrelin peptide is reacted with the core particle and the VLP, respectively, previously activated with a hetero-bifunctional cross-linker comprising a cysteine reactive moiety, and therefore displaying at least one or several functional groups reactive towards cysteine residues, to which the thiolated ghrelin or ghrelin peptide  
25 can react, such as described above. Optionally, low amounts of a reducing agent are included in the reaction mixture. In further methods, the ghrelin or ghrelin peptide is attached to the core particle and the VLP, respectively, using a homo-bifunctional cross-linker such as glutaraldehyde, DSG, BM[PEO]<sub>4</sub>, BS<sup>3</sup>, (Pierce Chemical Company, Rockford, IL, USA) or other known homo-bifunctional cross-linkers with  
30 functional groups reactive towards amine groups or carboxyl groups of the core particle and the VLP, respectively,.

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Other methods of binding the VLP to a ghrelin or ghrelin peptide include methods where the core particle and the VLP, respectively, is biotinylated, and the ghrelin or ghrelin peptide expressed as a streptavidin-fusion protein, or methods wherein both the ghrelin or ghrelin peptides and the core particle and the VLP, respectively, are biotinylated, for example as described in WO 00/23955. In this case, the ghrelin or ghrelin peptide may be first bound to streptavidin or avidin by adjusting the ratio of ghrelin or ghrelin peptide to streptavidin such that free binding sites are still available for binding of the core particle and the VLP, respectively, which is added in the next step. Alternatively, all components may be mixed in a “one pot” reaction. Other ligand-receptor pairs, where a soluble form of the receptor and of the ligand is available, and are capable of being cross-linked to the core particle and the VLP, respectively, or the ghrelin or ghrelin peptide, may be used as binding agents for binding the ghrelin or ghrelin peptide to the core particle and the VLP, respectively. Alternatively, either the ligand or the receptor may be fused to the ghrelin or ghrelin peptide, and so mediate binding to the core particle and the VLP, respectively, chemically bound or fused either to the receptor, or the ligand respectively. Fusion may also be effected by insertion or substitution.

As already indicated, in a favored embodiment of the present invention, the VLP is the VLP of a RNA phage, and in a more preferred embodiment, the VLP is the VLP of RNA phage Q $\beta$  coat protein.

One or several antigen molecules, i.e. a ghrelin or a ghrelin peptide, can be attached to one subunit of the capsid or VLP of RNA phages coat proteins, preferably through the exposed lysine residues of the VLP of RNA phages, if sterically allowable. A specific feature of the VLP of the coat protein of RNA phages and in particular of the Q $\beta$  coat protein VLP is thus the possibility to couple several antigens per subunit. This allows for the generation of a dense antigen array.

In a preferred embodiment of the invention, the binding and attachment, respectively, of the at least one ghrelin or ghrelin peptide to the core particle and the virus-like particle, respectively, is by way of interaction and association, respectively, between at least one first attachment site of the virus-like particle and at least one second attachment of the antigen or antigenic determinant.

VLPs or capsids of Q $\beta$  coat protein display a defined number of lysine residues on their surface, with a defined topology with three lysine residues pointing towards the interior of the capsid and interacting with the RNA, and four other lysine residues exposed to the exterior of the capsid. These defined properties favor the attachment  
5 of antigens to the exterior of the particle, rather than to the interior of the particle where the lysine residues interact with RNA. VLPs of other RNA phage coat proteins also have a defined number of lysine residues on their surface and a defined topology of these lysine residues.

In further preferred embodiments of the present invention, the first attachment  
10 site is a lysine residue and/or the second attachment comprises sulfhydryl group or a cysteine residue. In a very preferred embodiment of the present invention, the first attachment site is a lysine residue and the second attachment is a cysteine residue.

In very preferred embodiments of the invention, the ghrelin or ghrelin peptide is bound via a cysteine residue, either naturally present on the ghrelin or ghrelin  
15 peptide or engineered, to lysine residues of the VLP of RNA phage coat protein, and in particular to the VLP of Q $\beta$  coat protein.

Another advantage of the VLPs derived from RNA phages is their high expression yield in bacteria that allows production of large quantities of material at affordable cost.

As indicated, the inventive conjugates and arrays, respectively, differ from  
20 prior art conjugates in their highly organized structure, dimensions, and in the repetitiveness of the antigen on the surface of the array. Moreover, the use of the VLPs as carriers allow the formation of robust antigen arrays and conjugates, respectively, with variable antigen density. In particular, the use of VLPs of RNA  
25 phages, and hereby in particular the use of the VLP of RNA phage Q $\beta$  coat protein allows achievement of a very high epitope density. The preparation of compositions of VLPs of RNA phage coat proteins with a high epitope density can be effected by using the teaching of this application. In preferred embodiment of the invention, when a ghrelin peptide is coupled to, preferably the VLP of Q $\beta$  coat protein, an average  
30 number of ghrelin peptide per subunit of 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4,

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1.5, 1.6 , 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4 2.5, 2.6, 2.7, 2.8, 2.9, or higher is preferred.

The second attachment site, as defined herein, may be either naturally or non-naturally present with the antigen or the antigenic determinant. In the case of the  
5 absence of a suitable natural occurring second attachment site on the antigen or antigenic determinant, such a then non-natural second attachment has to be engineered to the antigen.

As described above, four lysine residues are exposed on the surface of the VLP of Q $\beta$  coat protein. Typically these residues are derivatized upon reaction with a  
10 cross-linker molecule. In the instance where not all of the exposed lysine residues can be coupled to an antigen, the lysine residues which have reacted with the cross-linker are left with a cross-linker molecule attached to the  $\epsilon$ -amino group after the derivatization step. This leads to disappearance of one or several positive charges, which may be detrimental to the solubility and stability of the VLP. By replacing  
15 some of the lysine residues with arginines, as in the disclosed Q $\beta$  coat protein mutants described below, we prevent the excessive disappearance of positive charges since the arginine residues do not react with the cross-linker. Moreover, replacement of lysine residues by arginines may lead to more defined antigen arrays, as fewer sites are available for reaction to the antigen.

20 Accordingly, exposed lysine residues were replaced by arginines in the following Q $\beta$  coat protein mutants and mutant Q $\beta$  VLPs disclosed in this application: Q $\beta$ -240 (Lys13-Arg; SEQ ID NO:17), Q $\beta$ -250 (Lys 2-Arg, Lys13-Arg; SEQ ID NO:19) and Q $\beta$ -259 (Lys 2-Arg, Lys16-Arg; SEQ ID NO:21). The constructs were cloned, the proteins expressed, the VLPs purified and used for coupling to peptide  
25 and protein antigens. Q $\beta$ -251; (SEQ ID NO:20) was also constructed, and guidance on how to express, purify and couple the VLP of Q $\beta$ -251 coat protein can be found throughout the application.

In a further embodiment, we disclose a Q $\beta$  mutant coat protein with one additional lysine residue, suitable for obtaining even higher density arrays of  
30 antigens. This mutant Q $\beta$  coat protein, Q $\beta$ -243 (Asn 10-Lys; SEQ ID NO:18), was

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cloned, the protein expressed, and the capsid or VLP isolated and purified, showing that introduction of the additional lysine residue is compatible with self-assembly of the subunits to a capsid or VLP. Thus, ghrelin or ghrelin peptide arrays and conjugates, respectively, may be prepared using VLP of Q $\beta$  coat protein mutants. A particularly favored method of attachment of antigens to VLPs, and in particular to VLPs of RNA phage coat proteins is the linking of a lysine residue present on the surface of the VLP of RNA phage coat proteins with a cysteine residue naturally present or engineered on the antigen, i.e. the ghrelin or ghrelin peptide. In order for a cysteine residue to be effective as second attachment site, a sulfhydryl group must be available for coupling. Thus, a cysteine residue has to be in its reduced state, that is, a free cysteine or a cysteine residue with a free sulfhydryl group has to be available. In the instant where the cysteine residue to function as second attachment site is in an oxidized form, for example if it is forming a disulfide bridge, reduction of this disulfide bridge with *e.g.* DTT, TCEP or  $\beta$ -mercaptoethanol is required. The concentration of reductant, and the molar excess of reductant over antigen have to be adjusted for each antigen. A titration range, starting from concentrations as low as 10  $\mu$ M or lower, up to 10 to 20 mM or higher reductant if required is tested, and coupling of the antigen to the carrier assessed. Although low concentrations of reductant are compatible with the coupling reaction as described in WO 02/056905, higher concentrations inhibit the coupling reaction, as a skilled artisan would know, in which case the reductant has to be removed by dialysis or gel filtration. Advantageously, the pH of the dialysis or equilibration buffer is lower than 7, preferably 6. The compatibility of the low pH buffer with antigen activity or stability has to be tested.

Epitope density on the VLP of RNA phage coat proteins can be modulated by the choice of cross-linker and other reaction conditions. For example, the cross-linkers Sulfo-GMBS and SMPH typically allow reaching high epitope density. Derivatization is positively influenced by high concentration of reactands, and manipulation of the reaction conditions can be used to control the number of antigens coupled to VLPs of RNA phage coat proteins, and in particular to VLPs of Q $\beta$  coat protein.

Prior to the design of a non-natural second attachment site the position at which it should be fused, inserted or generally engineered has to be chosen. Thus, the location of the second attachment site will be selected such that steric hindrance from the second attachment site or any amino acid linker containing the same is avoided.

5 In further embodiments, an antibody response directed at a site distinct from the interaction site of the self-antigen with its natural ligand is desired. In such embodiments, the second attachment site may be selected such that it prevents generation of antibodies against the interaction site of the self-antigen with its natural ligands.

10 In the most preferred embodiments, the ghrelin or ghrelin peptide comprises a single second attachment site or a single reactive attachment site capable of association with the first attachment sites on the core particle and the VLPs or VLP subunits, respectively. This ensures a defined and uniform binding and association, respectively, of the at least one, but typically more than one, preferably more than  
15 10, 20, 40, 80, 120, 150, 180, 210, 240, 270, 300, 360, 400, 450 antigens to the core particle and VLP, respectively. The provision of a single second attachment site or a single reactive attachment site on the antigen, thus, ensures a single and uniform type of binding and association, respectively leading to a very highly ordered and repetitive array. For example, if the binding and association, respectively, is effected  
20 by way of a lysine- (as the first attachment site) and cysteine- (as a second attachment site) interaction, it is ensured, in accordance with this preferred embodiment of the invention, that only one cysteine residue per antigen, independent whether this cysteine residue is naturally or non-naturally present on the antigen, is capable of binding and associating, respectively, with the VLP and the first  
25 attachment site of the core particle, respectively.

In some embodiments, engineering of a second attachment site onto the antigen require the fusion of an amino acid linker containing an amino acid suitable as second attachment site according to the disclosures of this invention. Therefore, in a preferred embodiment of the present invention, an amino acid linker is bound to the  
30 antigen or the antigenic determinant by way of at least one covalent bond. Preferably, the amino acid linker comprises, or alternatively consists of, the second attachment

site. In a further preferred embodiment, the amino acid linker comprises a sulfhydryl group or a cysteine residue. In another preferred embodiment, the amino acid linker is cysteine. Some criteria of selection of the amino acid linker as well as further preferred embodiments of the amino acid linker according to the invention have  
5 already mentioned above.

In a further preferred embodiment of the invention, the at least one antigen or antigenic determinant, i.e. the ghrelin or ghrelin peptide is fused to the core particle and the virus-like particle, respectively. As outlined above, a VLP is typically composed of at least one subunit assembling into a VLP. Thus, in again a further  
10 preferred embodiment of the invention, the antigen or antigenic determinant, preferably the at least one ghrelin or ghrelin peptide, is fused to at least one subunit of the virus-like particle or of a protein capable of being incorporated into a VLP generating a chimeric VLP-subunit-ghrelin or ghrelin peptide protein fusion.

Fusion of ghrelin or ghrelin peptides can be effected by insertion into the VLP  
15 subunit sequence, or by fusion to either the N- or C-terminus of the VLP-subunit or protein capable of being incorporated into a VLP. Hereinafter, when referring to fusion proteins of a peptide to a VLP subunit, the fusion to either ends of the subunit sequence or internal insertion of the peptide within the subunit sequence are encompassed.

Fusion may also be effected by inserting the ghrelin or ghrelin peptide  
20 sequences into a variant of a VLP subunit where part of the subunit sequence has been deleted, that are further referred to as truncation mutants. Truncation mutants may have N- or C-terminal, or internal deletions of part of the sequence of the VLP subunit. For example, the specific VLP HBcAg with, for example, deletion of amino  
25 acid residues 79 to 81 is a truncation mutant with an internal deletion. Fusion of ghrelin or ghrelin peptide to either the N- or C-terminus of the truncation mutants VLP-subunits also lead to embodiments of the invention. Likewise, fusion of an epitope into the sequence of the VLP subunit may also be effected by substitution, where for example for the specific VLP HBcAg, amino acids 79-81 are replaced with  
30 a foreign epitope. Thus, fusion, as referred to hereinafter, may be effected by insertion of the ghrelin or ghrelin peptide sequence in the sequence of a VLP subunit,



by substitution of part of the sequence of the VLP subunit with the ghrelin or ghrelin peptide sequence, or by a combination of deletion, substitution or insertions.

The chimeric ghrelin or ghrelin peptide VLP subunit will be in general capable of self-assembly into a VLP. VLP displaying epitopes fused to their subunits are also herein referred to as chimeric VLPs. As indicated, the virus-like particle comprises or alternatively is composed of at least one VLP subunit. In a further embodiment of the invention, the virus-like particle comprises or alternatively is composed of a mixture of chimeric VLP subunits and non-chimeric VLP subunits, i.e. VLP subunits not having an antigen fused thereto, leading to so called mosaic particles. This may be advantageous to ensure formation of and assembly to a VLP. In those embodiments, the proportion of chimeric VLP-subunits may be 1, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95% or higher.

Flanking amino acid residues may be added to either end of the sequence of the peptide or epitope to be fused to either end of the sequence of the subunit of a VLP, or for internal insertion of such peptidic sequence into the sequence of the subunit of a VLP. Glycine and serine residues are particularly favored amino acids to be used in the flanking sequences added to the ghrelin or ghrelin peptide to be fused. Glycine residues confer additional flexibility, which may diminish the potentially destabilizing effect of fusing a foreign sequence into the sequence of a VLP subunit.

In a specific embodiment of the invention, the VLP is a Hepatitis B core antigen VLP. Fusion proteins to either the N-terminus of HBcAg (Neyrinck, S. *et al.*, *Nature Med.* 5:1157-1163 (1999)) or insertions in the so called major immunodominant region (MIR) have been described (Pumpens, P. and Grens, E., *Intervirology* 44:98-114 (2001)), WO 01/98333), and are preferred embodiments of the invention. Naturally occurring variants of HBcAg with deletions in the MIR have also been described (Pumpens, P. and Grens, E., *Intervirology* 44:98-114 (2001)), which is expressly incorporated by reference in their entirety), and fusions to the N- or C-terminus, as well as insertions at the position of the MIR corresponding to the site of deletion as compared to a wt HBcAg are further embodiments of the invention. Fusions to the C-terminus have also been described (Pumpens, P. and Grens, E., *Intervirology* 44:98-114 (2001)). One skilled in the art will easily find

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guidance on how to construct fusion proteins using classical molecular biology techniques (Sambrook, J. *et al.*, eds., *Molecular Cloning, A Laboratory Manual*, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989), Ho *et al.*, *Gene* 77:51 (1989)). Vectors and plasmids encoding HBcAg and HBcAg fusion proteins and useful for the expression of a HBcAg and HBcAg fusion proteins have been described (Pumpens, P. & Grens, E. *Intervirology* 44: 98-114 (2001), Neyrinck, S. *et al.*, *Nature Med.* 5:1157-1163 (1999)) and can be used in the practice of the invention. We also describe by way of example (Example 6) the insertion of an epitope into the MIR of HBcAg, resulting in a chimeric self-assembling HBcAg.

10 An important factor for the optimization of the efficiency of self-assembly and of the display of the epitope to be inserted in the MIR of HBcAg is the choice of the insertion site, as well as the number of amino acids to be deleted from the HBcAg sequence within the MIR (Pumpens, P. and Grens, E., *Intervirology* 44:98-114 (2001); EP 421'635; US 6'231'864) upon insertion, or in other words, which amino

15 acids from HBcAg are to be substituted with the new epitope. For example, substitution of HBcAg amino acids 76-80, 79-81, 79-80, 75-85 or 80-81 with foreign epitopes has been described (Pumpens, P. and Grens, E., *Intervirology* 44:98-114 (2001); EP0421635; US 6'231'864). HBcAg contains a long arginine tail (Pumpens, P. and Grens, E., *Intervirology* 44:98-114 (2001)) which is dispensable for capsid

20 assembly and capable of binding nucleic acids (Pumpens, P. and Grens, E., *Intervirology* 44:98-114 (2001)). HBcAg either comprising or lacking this arginine tail are both embodiments of the invention.

In a further preferred embodiment of the invention, the VLP is a VLP of a RNA phage. The major coat proteins of RNA phages spontaneously assemble into

25 VLPs upon expression in bacteria, and in particular in *E. coli*. Specific examples of bacteriophage coat proteins which can be used to prepare compositions of the invention include the coat proteins of RNA bacteriophages such as bacteriophage Q $\beta$  (SEQ ID NO:4; PIR Database, Accession No. VCBPQ $\beta$  referring to Q $\beta$  CP and SEQ ID NO: 5; Accession No. AAA16663 referring to Q $\beta$  A1 protein) and bacteriophage

30 fr (SEQ ID NO:7; PIR Accession No. VCBPFR).

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In a more preferred embodiment, the at least one ghrelin or ghrelin peptide is fused to a Q $\beta$  coat protein. Fusion protein constructs wherein epitopes have been fused to the C-terminus of a truncated form of the A1 protein of Q $\beta$ , or inserted within the A1 protein have been described (Kozlovskaja, T. M., *et al.*, *Intervirology*, 39:9-15 (1996)). The A1 protein is generated by suppression at the UGA stop codon and has a length of 329 aa, or 328 aa, if the cleavage of the N-terminal methionine is taken into account. Cleavage of the N-terminal methionine before an alanine (the second amino acid encoded by the Q $\beta$  CP gene) usually takes place in *E. coli*, and such is the case for N-termini of the Q $\beta$  coat proteins CP. The part of the A1 gene, 3' of the UGA amber codon encodes the CP extension, which has a length of 195 amino acids. Insertion of the at least one ghrelin or ghrelin peptide between position 72 and 73 of the CP extension leads to further embodiments of the invention (Kozlovskaja, T. M., *et al.*, *Intervirology* 39:9-15 (1996)). Fusion of a ghrelin or ghrelin peptide at the C-terminus of a C-terminally truncated Q $\beta$  A1 protein leads to further preferred embodiments of the invention. For example, Kozlovskaja *et al.*, (*Intervirology*, 39: 9-15 (1996)) describe Q $\beta$  A1 protein fusions where the epitope is fused at the C-terminus of the Q $\beta$  CP extension truncated at position 19.

As described by Kozlovskaja *et al.* (*Intervirology*, 39: 9-15 (1996)), assembly of the particles displaying the fused epitopes typically requires the presence of both the A1 protein-ghrelin or ghrelin peptide fusion and the wt CP to form a mosaic particle. However, embodiments comprising virus-like particles, and hereby in particular the VLPs of the RNA phage Q $\beta$  coat protein, which are exclusively composed of VLP subunits having at least one ghrelin or ghrelin peptide fused thereto, are also within the scope of the present invention.

The production of mosaic particles may be effected in a number of ways. Kozlovskaja *et al.*, *Intervirology*, 39:9-15 (1996), describe two methods, which both can be used in the practice of the invention. In the first approach, efficient display of the fused epitope on the VLPs is mediated by the expression of the plasmid encoding the Q $\beta$  A1 protein fusion having a UGA stop codon between CP and CP extension in a *E. coli* strain harboring a plasmid encoding a cloned UGA suppressor tRNA which

leads to translation of the UGA codon into Trp (pISM3001 plasmid (Smiley B.K., *et al.*, *Gene* 134:33-40 (1993))). In another approach, the CP gene stop codon is modified into UAA, and a second plasmid expressing the A1 protein-ghrelin or ghrelin peptide fusion is cotransformed. The second plasmid encodes a different antibiotic resistance and the origin of replication is compatible with the first plasmid (Kozlovska, T. M., *et al.*, *Intervirology* 39:9-15 (1996)). In a third approach, CP and the A1 protein-ghrelin or ghrelin peptide fusion are encoded in a bicistronic manner, operatively linked to a promoter such as the Trp promoter, as described in FIG. 1 of Kozlovska *et al.*, *Intervirology*, 39:9-15 (1996).

In a further embodiment, the ghrelin or ghrelin peptide is inserted between amino acid 2 and 3 (numbering of the cleaved CP, that is wherein the N-terminal methionine is cleaved) of the fr CP, thus leading to a ghrelin or ghrelin peptide-fr CP fusion protein. Vectors and expression systems for construction and expression of fr CP fusion proteins self-assembling to VLP and useful in the practice of the invention have been described (Pushko P. *et al.*, *Prot. Eng.* 6:883-891 (1993)). In a specific embodiment, the ghrelin or ghrelin peptide sequence is inserted into a deletion variant of the fr CP after amino acid 2, wherein residues 3 and 4 of the fr CP have been deleted (Pushko P. *et al.*, *Prot. Eng.* 6:883-891 (1993)).

Fusion of epitopes in the N-terminal protuberant  $\beta$ -hairpin of the coat protein of RNA phage MS-2 and subsequent presentation of the fused epitope on the self-assembled VLP of RNA phage MS-2 has also been described (WO 92/13081), and fusion of ghrelin or ghrelin peptide by insertion or substitution into the coat protein of MS-2 RNA phage is also falling under the scope of the invention.

In another embodiment of the invention, the ghrelin or ghrelin peptides are fused to a capsid protein of papillomavirus. In a more specific embodiment, the ghrelin or ghrelin peptides are fused to the major capsid protein L1 of bovine papillomavirus type 1 (BPV-1). Vectors and expression systems for construction and expression of BPV-1 fusion proteins in a baculovirus/insect cells systems have been described (Chackerian, B. *et al.*, *Proc. Natl. Acad. Sci. USA* 96:2373-2378 (1999), WO 00/23955). Substitution of amino acids 130-136 of BPV-1 L1 with a ghrelin or ghrelin peptide leads to a BPV-1 L1- ghrelin or ghrelin peptide fusion protein, which

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is a preferred embodiment of the invention. Cloning in a baculovirus vector and expression in baculovirus infected Sf9 cells has been described, and can be used in the practice of the invention (Chackerian, B. *et al.*, *Proc. Natl. Acad. Sci. USA* 96:2373-2378 (1999), WO 00/23955). Purification of the assembled particles displaying the fused ghrelin or ghrelin peptides can be performed in a number of ways, such as for example gel filtration or sucrose gradient ultracentrifugation (Chackerian, B. *et al.*, *Proc. Natl. Acad. Sci. USA* 96:2373-2378 (1999), WO 00/23955).

In a further embodiment of the invention, the ghrelin or ghrelin peptides are fused to a Ty protein capable of being incorporated into a Ty VLP. In a more specific embodiment, the ghrelin or ghrelin peptides are fused to the p1 or capsid protein encoded by the TYA gene (Roth, J.F., *Yeast* 16:785-795 (2000)). The yeast retrotransposons Ty1, 2, 3 and 4 have been isolated from *Saccharomyces Cerevisiae*, while the retrotransposon Tf1 has been isolated from *Schizosaccharomyces Pombae* (Boeke, J.D. and Sandmeyer, S.B., "Yeast Transposable elements," in *The molecular and Cellular Biology of the Yeast Saccharomyces: Genome dynamics, Protein Synthesis, and Energetics.*, p. 193, Cold Spring Harbor Laboratory Press (1991)). The retrotransposons Ty1 and 2 are related to the *copia* class of plant and animal elements, while Ty3 belongs to the *gypsy* family of retrotransposons, which is related to plants and animal retroviruses. In the Ty1 retrotransposon, the p1 protein, also referred to as Gag or capsid protein has a length of 440 amino acids. P1 is cleaved during maturation of the VLP at position 408, leading to the p2 protein, the essential component of the VLP.

Fusion proteins to p1 and vectors for the expression of said fusion proteins in Yeast have been described (Adams, S.E., *et al.*, *Nature* 329:68-70 (1987)). So, for example, a ghrelin or ghrelin peptide may be fused to p1 by inserting a sequence coding for the ghrelin or ghrelin peptide into the BamH1 site of the pMA5620 plasmid (Adams, S.E., *et al.*, *Nature* 329:68-70 (1987)). The cloning of sequences coding for foreign epitopes into the pMA5620 vector leads to expression of fusion proteins comprising amino acids 1-381 of p1 of Ty1-15, fused C-terminally to the N-terminus of the foreign epitope. Likewise, N-terminal fusion of ghrelin or ghrelin

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peptides, or internal insertion into the p1 sequence, or substitution of part of the p1 sequence is also meant to fall within the scope of the invention. In particular, insertion of ghrelin or ghrelin peptides into the Ty sequence between amino acids 30-31, 67-68, 113-114 and 132-133 of the Ty protein p1 (EP0677111) leads to preferred  
5 embodiments of the invention.

Further VLPs suitable for fusion of ghrelin or ghrelin peptides are, for example, Retrovirus-like-particles (WO9630523), HIV2 Gag (Kang, Y.C., *et al*, *Biol. Chem.* 380:353-364 (1999)), Cowpea Mosaic Virus (Taylor, K.M.*et al.*, *Biol. Chem.* 380:387-392 (1999)), parvovirus VP2 VLP (Rueda, P. *et al.*, *Virology* 263:89-99  
10 (1999)), HBsAg (US 4,722,840, EP0020416B1).

Examples of chimeric VLPs suitable for the practice of the invention are also those described in *Intervirology* 39:1 (1996). Further examples of VLPs contemplated for use in the invention are: HPV-1, HPV-6, HPV-11, HPV-16, HPV-18, HPV-33, HPV-45, CRPV, COPV, HIV GAG, Tobacco Mosaic Virus. Virus-like  
15 particles of SV-40, Polyomavirus, Adenovirus, Herpes Simplex Virus, Rotavirus and Norwalk virus have also been made, and chimeric VLPs of those VLPs are also within the scope of the present invention.

In a further preferred embodiment of the present invention, the antigen or antigenic determinant is ghrelin or a ghrelin peptide.

20 In a further very preferred embodiment of the invention, the antigen or antigenic determinant is selected from the group consisting of a) human ghrelin; b) cat ghrelin; c) dog ghrelin; d) bovine ghrelin; e) sheep ghrelin; f) horse ghrelin g) pig ghrelin; h) a peptide or a fragment thereof of any ghrelin of a)-g).

In a further very preferred embodiment of the invention, the antigen or  
25 antigenic determinant comprises, alternatively essentially consists of, or alternatively consists of an amino acid sequence selected from the group consisting of:

- (a) GSSFLSPEHQRVQRKESKKPPAKLQPR (SEQ ID NO: 48)
- (b) GSSFLSPEHQRVQQRKESKKPPAKLQPR (SEQ ID NO: 31)
- (c) GSSFLSPEHQKLQQRKESKKPPAKLQPR (SEQ ID NO: 49)
- 30 (d) GSSFLSPEHQKLQRKESKKPPAKLQPR (SEQ ID NO: 50)
- (e) GSSFLSPEHQKAQQRKESKKPPAKLQPR (SEQ ID NO: 32)

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GSSFLSPEHQKAQRKESKKPPAKLQPR (SEQ ID NO: 51)

KKPPAKLQPR (SEQ ID NO: 52)

PPAKLQPR (SEQ ID NO: 53)

AKLQPR (SEQ ID NO: 54)

5 GSSFLSPEHQ (SEQ ID NO: 55)

EHQRVQQRKE (SEQ ID NO: 56)

EHQRVQQRKES (SEQ ID NO: 111)

EHQKAQQRKE (SEQ ID NO: 112)

EHQKAQQRKES (SEQ ID NO: 113)

10 EHQKLQQRKE (SEQ ID NO: 114)

EHQKLQQRKES (SEQ ID NO: 115)

LSPEHQRVQQ (SEQ ID NO: 116)

LSPEHQKAQQ (SEQ ID NO: 117)

(s) LSPEHQKLQQ (SEQ ID NO: 118).

15 In a further very preferred embodiment of the invention, the antigen or antigenic determinant is the human, cat, pig, horse, sheep, bovine, guinea pig, dog or mouse ghrelin and ghrelin peptide, respectively. Ghrelins and ghrelin peptides, respectively can be produced by expression of DNA encoding ghrelin and ghrelin peptide, respectively, under the control of a strong promotor. Preferably, the DNA  
20 does not encode the preproghrelin but only the peptidic backbone of the active n-octanoyl-modified peptide. Various examples hereto have been described in the literature and can be used, possibly after modifications, to express ghrelin of any desired species.

In a further preferred embodiment of the invention, the antigen or antigenic  
25 determinant is a ghrelin-derived peptide or a fragment thereof. Preferably the ghrelin peptide is selected from the group consisting: a) human ghrelin peptide; b) bovine ghrelin peptide; c) cat ghrelin peptide; d) dog ghrelin peptide e) pig ghrelin peptide; f) chicken ghrelin peptide; g) mouse ghrelin peptide; h) horse ghrelin peptide; and i) sheep ghrelin and j) a fragment of any ghrelin peptide of a)-i).

30 Such ghrelin peptides or fragments thereof can be produced using standard molecular biological technologies where the nucleotide sequence coding for the

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fragment of interest is amplified by PCR and is cloned as a fusion to a polypeptide tag, such as the histidine tag, the Flag tag, myc tag or the constant region of an antibody (Fc region). By introducing an enterokinase cleavage site between the ghrelin fragment and the tag, the ghrelin fragment can be separated from the tag after  
5 purification by digestion with enterokinase. In another approach the ghrelin fragment can be synthesized in vitro with or without n-octanoyl-modification using standard peptide synthesis reactions known to a person skilled in the art. In a further approach ghrelin fragments can be produced by protease digestion or chemical cleavage of the full length ghrelin, both methods which are well known to people trained in the art.

10 In a further preferred embodiment of the present invention, the ghrelin peptide comprises at least one antigenic site of ghrelin. The skilled person in the art knows how to identify the corresponding peptides and amino acid sequences, respectively. Preferable fragments may encompass the N or C terminus of ghrelin. The C-terminus may be particularly useful since the n-octanoyl-modification, which could perhaps  
15 interfere with the binding of antibodies specific for the non-modified form, is close to the N-terminus.

Preferred human ghrelin peptides would encompass:

KKPPAKLQPR (SEQ ID NO: 52)

PPAKLQPR (SEQ ID NO: 53)

20 AKLQPR (SEQ ID NO: 54)

KLQPR (SEQ ID NO: 59)

GSSFLSPEHQ (SEQ ID NO: 55)

EHQRVQQRKES (SEQ ID NO: 111)

LSPEHQRVQQ (SEQ ID NO: 116)

25 GSSFLSP (SEQ ID NO: 119)

However, peptides from ghrelin internal sites may also be attractive candidates for vaccines. Since ghrelins of various species are highly homologous, it is likely that cross-reactive antibody responses can be induced. Thus, antibody responses against dog or mouse ghrelin may also recognize human ghrelin and vice versa. It is  
30 therefore within the scope of this invention that all ghrelin molecules and peptides derived thereof with amino acid identities > than 80 %, preferably higher than 85%,



more preferably higher than 90%, or even more preferably higher than 95%, 97% or even 99% to human ghrelin may be used for vaccination.

Guidance on how to modify human ghrelin or ghrelin peptide, in particular, for binding to the virus-like particle is given throughout the application. Immunization  
5 against ghrelin using the inventive compositions comprising, preferably human ghrelin or a ghrelin peptide bound to a core particle and VLP, respectively, may provide a way of treating obesity.

In a further very preferred embodiment of the present invention, the antigen or antigenic determinant is a ghrelin or a ghrelin peptide comprising, or alternatively  
10 essentially consisting of, or alternatively consisting of an amino acid sequence selected from the group consisting of:

- (a) GSSFLSPEHQRVQRKESKKPPAKLQPR (human) (SEQ ID NO: 48)
- (b) GSSFLSPEHQRVQQRKESKKPPAKLQPR (human) (SEQ ID NO: 31)
- (c) GSSFLSPEHQRVQ (human) (SEQ ID NO: 60)
- (d) QRKESKKPPAKLQPR (human) (SEQ ID NO: 61)
- (e) PPAKLQPR (human) (SEQ ID NO: 53)
- (f) GSSFLSPEHQKLQQRKESKKPPAKLQPR (dog) (SEQ ID NO: 49)
- 20 (g) GSSFLSPEHQKLQRKESKKPPAKLQPR (dog) (SEQ ID NO: 50)
- (h) GSSFLSPEHQKLQ (dog) (SEQ ID NO: 62)
- (i) QRKESKKPPAKLQPR (dog) (SEQ ID NO: 63)
- (k) EHQRVQQRKE (human) (SEQ ID NO: 56)
- (l) GSSFLSPEHQKAQQRKESKKPPAKLQPR (mouse) (SEQ ID NO: 32)
- 25 (n) the amino acid sequence of any fragment of any of (a)-(l).

In a still further preferred embodiment of the present invention, the antigen or antigenic determinant further comprise at least one second attachment site being selected from the group consisting of: (i) an attachment site not naturally occurring  
30 with said antigen or antigenic determinant; and (ii) an attachment site naturally occurring with said antigen or antigenic determinant. In a preferred embodiment, said

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attachment site comprises an amino acid linker of the invention, preferably a linker sequence of C, CG, or CGG. Preferably, the antigen or antigenic determinant with said at least second attachment site comprises, or alternatively consists of, or alternatively consists of an amino acid sequence selected from the group consisting

5 of:

CGSSFLSPEHQRVQRKESKKPPAKLQPR (human) (SEQ ID NO: 64)

CGSSFLSPEHQRVQQRKESKKPPAKLQPR (human) (SEQ ID NO: 65)

GSSFLSPEHQRVQRKESKKPPAKLQPRC (human) (SEQ ID NO: 66)

GSSFLSPEHQRVQRKESKKPPAKLQPRGC (human) (SEQ ID NO: 120)

10 GSSFLSPEHQRVQQRKESKKPPAKLQPRC (human) (SEQ ID NO: 67)

GSSFLSPEHQRVQQRKESKKPPAKLQPRGC (human) (SEQ ID NO: 121)

GSSFLSPEHQRVQC (human) (SEQ ID NO: 68)

GSSFLSPEHQRVQGC (human) (SEQ ID NO: 122)

CQRKESKKPPAKLQPR (human) (SEQ ID NO: 69)

15 CPPAKLQPR (human) (SEQ ID NO: 70)

CGSSFLSPEHQKLQQRKESKKPPAKLQPR (dog) (SEQ ID NO: 71)

CGSSFLSPEHQKLQRKESKKPPAKLQPR (dog) (SEQ ID NO: 72)

GSSFLSPEHQKLQQRKESKKPPAKLQPRC (dog) (SEQ ID NO: 73)

GSSFLSPEHQKLQQRKESKKPPAKLQPRGC (dog) (SEQ ID NO: 123)

20 GSSFLSPEHQKLQRKESKKPPAKLQPRC (dog) (SEQ ID NO: 74)

GSSFLSPEHQKLQRKESKKPPAKLQPRGC (dog) (SEQ ID NO: 124)

GSSFLSPEHQKLQC (SEQ ID NO: 75)

GSSFLSPEHQKLQGC (SEQ ID NO: 125)

CEHQRVQQRKE (SEQ ID NO: 76)

25 CGSSFLSPEHQKAQQRKESKKPPAKLQPR (mouse) (SEQ ID NO: 77)

GSSFLSPEHQKAQRKESKKPPAKLQPRC (mouse) (SEQ ID NO: 126)

GSSFLSPEHQKAQRKESKKPPAKLQPRGC (mouse) (SEQ ID NO: 127)

GSSFLSPEHQKAQQRKESKKPPAKLQPRC (mouse) (SEQ ID NO: 128)

GSSFLSPEHQKAQQRKESKKPPAKLQPRGC (mouse) (SEQ ID NO: 129)

30 GSSFLSPEHQKAQC (mouse) (SEQ ID NO: 130)

GSSFLSPEHQKAQGC (mouse) (SEQ ID NO: 131)

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GGSSFLSPEHQGC (SEQ ID NO: 132)

CKKPPAKLQPR (SEQ ID NO: 133)

CEHQKAQQRKE (SEQ ID NO: 134)

CEHQKAQQRKES (SEQ ID NO: 135)

5 CLSPEHQKAQQ (SEQ ID NO: 136)

CEHQRVQQRKES (SEQ ID NO: 137)

CLSPEHQRVQQ (SEQ ID NO: 138)

the amino acid sequence of any fragment of any of (a)-(ee).

Some of the very preferred ghrelin peptides, are described in Example 13,  
10 wherein there, in particular, the corresponding murine peptides are provided. These  
peptides comprise an N- or C- terminal cysteine residue as a second attachment  
added for coupling to VLPs and Pili. These very preferred short ghrelin peptide  
fragments are capable of having a very enhanced immunogenicity when coupled to  
VLP and to a core particle, respectively. The preferred ghrelin fragments are,  
15 furthermore, capable of also overcoming safety issues that arise when targeting self-  
proteins as shorter fragment are much more less likely to contain T cell epitopes.  
Typically, the shorter the peptides, the safer with respect to T cell activation.  
However, too short peptides may fail to induce high-affinity antibodies that are able  
to strongly bind ghrelin in solution.

20 The very preferred ghrelin peptide fragments corresponding to the murine  
ghrelin fragment of residue 1-10 (GSSFLSPEHQ) (SEQ ID NO: 55) was chosen  
primarily because the N-terminal segment of ghrelin is identical among all known  
species. Additionally, it is likely to be identical in species where ghrelin has yet to be  
identified. Furthermore, the C-terminal residue, a glutamine, would enhance  
25 solubility, facilitating the production of a soluble vaccine product when coupled to  
VLP and to a core particle, respectively. In fact, the solubility of peptides is often a  
limiting factor for coupling efficiency and vaccine stability. Further reasoning  
includes avoiding a potential T cell epitope. The choice of a smaller peptide fragment  
reduces the probability of a T cell epitope being present. Coupling murine ghrelin  
30 residues 1-10 via the C-terminus to VLP will induce N-terminal specific antibodies

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when immunized into mice that are capable of binding active ghrelin and hence, prevent it's passing of the blood brain barrier, leading to reduced food intake.

The further very preferred ghrelin peptide fragments corresponding to the murine ghrelin peptide fragment of residues 42-51 (KKPPAKLQPR) (SEQ ID NO: 52) has been chosen for similar reasons to that above. The only difference being that this peptide fragment, coupled via the N-terminus, is capable of inducing antibodies specific for the C-terminal part of ghrelin when immunized into mice. Moreover, antibody recognition could be enhanced as the *n*-octanoyl group on serine 3 would be avoided, hence reducing its potential for interference. Coupling murine ghrelin residues 42-51 via the N-terminus to VLP will induce antibodies capable of neutralizing active ghrelin and, hence, prevent it's passing of the blood brain barrier, leading to reduced food intake.

Furthermore, the again further very preferred ghrelin peptide fragments corresponding to the murine ghrelin peptide fragments of residues 31-41 (EHQKAQQRKES) (SEQ ID NO: 113) and residues 28-37 (LSPEHQKAQQ) (SEQ ID NO: 117) were chosen and encompass the central segment of ghrelin. Similarly, these ghrelin fragments would avoid potential T cell epitopes and would also avoid the *n*-octanoyl group on serine 3. Coupling of these peptides via the N- or C-terminus to VLP will induce antibodies capable of neutralizing active ghrelin and, hence, prevent it's passing of the blood brain barrier, leading to reduced food intake.

Further preferred ghrelin peptides suitable for use for the present invention can be identified by using existing or future monoclonal or polyclonal antibodies.

Further preferred ghrelin molecules and peptides derived from the molecules may be discovered in the future in species where no sequence information is available yet.

It will be understood by one of ordinary skill in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein are readily apparent and may be made without departing from the scope of the invention or any embodiment thereof. Having now described the present invention in detail, the same will be more clearly understood by reference to the following

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examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention.

## EXAMPLES

5

### EXAMPLE 1

Construction and expression of mutant Q $\beta$  coat proteins, and purification of mutant Q $\beta$  coat protein VLPs or Capsids.

Plasmid construction and cloning of mutant coat proteins

Construction of pQ $\beta$ -240:

10 The plasmid pQ $\beta$ 10 (Kozlovska, TM, *et al.*, *Gene* 137:133-137) was used as an initial plasmid for the construction of pQ $\beta$ -240. The mutation Lys13 $\rightarrow$ Arg was created by inverse PCR. The inverse primers were designed in inverted tail-to-tail directions:

15 5'-GGTAACATCGGTCGAGATGGAAAACAACTCTGGTCC-3' (SEQ ID NO: 78)

and

5'-GGACCAGAGTTTGTTCATCTCGACCGATGTTACC-3' (SEQ ID NO: 79).

20 The products of the first PCR were used as templates for the second PCR reaction, in which an upstream primer

5'-AGCTCGCCCGGGATCCTCTAG-3' (SEQ ID NO: 80)

and a downstream primer

5'-CGATGCATTTTCATCCTTAGTTATCAATACGC-TGGGTTTCAG3'  
(SEQ ID NO: 81)

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were used. The product of the second PCR was digested with *XbaI* and *Mph1103I* and cloned into the pQ $\beta$ 10 expression vector, which was cleaved by the same restriction enzymes. The PCR reactions were performed with PCR kit reagents and according to producer protocol (MBI Fermentas, Vilnius, Lithuania).

5 Sequencing using the direct label incorporation method verified the desired mutations. *E.coli* cells harbouring pQ $\beta$ -240 supported efficient synthesis of 14-kD protein co migrating upon SDS-PAGE with control Q $\beta$  coat protein isolated from Q $\beta$  phage particles.

Resulting amino acid sequence: (SEQ ID NO: 17)

10 AKLETVTLGNIGRDGKQTLVLNPRGVNPTNGVASLSQAGAVP  
ALEKRVTVSVSQPSRNRKKNYKVQVKIQNPTACTANGSCDPSVTRQ  
KYADVTFSTQYSTDEERAFVRTELAALLASPLLIDAIDQLNPAY

Construction of pQ $\beta$ -243:

15 The plasmid pQ $\beta$ 10 was used as an initial plasmid for the construction of pQ $\beta$ -243. The mutation Asn10 $\rightarrow$ Lys was created by inverse PCR. The inverse primers were designed in inverted tail-to-tail directions:

5'-GGCAAATTAGAGACTGTTACTTTAGGTAAGATCGG -3' (SEQ ID  
NO: 82)

20 and

5'-CCGATCTTACCTAAAGTAACAGTCTCTAATTTTGCC -3' (SEQ ID  
NO: 83).

The products of the first PCR were used as templates for the second PCR reaction, in which an upstream primer

25 5'-AGCTCGCCCGGGGATCCTCTAG-3' (SEQ ID NO: 80)

and a downstream primer

5'-CGATGCATTTATCCTTAGTTATCAATACGCTGGGTTTCAG-3'  
(SEQ ID NO: 81)

30 were used. The product of the second PCR was digested with *XbaI* and *Mph1103I* and cloned into the pQ $\beta$ 10 expression vector, which was cleaved by the

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same restriction enzymes. The PCR reactions were performed with PCR kit reagents and according to producer protocol (MBI Fermentas, Vilnius, Lithuania).

Sequencing using the direct label incorporation method verified the desired mutations. *E.coli* cells harbouring pQ $\beta$ -243 supported efficient synthesis of 14-kD protein co migrating upon SDS-PAGE with control Q $\beta$  coat protein isolated from Q $\beta$  phage particles.

Resulting amino acid sequence: (SEQ ID NO: 18)

AKLETVTLGKIGKDGKQTLVLNPRGVNPTNGVASLSQAGAVP  
ALEKRVTVSVSQPSRNRKKNYKVQVKIQNPTACTANGSCDPSVTRQ  
KYADVTFSTQYSTDEERAFVRTELAALLASPLLIDAIDQLNPAY

Construction of pQ $\beta$ -250:

The plasmid pQ $\beta$ -240 was used as an initial plasmid for the construction of pQ $\beta$ -250. The mutation Lys2 $\rightarrow$ Arg was created by site-directed mutagenesis. An upstream primer

5'-GGCCATGGCACGACTCGAGACTGTTACTTTAGG-3' (SEQ ID NO: 84)

and a downstream primer

5'-GATTTAGGTGACACTATAG-3' (SEQ ID NO: 85)

were used for the synthesis of the mutant PCR-fragment, which was introduced into the pQ $\beta$ -185 expression vector at the unique restriction sites *NcoI* and *HindIII*. The PCR reactions were performed with PCR kit reagents and according to producer protocol (MBI Fermentas, Vilnius, Lithuania).

Sequencing using the direct label incorporation method verified the desired mutations. *E.coli* cells harbouring pQ $\beta$ -250 supported efficient synthesis of 14-kD protein co migrating upon PAGE with control Q $\beta$  coat protein isolated from Q $\beta$  phage particles.

Resulting amino acid sequence: (SEQ ID NO: 19)

ARLETVTLGNIGRDGKQTLVLNPRGVNPTNGVASLSQAGAVP  
ALEKRVTVSVSQPSRNRKKNYKVQVKIQNPTACTANGSCDPSVTRQ

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KYADVTFSTQYSTDEERAFVRTELAALLASPLLIDAIDQLNPAY

Construction of pQ $\beta$ -251:

The plasmid pQ $\beta$ 10 was used as an initial plasmid for the construction of pQ $\beta$ -  
5 251. The mutation Lys16→Arg was created by inverse PCR. The inverse primers  
were designed in inverted tail-to-tail directions:

5'-GATGGACGTCAAACTCTGGTCCTCAATCCGCGTGGGG -3' (SEQ  
ID NO: 86)

and

10 5'-CCCCACGCGGATTGAGGACCAGAGTTTGACGTCATC -3' (SEQ  
ID NO: 87).

The products of the first PCR were used as templates for the second PCR  
reaction, in which an upstream primer

5'-AGCTCGCCCGGGGATCCTCTAG-3' (SEQ ID NO: 80)

15 and a downstream primer

5'-CGATGCATTTATCCTTAGTTATCAATACGCTGGGTTTCAG-3'  
(SEQ ID NO: 81)

were used. The product of the second PCR was digested with *Xba*I and  
*Mph*1103I and cloned into the pQ $\beta$ 10 expression vector, which was cleaved by the  
20 same restriction enzymes. The PCR reactions were performed with PCR kit reagents  
and according to producer protocol (MBI Fermentas, Vilnius, Lithuania).

Sequencing using the direct label incorporation method verified the desired  
mutations. *E.coli* cells harbouring pQ $\beta$ -251 supported efficient synthesis of 14-kD  
protein co migrating upon SDS-PAGE with control Q $\beta$  coat protein isolated from Q $\beta$   
25 phage particles. The resulting amino acid sequence encoded by this construct is  
shown in SEQ. ID NO: 20.



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Construction of pQ $\beta$ -259:

The plasmid pQ $\beta$ -251 was used as an initial plasmid for the construction of pQ $\beta$ -259. The mutation Lys2→Arg was created by site-directed mutagenesis. An upstream primer

5 5'-GGCCATGGCACGACTCGAGACTGTTACTTTAGG-3' (SEQ ID NO: 84)

and a downstream primer

5'-GATTTAGGTGACACTATAG-3' (SEQ ID NO: 85)

10 were used for the synthesis of the mutant PCR-fragment, which was introduced into the pQ $\beta$ -185 expression vector at the unique restriction sites *NcoI* and *HindIII*. The PCR reactions were performed with PCR kit reagents and according to producer protocol (MBI Fermentas, Vilnius, Lithuania).

15 Sequencing using the direct label incorporation method verified the desired mutations. *E.coli* cells harbouring pQ $\beta$ -259 supported efficient synthesis of 14-kD protein co migrating upon SDS-PAGE with control Q $\beta$  coat protein isolated from Q $\beta$  phage particles.

Resulting amino acid sequence: (SEQ ID NO: 21)

AKLETVTLGNIGKDGKQTLVNLNPRGVNPTNGVASLSQAGAVP  
 ALEKRVTVSVSQPSRNRKKNYKVQVKIQNPTACTANGSCDPSVTRQ  
 20 KYADVTF'SFTQYSTDEERAFVRELAALLASPLLI DAIDQLNPAY

General procedures for Expression and purification of Q $\beta$  and Q $\beta$  mutants

## Expression

25 *E.coli* JM109 was transformed with Q $\beta$  coat protein expression plasmids. 5 ml of LB liquid medium containing 20  $\mu$ g/ml ampicillin were inoculated with clones transformed with with Q $\beta$  coat protein expression plasmids. The inoculated culture was incubated at 37 °C for 16-24 h without shaking. The prepared inoculum was subsequently diluted 1:100 in 100-300 ml of fresh LB medium, containing 20  $\mu$ g/ml ampicillin and incubated at 37°C overnight without shaking. The resulting second

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inoculum was diluted 1:50 in M9 medium containing 1 % Casamino acids and 0.2 % glucose in flasks, and incubated at 37 °C overnight under shaking.

### Purification

5 Solutions and buffers for the purification procedure:

1. Lysis buffer LB

50mM Tris-HCl pH8,0 with 5mM EDTA , 0,1%

tritonX100 and freshly prepared PMSF at a concentration of 5micrograms per ml. Without lysozyme and DNase.

10 2. SAS

Saturated ammonium sulphate in water

3. Buffer NET.

20 mM Tris-HCl, pH 7.8 with 5mM EDTA and

150 mM NaCl.

15 4. PEG

40% (w/v) polyethylenglycol 6000 in NET

### Disruption and lysis

Frozen cells were resuspended in LB at 2 ml/g cells. The mixture was  
20 sonicated with 22 kHz five times for 15 seconds, with intervals of 1min to cool the  
solution on ice. The lysate was then centrifuged at 14 000 rpm, for 1h using a Jannecki  
K 60 rotor. The centrifugation steps described below were all performed using the  
same rotor, except otherwise stated. The supernatant was stored at 4° C, while cell  
debris was washed twice with LB. After centrifugation, the supernatants of the lysate  
25 and wash fractions were pooled.

### Fractionation

A saturated ammonium sulphate solution was added dropwise under stirring to  
the above pooled lysate. The volume of the SAS was adjusted to be one fifth of total

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volume, to obtain 20% of saturation. The solution was left standing overnight, and was centrifuged the next day at 14 000 rpm, for 20 min. The pellet was washed with a small amount of 20% ammonium sulphate, and centrifuged again. The obtained supernatants were pooled, and SAS was added dropwise to obtain 40% of saturation.

5 The solution was left standing overnight, and was centrifuged the next day at 14 000 rpm, for 20 min. The obtained pellet was solubilised in NET buffer.

### Chromatography

The capsid or VLP protein resolubilized in NET buffer was loaded on a

10 Sepharose CL-4B column. Three peaks eluted during chromatography. The first one mainly contained membranes and membrane fragments, and was not collected. Capsids were contained in the second peak, while the third one contained other E.coli proteins.

The peak fractions were pooled, and the NaCl concentration was adjusted to a

15 final concentration of 0.65 M. A volume of PEG solution corresponding to one half of the pooled peak fraction was added dropwise under stirring. The solution was left to stand overnight without stirring. The capsid protein was sedimented by centrifugation at 14 000 rpm for 20 min. It was then solubilized in a minimal volume of NET and loaded again on the Sepharose CL-4B column. The peak fractions were

20 pooled, and precipitated with ammonium sulphate at 60% of saturation (w/v). After centrifugation and resolubilization in NET buffer, capsid protein was loaded on a Sepharose CL-6B column for rechromatography.

### Dialysis and drying

25 The peak fractions obtained above were pooled and extensively dialysed against sterile water, and lyophilized for storage.

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Expression and purification Q $\beta$ -240

Cells (*E. coli* JM 109, transformed with the plasmid pQ $\beta$ -240) were resuspended in LB, sonicated five times for 15 seconds (water ice jacket) and centrifuged at 13000 rpm for one hour. The supernatant was stored at 4°C until  
5 further processing, while the debris were washed 2 times with 9 ml of LB, and finally with 9 ml of 0,7 M urea in LB. All supernatants were pooled, and loaded on the Sepharose CL-4B column. The pooled peak fractions were precipitated with ammonium sulphate and centrifuged. The resolubilized protein was then purified further on a Sepharose 2B column and finally on a Sepharose 6B column. The capsid  
10 peak was finally extensively dialyzed against water and lyophilized as described above. The assembly of the coat protein into a capsid was confirmed by electron microscopy.

Expression and purification Q $\beta$ -243

15 Cells (*E. coli* RR1) were resuspended in LB and processed as described in the general procedure. The protein was purified by two successive gel filtration steps on the sepharose CL-4B column and finally on a sepharose CL-2B column. Peak fractions were pooled and lyophilized as described above. The assembly of the coat protein into a capsid was confirmed by electron microscopy.

20

Expression and purification of Q $\beta$ -250

Cells (*E. coli* JM 109, transformed with pQ $\beta$ -250) were resuspended in LB and processed as described above. The protein was purified by gel filtration on a Sepharose CL-4B and finally on a Sepharose CL-2B column, and lyophilized as  
25 described above. The assembly of the coat protein into a capsid was confirmed by electron microscopy.

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Expression and purification of Q $\beta$ -259

Cells (*E. coli* JM 109, transformed with pQ $\beta$ -259) were resuspended in LB and sonicated. The debris were washed once with 10 ml of LB and a second time with 10 ml of 0,7 M urea in LB. The protein was purified by two gel-filtration chromatography steps, on a Sepharose CL-4 B column. The protein was dialyzed and lyophilized, as described above. The assembly of the coat protein into a capsid was confirmed by electron microscopy.

## EXAMPLE 2

10 Insertion of a peptide containing a Lysine residue into the c/e1 epitope of HBcAg (1-149).

The c/e1 epitope (residues 72 to 88) of HBcAg is located in the tip region on the surface of the Hepatitis B virus capsid (HBcAg). A part of this region (Proline 79 and Alanine 80) was genetically replaced by the peptide Gly-Gly-Lys-Gly-Gly (SEQ ID NO: 33), resulting in the HBcAg-Lys construct (SEQ ID NO: 26). The introduced Lysine residue contains a reactive amino group in its side chain that can be used for intermolecular chemical crosslinking of HBcAg particles with any antigen containing a free cysteine group.

HBcAg-Lys DNA, having the amino acid sequence shown in SEQ ID NO:78, was generated by PCR: The two fragments encoding HBcAg fragments (amino acid residues 1 to 78 and 81 to 149) were amplified separately by PCR. The primers used for these PCRs also introduced a DNA sequence encoding the Gly-Gly-Lys-Gly-Gly peptide (SEQ ID NO: 33). The HBcAg (1 to 78) fragment was amplified from pEco63 using primers EcoRIHBcAg(s) and Lys-HBcAg(as). The HBcAg (81 to 149) fragment was amplified from pEco63 using primers Lys-HBcAg(s) and HBcAg(1-149)Hind(as). Primers Lys-HBcAg(as) and Lys-HBcAg(s) introduced complementary DNA sequences at the ends of the two PCR products allowing fusion of the two PCR products in a subsequent assembly PCR. The assembled fragments were amplified by PCR using primers EcoRIHBcAg(s) and HbcAg(1-149) Hind(as).

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For the PCRs, 100 pmol of each oligo and 50 ng of the template DNAs were used in the 50 ml reaction mixtures with 2 units of Pwo polymerase, 0.1 mM dNTPs and 2 mM MgSO<sub>4</sub>. For both reactions, temperature cycling was carried out as follows: 94°C for 2 minutes; 30 cycles of 94°C (1 minute), 50°C (1 minute), 72°C (2 minutes).

Primer sequences:

EcoRIHBcAg(s):

(5'-CCGGAATTCATGGACATTGACCCCTATAAAG-3') (SEQ ID NO: 88);

10

Lys-HBcAg(as):

(5'-

CCTAGAGCCACCTTTGCCACCATCTTCTAAATTAGTACCCACCCAG

GTAGC-3') (SEQ ID NO: 89);

15

Lys-HBcAg(s):

(5'-

GAAGATGGTGGCAAAGGTGGCTCTAGGGACCTAGTAGTCAGTTAT

GTC -3') (SEQ ID NO: 90);

20

HBcAg(1-149)Hind(as):

(5'-CGCGTCCCAAGCTTCTAAACAACAGTAGTCTCCGGAAG-3') (SEQ ID NO: 91).

For fusion of the two PCR fragments by PCR 100 pmol of primers EcoRIHBcAg(s) and HBcAg(1-149)Hind(as) were used with 100 ng of the two purified PCR fragments in a 50 ml reaction mixture containing 2 units of Pwo polymerase, 0.1 mM dNTPs and 2 mM MgSO<sub>4</sub>. PCR cycling conditions were: 94°C for 2 minutes; 30 cycles of 94°C (1 minute), 50°C (1 minute), 72°C (2 minutes). The assembled PCR product was analyzed by agarose gel electrophoresis, purified and digested for 19 hours in an appropriate buffer with EcoRI and HindIII restriction

30

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enzymes. The digested DNA fragment was ligated into EcoRI/HindIII-digested pKK vector to generate pKK-HBcAg-Lys expression vector. Insertion of the PCR product into the vector was analyzed by EcoRI/HindIII restriction analysis and DNA sequencing of the insert.

5

## EXAMPLE 3

## Expression and purification of HBcAg-Lys.

*E. coli* strains K802 or JM109 were transformed with pKK-HBcAg-Lys. 1 ml of an overnight culture of bacteria was used to inoculate 100 ml of LB medium containing 100 µg/ml ampicillin. This culture was grown for 4 hours at 37°C until an OD at 600 nm of approximately 0.8 was reached. Induction of the synthesis of HBcAg-Lys was performed by addition of IPTG to a final concentration of 1 mM. After induction, bacteria were further shaken at 37°C for 4 hours. Bacteria were harvested by centrifugation at 5000 x g for 15 minutes. The pellet was frozen at -80°C. The pellet was thawed and resuspended in bacteria lysis buffer (10 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.0, 30 mM NaCl, 0.25% Tween-20, 10 mM EDTA) supplemented with 200 µg/ml lysozyme and 10 µl of Benzonase (Merck). Cells were incubated for 30 minutes at room temperature and disrupted by sonication. *E. coli* cells harboring pKK-HBcAg-Lys expression plasmid or a control plasmid were used for induction of HBcAg-Lys expression with IPTG. Prior to the addition of IPTG, a sample was removed from the bacteria culture carrying the pKK-HBcAg-Lys plasmid and from a culture carrying the control plasmid. Four hours after addition of IPTG, samples were again removed from the culture containing pKK-HBcAg-Lys and from the control culture. Protein expression was monitored by SDS-PAGE followed by Coomassie staining.

25

The lysate was then centrifuged for 30 minutes at 12,000 x g in order to remove insoluble cell debris. The supernatant and the pellet were analyzed by Western blotting using a monoclonal antibody against HBcAg (YVS1841, purchased from Accurate Chemical and Scientific Corp., Westbury, NY, USA), indicating that a significant amount of HBcAg-Lys protein was soluble. Briefly, lysates from

*E. coli* cells expressing HBcAg-Lys and from control cells were centrifuged at 14,000 x g for 30 minutes. Supernatant (= soluble fraction) and pellet (= insoluble fraction) were separated and diluted with SDS sample buffer to equal volumes. Samples were analyzed by SDS-PAGE followed by Western blotting with anti-  
5 HBcAg monoclonal antibody YVS 1841.

The cleared cell lysate was used for step-gradient centrifugation using a sucrose step gradient consisting of a 4 ml 65% sucrose solution overlaid with 3 ml 15% sucrose solution followed by 4 ml of bacterial lysate. The sample was centrifuged for 3 hrs with 100,000 x g at 4°C. After centrifugation, 1 ml fractions  
10 from the top of the gradient were collected and analyzed by SDS-PAGE followed by Coomassie staining. The HBcAg-Lys protein was detected by Coomassie staining.

The HBcAg-Lys protein was enriched at the interface between 15 and 65% sucrose indicating that it had formed a capsid particle. Most of the bacterial proteins remained in the sucrose-free upper layer of the gradient, therefore step-gradient  
15 centrifugation of the HBcAg-Lys particles led both to enrichment and to a partial purification of the particles.

Expression and purification of HBcAg-Lys in large scale was performed as follows. An overnight culture was prepared by inoculating a single colony in 100 ml LB, 100 µg/ml Ampicillin and growing the culture overnight at 37°C. 25 ml of the  
20 preculture were diluted in 800 ml LB Ampicillin medium the next day, and the culture grown to an optical density OD<sup>600</sup> of 0.6-0.8. The culture was then induced with 1 mM IPTG, and left to grow for another 4 hours. The cells were harvested and lysed essentially as described above.

HBcAg-Lys was then purified by first precipitating the protein with ammonium sulphate (30% saturation) from the cleared cell lysate, then loading the resolubilized  
25 pellet on a gel filtration column (Sephacryl S-400, Pharmacia). The pooled fractions were precipitated again with ammonium sulphate, the pellet resolubilized and loaded a second time on the same gel filtration column. The fractions were finally pooled and concentrated, and the concentration assessed using a Bradford test (BioRad).



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## EXAMPLE 4

Construction of an HBcAg devoid of free cysteine residues and containing an inserted lysine residue.

5 A Hepatitis core Antigen (HBcAg), referred to herein as HBcAg-lys-2cys-Mut, devoid of cysteine residues at positions corresponding to 48 and 107 in SEQ ID NO:25 and containing an inserted lysine residue was constructed using the following methods.

The two mutations were introduced by first separately amplifying three fragments of the HBcAg-Lys gene prepared as described above in Example 2 with the following PCR primer combinations. PCR methods and conventional cloning techniques were used to prepare the HBcAg-lys-2cys-Mut gene.

In brief, the following primers were used to prepare fragment 1:

Primer 1: EcoRIHBcAg(s)

CCGGAATTCATGGACATTGACCCTTATAAAG (SEQ ID NO: 88)

15

Primer 2: 48as

GTGCAGTATGGTGAGGTGAGGAATGCTCAGGAGACTC (SEQ ID NO:

92)

20

The following primers were used to prepare fragment 2:

Primer 3: 48s

GAGTCTCCTGAGCATTCCCTCACCTCACCATACTGCAC (SEQ ID NO:

93)

25

Primer 4: 107as

CTTCCAAAAGTGAGGGAAGAAATGTGAAACCAC (SEQ ID NO: 94)

The following primers were used to prepare fragment 3:

- 88 -

Primer 5: HBcAg149hind-as  
CGCGTCCCAAGCTTCTAAACAACAGTAGTCTCCGGAAGC-  
GTTGATAG (SEQ ID NO: 95)

5 Primer 6: 107s  
GTGGTTTCACATTTCTTCCCTCACTTTTGGGAAG (SEQ ID NO: 96)

Fragments 1 and 2 were then combined with PCR primers EcoRIHBcAg(s) and 107as to give fragment 4. Fragment 4 and fragment 3 were then combined with  
10 primers EcoRIHBcAg(s) and HBcAg149hind-as to produce the full length gene. The full length gene was then digested with the EcoRI (GAATTC) and HindIII (AAGCTT) enzymes and cloned into the pKK vector (Pharmacia) cut at the same restriction sites. Expression and purification of HBcAg-lys-2cys-Mut were performed as set out in Example 3.

15 EXAMPLE 5

Construction of HBcAg1-185-Lys.

Hepatitis core Antigen (HBcAg) 1-185 was modified as described in Example 2. A part of the c/e1 epitope (residues 72 to 88) region (Proline 79 and Alanine 80) was genetically replaced by the peptide Gly-Gly-Lys-Gly-Gly (SEQ ID NO: 33),  
20 resulting in the HBcAg-Lys construct (SEQ ID NO: 26). The introduced Lysine residue contains a reactive amino group in its side chain that can be used for intermolecular chemical crosslinking of HBcAg particles with any antigen containing a free cysteine group. PCR methods and conventional cloning techniques were used to prepare the HBcAg1-185-Lys gene.

25 The Gly-Gly-Lys-Gly-Gly sequence (SEQ ID NO: 33) was inserted by amplifying two separate fragments of the HBcAg gene from pEco63, as described above in Example 2 and subsequently fusing the two fragments by PCR to assemble the full length gene. The following PCR primer combinations were used:

fragment 1:

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Primer 1: EcoRIHBcAg(s) ((SEQ ID NO: 88), see Example 2)

Primer 2: Lys-HBcAg(as) ((SEQ ID NO: 89), see Example 2)

fragment 2:

Primer 3: Lys-HBcAg(s) ((SEQ ID NO: 90), see Example 2)

5 Primer 4: HBcAgwtHindIII

CGCGTCCCAAGCTTCTAACATTGAGATTCCCGAGATTG (SEQ ID NO:  
97)

Assembly:

Primer 1: EcoRIHBcAg(s) ((SEQ ID NO: 88), see example 2)

10 Primer 2: HBcAgwtHindIII (SEQ ID NO: 97)

The assembled full length gene was then digested with the EcoRI (GAATTC) and HindIII (AAGCTT) enzymes and cloned into the pKK vector (Pharmacia) cut at the same restriction sites.

15

## EXAMPLE 6

Fusion of a peptide epitope in the MIR region of HbcAg.

The residues 79 and 80 of HBcAg1-185 were substituted with the epitope CεH3 of sequence VNLTWSRASG (SEQ ID NO: 98). The CεH3 sequence stems from the sequence of the third constant domain of the heavy chain of human IgE. The epitope was inserted in the HBcAg1-185 sequence using an assembly PCR method. In the first PCR step, the HBcAg1-185 gene originating from ATCC clone pEco63 and amplified with primers HBcAg-wt EcoRI fwd and HBcAg-wt Hind III rev was used as template in two separate reactions to amplify two fragments containing sequence elements coding for the CεH3 sequence. These two fragments were then assembled in a second PCR step, in an assembly PCR reaction.

20

25

Primer combinations in the first PCR step: CεH3fwd with HBcAg-wt Hind III rev, and HBcAg-wt EcoRI fwd with CεH3rev. In the assembly PCR reaction, the two fragments isolated in the first PCR step were first assembled during 3 PCR cycles

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without outer primers, which were added afterwards to the reaction mixture for the next 25 cycles. Outer primers: HBcAg-wt EcoRI fwd and HBcAg-wt Hind III rev.

The PCR product was cloned in the pKK223.3 using the EcoRI and HindIII sites, for expression in *E. coli* (see Example 2). The chimeric VLP was expressed in *E. coli* and purified as described in Example 2. The elution volume at which the HBcAg1-185- CεH3 eluted from the gel filtration showed assembly of the fusion proteins to a chimeric VLP.

Primer sequences:

10 CεH3fwd:

5' GTT AAC TTG ACC TGG TCT CGT GCT TCT GGT GCA TCC AGG GAT CTA GTA GTC  
3' (SEQ ID NO: 99)

V N L T W S R A S G A80 S R D L V V86  
(SEQ ID NO: 100)

15

CεH3rev:

5' ACC AGA AGC ACG AGA CCA GGT CAA GTT AAC ATC TTC CAA ATT ATT ACC CAC 3'  
(SEQ ID NO: 101)

20 (SEQ ID NO: 102)

D78 E L N N G V72

HBcAg-wt EcoRI fwd:

5' CCGgaattcATGGACATTGACCCTTATAAAG (SEQ ID NO: 103)

HBcAg-wt Hind III rev:

5' CGCGTCCCaaagcttCTAACATTGAGATTCCCGAGATTG (SEQ ID NO:  
25 104)

## EXAMPLE 7

Fusion of a ghrelin peptide epitope in the MIR region of HbcAg.

The residues 79 and 80 of HBcAg1-185 are substituted with the ghrelin peptide  
30 epitope of sequence: EHQRVQQRKE (SEQ ID NO: 56). Two overlapping primers

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are designed using the same strategy described in Example 6, and the fusion protein constructed by assembly PCR. The PCR product is cloned in the pKK223.3 vector, and expressed in *E. coli* K802. The chimeric VLPs are expressed and purified as described in Example 3.

5

## EXAMPLE 8

Fusion of a ghrelin peptide epitope to the C-terminus of the Q $\beta$  A1 protein truncated at position 19 of the CP extension.

A primer annealing to the 5' end of the Q $\beta$  A1 gene and a primer annealing to the 3' end of the A1 gene and comprising additionally a sequence element coding a ghrelin fragment, of sequence KKPPAKLQPR (SEQ ID NO: 52), are used in a PCR  
10 reaction with pQ $\beta$ 10 as template. The PCR product is cloned in pQ $\beta$ 10 (Kozłowska T.M. *et al.*, *Gene* 137: 133-37 (1993)), and the chimeric VLP expressed and purified as described in Example 1.

## EXAMPLE 9

15

Insertion of a ghrelin peptide epitope between positions 2 and 3 of fr coat protein.

Complementary primers coding for the sequence of the ghrelin peptide of sequence EHQRVQQRKE (SEQ ID NO: 56), and containing *Bsp119I* compatible ends and additional nucleotides enabling in frame insertion, are inserted in the  
20 *Bsp119I* site of the pFrd8 vector (Pushko, P. *et al.*, *Prot. Eng.* 6: 883-91 (1993)) by standard molecular biology techniques. Alternatively, the overhangs of the pFrd8 vector are filled in with Klenow after digestion with *Bsp119I*, and oligonucleotides coding for the sequence of the ghrelin peptide murine ghrelin peptide and additional nucleotides for in frame cloning are ligated in pFrd8 after the Klenow treatment.  
25 Clones with the insert in the right orientation are analyzed by sequencing. Expression and purification of the chimeric fusion protein in *E. coli* JM109 or *E. coli* K802 is performed as described in Pushko, P. *et al.*, *Prot. Eng.* 6:883-91 (1993), but for the

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chromatography steps which are performed using a Sepharose CL-4B or Sephacryl S-400 (Pharmacia). The cell lysate is precipitated with ammonium sulphate, and purified by two successive gel filtration purification steps, similarly to the procedure described for Q $\beta$  in Example 1.

5

## EXAMPLE 10

Insertion of a ghrelin peptide epitope between positions 67 and 68 of Ty1 protein p1 in the vector pOGS8111.

Two complementary oligonucleotides coding for the human ghrelin peptide sequence EHQRVQQRKE (SEQ ID NO: 56), with ends compatible with the NheI site of pOGS8111 are synthesized. Additional nucleotides are added to allow for in frame insertion of a sequence coding for the murine ghrelin epitope according to the description of EP06777111. The amino acids AS and SS flanking the inserted epitope are encoded by the altered NheI sites resulting from the insertion of the oligonucleotide in the TyA(d) gene of pOGS8111.

15 pOGS8111 is transformed into *S. cerevisiae* strain MC2, for expression of the chimeric Ty VLP as described in EP0677111 and references therein. The chimeric Ty VLP is purified by sucrose gradient ultracentrifugation as described in EP0677111.

## EXAMPLE 11

20 Insertion of a ghrelin peptide epitope in to the major capsid protein L1 of papillomavirus type 1 (BPV-1).

A sequence coding for the ghrelin epitope having the sequence EHQRVQQRKE (SEQ ID NO: 56) is substituted to the sequence coding for amino acids 130-136 of the BPV-1 L1 gene cloned in the pFastBac1 (GIBCO/BRL) vector as described (Chackerian, B. *et al.*, *Proc. Natl. Acad. USA* 96: 2373-2378 (1999)).  
25 The sequence of the construct is verified by nucleotide sequence analysis. Recombinant baculovirus is generated using the GIBCO/BRL baculovirus system as described by the manufacturer. The chimeric VLPs are purified from baculovirus

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infected Sf9 cells as described by Kirnbauer, R. *et al.*, *Proc. Natl. Acad. Sci.* 89:12180-84 (1992) and Greenstone, H.L., *et al.*, *Proc. Natl. Acad. Sci.* 95:1800-05 (1998).

## EXAMPLE 12

5                   Immunization of mice with ghrelin-peptides fused to VLPs.

Chimeric VLPs displaying the murine ghrelin epitope of sequence EHQRVQQRKE (SEQ ID NO: 56) generated in Examples 7, 9, 10 and 11 are used for immunization of mice as described in Example 15. The sera obtained from the immunized mice are analyzed in a ghrelin -specific ELISA as described in Example  
10   14.

The effect of the vaccine is examined by following the weight increase of the mice and by measuring food uptake.

## EXAMPLE 13

15                   Coupling of murine ghrelin and murine ghrelin peptides to Q $\beta$  capsid protein:  
  ghrelin peptide vaccines.

The following ghrelin peptides are chemically synthesized:

cGhrel: CGSSFLSPEHQKAQQRKESKKPPAKLQPR (SEQ ID NO: 77)

GhrelC: GSSFLSPEHQKAQQRKESKKPPAKLQPRGC (SEQ ID NO: 105)

cGhrQ14: CGSSFLSPEHQKAQQRKESKKPPAKLQPR (SEQ ID NO: 106)

20   GhrQ14C: GSSFLSPEHQKAQQRKESKKPPAKLQPRGC (SEQ ID NO: 107)

Ghrel24-33C   GGSSFLSPEHQGC (SEQ ID NO: 132)

cGhrel42-51   CKKPPAKLQPR (SEQ ID NO: 133)

cGhrel31-40   CEHQKAQQRKE (SEQ ID NO: 134)

cGhrel31-41   CEHQKAQQRKES (SEQ ID NO: 135)

25   cGhrel28-37   CLSPEHQKAQQ (SEQ ID NO: 136)

which comprise an added N- or C- terminal cysteine residue for coupling to VLPs and Pili, and used for chemical coupling to Q $\beta$  as described in the following.

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A solution of 5 ml of 140  $\mu$ M Q $\beta$  capsid protein in 20 mM Hepes, 150 mM NaCl pH 7.4 is reacted for 30 minutes with 108  $\mu$ l of a 65 mM solution of SMPH (Pierce) in H<sub>2</sub>O at 25°C on a rocking shaker. The reaction solution is subsequently dialyzed twice for 2 hours against 5 L of 20 mM Hepes, 150 mM NaCl, pH 7.2 at 4°C. 100  $\mu$ l of the dialyzed reaction mixture is then reacted either with 28.6  $\mu$ l of a 10 mM stock solution (in DMSO) of the ghrelin peptide (1:10 peptide/ Q $\beta$  capsid protein ratio). The coupling reaction is performed for 2h at 15 °C in a water bath. The reaction mixture is subsequently dialyzed for 24 h against 2x 5 L of 20 mM Hepes, 150 mM NaCl, pH 7.2 at 4°C.

The coupled products are centrifuged and supernatants and pellets are analysed on 16% SDS-PAGE gels under reducing conditions. Gels are stained with Coomassie Brilliant Blue.

#### Coupling of ghrelin peptides to fr capsid protein

A solution of 120  $\mu$ M fr capsid protein in 20 mM Hepes, 150 mM NaCl pH 7.2 is reacted for 30 minutes with a 10 fold molar excess of SMPH (Pierce) ), diluted from a stock solution in DMSO, at 25 °C on a rocking shaker. The reaction solution is subsequently dialyzed twice for 2 hours against 1 L of 20 mM Hepes, 150 mM NaCl, pH 7.2 at 4 °C. The dialyzed fr reaction mixture is then reacted with equimolar concentration of ghrelin peptide or a ration of 1:2 ghrelin peptide / fr over night at 16 °C on a rocking shaker. Coupling products are analysed by SDS-PAGE.

#### Coupling of ghrelin peptides to HBcAg-Lys-2cys-Mut

A solution of 120  $\mu$ M HBcAg-Lys-2cys-Mut in 20 mM Hepes, 150 mM NaCl pH 7.2 is reacted for 30 minutes with a 10 fold molar excess of SMPH (Pierce), diluted from a stock solution in DMSO, at 25 °C on a rocking shaker. The reaction solution is subsequently dialyzed twice for 2 hours against 1 L of 20 mM Hepes, 150 mM NaCl, pH 7.2 at 4 °C. The dialyzed HBcAg-Lys-2cys-Mut reaction mixture is then reacted with equimolar concentration of ghrelin peptide or a ration of 1:2



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ghrelin peptide / HBcAg-Lys-2cys-Mut over night at 16 °C on a rocking shaker. Coupling products are analysed by SDS-PAGE.

#### Coupling of ghrelin peptides to Pili

5 A solution of 125  $\mu$ M Type-1 pili of *E.coli* in 20 mM Hepes, pH 7.2, is reacted for 60 minutes with a 50-fold molar excess of cross-linker SMPH (Pierce), diluted from a stock solution in DMSO, at RT on a rocking shaker. The reaction mixture is desalted on a PD-10 column (Amersham-Pharmacia Biotech). The protein-containing fractions eluting from the column are pooled, and the desalted derivatized pili  
10 protein is reacted with the ghrelin peptides in equimolar or in a ratio of 1:2 peptide pili over night at 16 °C on a rocking shaker. Coupling products are analysed by SDS-PAGE.

#### EXAMPLE 14

##### Immunization of mice with VLP-ghrelin peptide conjugates.

15 Immunization of mice with Ghrelin-peptides coupled to Q $\beta$

Female C57BL/6 mice are immunized with the murine ghrelin peptides listed in example 13 coupled to Q $\beta$  mixed with alum, emulsified in IFA or with no adjuvant. 50  $\mu$ g are injected subcutaneously on day 0 and on day 14 and day 28. Mice are bled retroorbitally on day 14, 21, 28, 42 and subsequently at monthly  
20 intervals thereafter, after immunization. Serum is analyzed using a ghrelin protein-specific ELISA.

Having now fully described the present invention in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious to one of ordinary skill in the art that the same can be performed by modifying or  
25 changing the invention within a wide and equivalent range of conditions, formulations and other parameters without affecting the scope of the invention or any specific embodiment thereof and that such modifications or changes are intended to be encompassed within the scope of the appended claims.

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All publications, patents and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains, and are herein incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually  
5 indicated to be incorporated by reference.

#### EXAMPLE 15

##### Coupling of C-Ghrelin and Ghrelin-GC to Q $\beta$ capsid protein

10

A solution of 2 ml of 2.0 mg/ml Q $\beta$  capsid protein in 20 mM Hepes, 150 mM NaCl pH 7.2 was reacted for 30 minutes with 114.4  $\mu$ l of a SMPH (Pierce) solution (from a 50 mM stock solution dissolved in DMSO) at 25°C. The reaction solution was subsequently dialyzed twice for 2 hours against 2 L of 20 mM Hepes, 150 mM  
15 NaCl, pH 7.2 at 4°C.

The dialysed, derivatized Q $\beta$  VLP was subsequently reacted with the murine C-Ghrelin (SEQ ID: No. 77) or murine Ghrelin-GC peptide (SEQ ID: No. 105) for 2 hours at 15°C in 20 mM Hepes, 150 mM NaCl, pH 7.2. The coupling reaction was performed in multiple aliquots, where 1 ml of derivatized Q $\beta$  VLP (at a  
20 concentration of 2mg/ml) was reacted with 286 $\mu$ l of a 10mM peptide solution. 10% acetonitrile was added to the coupling reaction. The reaction solution was dialyzed once for 2 hours and then overnight against 2 L of 20 mM Hepes, 150 mM NaCl, pH 7.2 at 4°C.

Coupled products were analyzed on 16% SDS-PAGE gels. Gels were stained  
25 with Coomassie Brilliant Blue. Results are shown in Fig. 1. Coupled products could be detected in the Coomassie-stained gels and clearly demonstrate the covalent coupling of C-Ghrelin or Ghrelin-GC to Q $\beta$  capsid protein. Coupling bands corresponding to one, two, three or four peptides coupled per subunit are indicated by arrows.

30 Figure 1 shows the coupling products from the reaction of murine C-Ghrelin or murine Ghrelin-GC to Q $\beta$  capsid protein. Lane 2 and 4 show the coupling products

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in the soluble fraction of Q $\beta$ -C-Ghrelin and Ghrelin-GC-Q $\beta$ , respectively. Very little product is in the insoluble fraction.

#### EXAMPLE 16

##### 5           Immunization of mice with C-Ghrelin or Ghrelin-GC coupled to Q $\beta$ capsid protein

Female, C57BL/6 mice (5 per group) were vaccinated with either murine C-Ghrelin (SEQ ID: No. 77) or murine Ghrelin-GC (SEQ ID: No. 105) coupled to Q $\beta$  capsid protein. 50  $\mu$ g of dialyzed vaccine from each sample was diluted in PBS to a volume of 200  $\mu$ l and injected subcutaneously (100  $\mu$ l on two ventral sides) on days 0, 14 and 42. The vaccine was administered without any additional adjuvant. As a control, a group of mice were immunized with PBS. Mice were bled retro-orbitally on day 0, 14, 21, 42 and their serum was analyzed using a Ghrelin-specific ELISA (outlined in example 17). Furthermore, body weight and food consumption of mice was monitored at regular intervals over the course of the experiment (outlined in example 18).

20

#### EXAMPLE 17

##### Detection of ghrelin-specific antibodies in an ELISA

ELISA plates were coated with serine-octanylated, murine ghrelin (Bachem, Product No. H-4862) at a concentration of 20 $\mu$ g/ml. The plates were blocked and then incubated with serially diluted mouse sera from day 14, 21 and 42. Bound antibodies were detected with enzymatically labeled anti-mouse IgG antibody. As a control, pre-immune serum of the same mice was also tested (Figure 2).

In mice immunized with Q $\beta$ -C-Ghrelin or Ghrelin-CG-Q $\beta$ , an average titer on day 21 of 13000 and 8000 respectively, was reached (Figure 2). Pre-immune sera or sera from mice immunized with PBS did not show any reactivity with ghrelin. This

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clearly demonstrates that a ghrelin-VLP conjugate is able to induce a high antibody titer against ghrelin even when it is a self protein.

5

#### EXAMPLE 18

Efficacy experiments with C-Ghrelin or Ghrelin-GC coupled to Q $\beta$  capsid protein

Female, C57BL/6 mice (5 per group) were vaccinated with either murine C-  
10 Ghrelin or Ghrelin-GC coupled to Q $\beta$  capsid protein as described in example 16. As  
a control, mice were immunized with PBS. All mice were placed on a normal diet  
between days 0 and 14. Subsequently, all mice were given a high fat (45%) diet to  
facilitate the development of diet-induced obesity. Food and water was administered  
*ad libitum*. Individual mice were monitored for body weight changes and food and  
15 water consumption per cage (i.e. group) was also monitored at regular intervals after  
immunization (at day 5, 11, 14, 21, 28, 35, 40, 49, and 55 after immunization) (see  
Fig. 3).

In mice immunized with murine Q $\beta$ -C-Ghrelin or murine Ghrelin-CG-Q $\beta$ , an  
average decrease of approximately 30-40% in food intake was achieved, compared to  
20 control mice immunized with PBS (Fig. 3). This clearly demonstrates that a ghrelin-  
VLP conjugate is able to suppress appetite and reduce food consumption.

#### EXAMPLE 19

25

Efficacy experiments with C-Ghrelin or Ghrelin-GC coupled to Q $\beta$  capsid protein in an animal model of obesity

Female, C57BL/6 *ob/ob* mice (5 per group) are vaccinated with either murine  
C-Ghrelin or Ghrelin-GC coupled to Q $\beta$  capsid protein. Control mice are immunized  
30 with PBS or Q $\beta$  VLP alone. 50  $\mu$ g of dialyzed vaccine or control protein is diluted in

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a 200 µl volume of PBS and injected subcutaneously (100 µl on two ventral sides), with or without alum, on day 0. Mice are boosted with the corresponding formulation on day 14, 28 and 42.

Mice are bled retro-orbitally on day 0, 14, 21, 42, 56, 70 and then at monthly intervals. The sera are analyzed for ghrelin-specific antibodies in a Ghrelin-specific ELISA, as described in example 17. Mice will be boosted accordingly if ghrelin-specific antibody titers significantly decline over the period of the experiment. The effect of the vaccine is examined by measuring food consumption under *ad libitum* conditions and by following the weight increase of the mice as described in example 18.

#### EXAMPLE 20:

##### Cloning of the AP205 Coat Protein gene

The cDNA of AP205 coat protein (CP) (SEQ ID NO: 28) was assembled from two cDNA fragments generated from phage AP205 RNA by using a reverse transcription-PCR technique and cloning in the commercial plasmid pCR 4-TOPO for sequencing. Reverse transcription techniques are well known to those of ordinary skill in the relevant art. The first fragment, contained in plasmid p205-246, contained 269 nucleotides upstream of the CP sequence and 74 nucleotides coding for the first 24 N-terminal amino acids of the CP. The second fragment, contained in plasmid p205-262, contained 364 nucleotides coding for amino acids 12-131 of CP and an additional 162 nucleotides downstream of the CP sequence. Both p205-246 and p205-262 were a generous gift from J. Klovins.

The plasmid 283.-58 was designed by two-step PCR, in order to fuse both CP fragments from plasmids p205-246 and p205-262 in one full-length CP sequence.

An upstream primer p1.44 containing the *NcoI* site for cloning into plasmid pQb185, or p1.45 containing the *XbaI* site for cloning into plasmid pQb10, and a downstream primer p1.46 containing the *HindIII* restriction site were used (recognition sequence of the restriction enzyme underlined):

p1.44 5'-AACC ATG GCA AAT AAG CCA ATG CAA CCG-3' (SEQ ID NO: 139)

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p1.45 5'-AATCTAGAATTTTCTGCGCACCCATCCCGG-3' (SEQ ID NO: 140)

p1.46 5'-AAAAGC TTA AGC AGT AGT ATC AGA CGA TAC G-3' (SEQ ID NO: 141)

5 Two additional primers, p1.47, annealing at the 5' end of the fragment contained in p205-262, and p1.48, annealing at the 3' end of the fragment contained in plasmid p205-246 were used to amplify the fragments in the first PCR. Primers p1.47 and p1.48 are complementary to each other.

p1.47: 5'-GAGTGATCCAACCTCGTTTATCAACTACATTT-  
10 TCAGCAAGTCTG-3' (SEQ ID NO: 142)

p1.48: 5'-CAGACTTGCTGAAAATGTAGTTGATAAACGA-  
GTTGGATCACTC-3' (SEQ ID NO: 143)

In the first two PCR reactions, two fragments were generated. The first fragment was generated with primers p1.45 and p1.48 and template p205-246. The  
15 second fragment was generated with primers p1.47 and p1.46, and template p205-262. Both fragments were used as templates for the second PCR reaction, a splice-overlap extension, with the primer combination p1.45 and p1.46 or p1.44 and p1.46. The product of the two second-step PCR reactions were digested with *XbaI* or *NcoI* respectively, and *HindIII*, and cloned with the same restriction sites into pQb10 or  
20 pQb185 respectively, two pGEM-derived expression vectors under the control of *E.coli* tryptophan operon promoter.

Two plasmids were obtained, pAP283-58 (SEQ ID NO: 27), containing the gene coding for wt AP205 CP (SEQ ID NO: 28) in pQb10, and pAP281-32 (SEQ ID NO: 30) with mutation Pro5→Thr (SEQ ID NO: 29), in pQb185. The coat protein  
25 sequences were verified by DNA sequencing. PAP283-58 contains 49 nucleotides upstream of the ATG codon of the CP, downstream of the *XbaI* site, and contains the putative original ribosomal binding site of the coat protein mRNA.

#### EXAMPLE 21:

30 Expression and Purification of Recombinant AP205 VLP

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#### A. Expression of recombinant AP205 VLP

*E.coli* JM109 was transformed with plasmid pAP283-58. 5 ml of LB liquid medium with 20 µg/ml ampicillin were inoculated with a single colony, and incubated at 37 °C for 16-24 h without shaking.

5 The prepared inoculum was diluted 1:100 in 100-300 ml of LB medium, containing 20 µg/ml ampicillin and incubated at 37 °C overnight without shaking. The resulting second inoculum was diluted 1:50 in 2TY medium, containing 0.2 % glucose and phosphate for buffering, and incubated at 37 °C overnight on a shaker. Cells were harvested by centrifugation and frozen at -80°C.

10

#### B. Purification of recombinant AP205 VLP

Solutions and buffers:

Lysis buffer

50mM Tris-HCl pH 8.0 with 5mM EDTA , 0.1%  
15 tritonX100 and PMSF at 5 micrograms per  
ml.

SAS

Saturated ammonium sulphate in water

Buffer NET.

20 20 mM Tris-HCl, pH 7.8 with 5mM EDTA and  
150 mM NaCl.

PEG

40% (w/v) polyethylenglycol 6000 in NET

25 Lysis:

Frozen cells were resuspended in lysis buffer at 2 ml/g cells. The mixture was sonicated with 22 kH five times for 15 seconds, with intervals of 1min to cool the solution on ice. The lysate was then centrifuged for 20 minutes at 12 000 rpm, using a F34-6-38 rotor (Ependorf). The centrifugation steps described below were all  
30 performed using the same rotor, except otherwise stated. The supernatant was stored

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at 4° C, while cell debris were washed twice with lysis buffer. After centrifugation, the supernatants of the lysate and wash fractions were pooled.

Ammonium-sulphate precipitation can be further used to purify AP205 VLP. In a first step, a concentration of ammonium-sulphate at which AP205 VLP does not precipitate is chosen. The resulting pellet is discarded. In the next step, an ammonium sulphate concentration at which AP205 VLP quantitatively precipitates is selected, and AP205 VLP is isolated from the pellet of this precipitation step by centrifugation (14 000 rpm, for 20 min). The obtained pellet is solubilised in NET buffer.

10

#### Chromatography:

The capsid protein from the pooled supernatants was loaded on a Sepharose 4B column (2.8 X 70 cm), and eluted with NET buffer, at 4 ml/hour/fraction. Fractions 28-40 were collected, and precipitated with ammonium sulphate at 60% saturation. The fractions were analyzed by SDS-PAGE and Western Blot with an antiserum specific for AP205 prior to precipitation. The pellet isolated by centrifugation was resolubilized in NET buffer, and loaded on a Sepharose 2B column (2.3 X 65 cm), eluted at 3 ml/h/fraction. Fractions were analysed by SDS-PAGE, and fractions 44-50 were collected, pooled and precipitated with ammonium sulphate at 60% saturation. The pellet isolated by centrifugation was resolubilized in NET buffer, and purified on a Sepharose 6B column (2.5 X 47 cm), eluted at 3 ml/hour/fraction. The fractions were analysed by SDS-PAGE. Fractions 23-27 were collected, the salt concentration adjusted to 0.5 M, and precipitated with PEG 6000, added from a 40% stock in water and to a final concentration of 13.3%. The pellet isolated by centrifugation was resolubilized in NET buffer, and loaded on the same Sepharose 2B column as above, eluted in the same manner. Fractions 43-53 were collected, and precipitated with ammonium sulphate at a saturation of 60%. The pellet isolated by centrifugation was resolubilized in water, and the obtained protein solution was extensively dialyzed against water. About 10 mg of purified protein per gram of cells could be isolated.

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Examination of the virus-like particles in Electron microscopy showed that they were identical to the phage particles.

Having now fully described the present invention in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious  
5 to one of ordinary skill in the art that the same can be performed by modifying or changing the invention within a wide and equivalent range of conditions, formulations and other parameters without affecting the scope of the invention or any specific embodiment thereof, and that such modifications or changes are intended to  
10 be encompassed within the scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains, and are herein incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually  
15 indicated to be incorporated by reference.

## CLAIMS:

1. A composition comprising:

- 5 (a) a core particle with at least one first attachment site; and  
(b) at least one antigen or antigenic determinant with at least one second attachment site,

wherein said antigen or antigenic determinant is ghrelin or a ghrelin peptide, and wherein said second attachment site being selected from the group consisting of:

- 10 (i) an attachment site not naturally occurring with said antigen or antigenic determinant; and  
(ii) an attachment site naturally occurring with said antigen or antigenic determinant,

15 wherein said second attachment site is capable of association to said first attachment site; and wherein said ghrelin or a ghrelin peptide and said core particle interact through said association to form an ordered and repetitive antigen array.

2. The composition of claim 1, wherein said core particle is selected  
20 from the group consisting of:

- i) a virus;  
ii) a virus-like particle;  
iii) a bacteriophage;  
iv) a virus-like particle of a RNA-phage;  
25 v) a bacterial pilus;  
vi) a viral capsid particle; and  
vii) a recombinant form of (i), (ii), (iii), (iv), (v) or (vi).

3. The composition of claim 1, wherein said core particle comprises a virus-like particle.

5 4. The composition of claim 1, wherein said core particle is a virus-like particle.

5. The composition of claim 4, wherein said virus-like particle is a recombinant virus-like particle.

10

6. The composition of any one of claim 2 or 3, wherein said virus-like particle comprises recombinant proteins, or fragments thereof, selected from the group consisting of:

- (a) recombinant proteins of Hepatitis B virus;
- 15 (b) recombinant proteins of measles virus;
- (c) recombinant proteins of Sindbis virus;
- (d) recombinant proteins of Rotavirus;
- (e) recombinant proteins of Foot-and-Mouth-Disease virus;
- (f) recombinant proteins of Retrovirus;
- 20 (g) recombinant proteins of Norwalk virus;
- (h) recombinant proteins of Alphavirus;
- (i) recombinant proteins of human Papilloma virus;
- (j) recombinant proteins of Polyoma virus;
- (k) recombinant proteins of bacteriophages;
- 25 (l) recombinant proteins of RNA-phages;
- (m) recombinant proteins of Ty;
- (n) recombinant proteins of Q $\beta$ -phage;
- (o) recombinant proteins of GA-phage;
- (p) recombinant proteins of fr-phage;
- 30 (q) recombinant proteins of AP205 phage; and

- (r) fragments of any of the recombinant proteins from (a) to (q).

7. The composition of any one of claim 2 or 3, wherein said virus-like  
5 particle is Hepatitis B virus core antigen.

8. The composition of any one of claim 2 or 3, wherein said virus-like  
particle comprises, or alternatively consists of, recombinant proteins, or fragments  
thereof, of a RNA-phage.

10

9. The composition of claim 8, wherein said RNA-phage is selected  
from the group consisting of:

- (a) bacteriophage Q $\beta$ ;  
(b) bacteriophage R17;  
15 (c) bacteriophage fr;  
(d) bacteriophage GA;  
(e) bacteriophage SP;  
(f) bacteriophage MS2;  
(g) bacteriophage M11;  
20 (h) bacteriophage MX1;  
(i) bacteriophage NL95;  
(k) bacteriophage f2;  
(l) bacteriophage PP7; and  
(m) bacteriophage AP205.

25

10. The composition of any one of claims 2 to 3, wherein said virus-like  
particle comprises, or alternatively consists of, recombinant proteins, or fragments  
thereof, of RNA-phage Q $\beta$ .

11. The composition of any one of claims 2 to 3, wherein said virus-like particle comprises, or alternatively consists of, recombinant proteins, or fragments thereof, of RNA-phage fr.

5 12. The composition of any one of claims 2 to 3, wherein said virus-like particle comprises, or alternatively consists of, recombinant proteins, or fragments thereof, of RNA-phage AP205.

10 13. The composition of any one of claims 3 to 12, wherein the recombinant proteins comprise, or alternatively consist essentially of, or alternatively consist of coat proteins of RNA phages.

14. The composition of claim 13, wherein said coat proteins of RNA phages having an amino acid are selected from the group consisting of:

- 15 (a) SEQ ID NO:4;  
(b) a mixture of SEQ ID NO:4 and SEQ ID NO:5;  
(c) SEQ ID NO:6;  
(d) SEQ ID NO:7;  
(e) SEQ ID NO:8;  
20 (f) SEQ ID NO:9;  
(g) a mixture of SEQ ID NO:9 and SEQ ID NO:10;  
(h) SEQ ID NO:11;  
(i) SEQ ID NO:12;  
(k) SEQ ID NO:13;  
25 (l) SEQ ID NO:14;  
(m) SEQ ID NO:15;  
(n) SEQ ID NO:16; and  
(o) SEQ ID NO:28.

15. The composition of any one of claims 3 to 12, wherein the recombinant proteins comprise, or alternatively consist essentially of, or alternatively consist of mutant coat proteins of RNA phages.

5 16. The composition of claim 15, wherein said RNA-phage is selected from the group consisting of:

- (a) bacteriophage Q $\beta$ ;
- (b) bacteriophage R17;
- (c) bacteriophage fr;
- 10 (d) bacteriophage GA;
- (e) bacteriophage SP;
- (f) bacteriophage MS2;
- (g) bacteriophage M11;
- (h) bacteriophage MX1;
- 15 (i) bacteriophage NL95;
- (k) bacteriophage f2;
- (l) bacteriophage PP7; and
- (m) bacteriophage AP205.

20 17. The composition of claim 15 or 16, wherein said mutant coat proteins of said RNA phage have been modified by removal of at least one lysine residue by way of substitution.

25 18. The composition of claim 15 or 16, wherein said mutant coat proteins of said RNA phage have been modified by addition of at least one lysine residue by way of substitution.

30 19. The composition of claim 15 or 16, wherein said mutant coat proteins of said RNA phage have been modified by deletion of at least one lysine residue.

20. The composition of claim 15 or 16, wherein said mutant coat proteins of said RNA phage have been modified by addition of at least one lysine residue by way of insertion.

5 21. The composition of any one of the preceding claims, wherein said second attachment site is capable of association to said first attachment site through at least one covalent bond.

10 22. The composition of any one of the preceding claims, wherein said second attachment site is capable of association to said first attachment site through at least one non-peptide bond.

23. The composition of any one of the preceding claims, wherein said ghrelin or said ghrelin peptide is fused to said core particle.

15

24. The composition of any one of the preceding claims, wherein said antigen or antigenic determinant is a ghrelin being selected from the group consisting of:

20

(a) human ghrelin;

(b) bovine ghrelin;

(c) sheep ghrelin;

(d) dog ghrelin;

(e) cat ghrelin;

(f) mouse ghrelin;

25

(g) pig ghrelin;

(h) horse ghrelin and

(i) a peptide or fragment thereof of any ghrelin of (a)- (h).

25. The composition of any one of the preceding claims, wherein said antigen or antigenic determinant is ghrelin peptide being selected from the group consisting of:

- (a) human ghrelin peptide;
- 5 (b) bovine ghrelin peptide;
- (c) sheep ghrelin peptide;
- (d) dog ghrelin peptide;
- (e) cat ghrelin peptide;
- (f) mouse ghrelin peptide;
- 10 (g) pig ghrelin peptide;
- (h) horse ghrelin peptide and
- (i) a peptide or fragment thereof of any ghrelin of (a)- (h).

26. The composition of any one of the preceding claims, wherein said  
15 antigen or antigenic determinant is a ghrelin peptide.

27. The composition of any one of the preceding claims, wherein said antigen or antigenic determinant is a human ghrelin peptide or a dog ghrelin peptide.

20 28. The composition of any one of the preceding claims, wherein said ghrelin or said ghrelin peptide comprises an amino acid sequence selected from the group consisting of:

- (a) GSSFLSPEHQRVQRKESKKPPAKLQPR (SEQ ID NO: 48)
- (b) GSSFLSPEHQRVQQRKESKKPPAKLQPR (SEQ ID NO: 31)
- 25 (c) GSSFLSPEHQKLQQRKESKKPPAKLQPR (SEQ ID NO: 49)
- (d) GSSFLSPEHQKLQRKESKKPPAKLQPR (SEQ ID NO: 50)
- (e) GSSFLSPEHQKAQQRKESKKPPAKLQPR (SEQ ID NO: 32)
- (f) GSSFLSPEHQKAQRKESKKPPAKLQPR (SEQ ID NO: 51)
- (g) KKPPAKLQPR (SEQ ID NO: 52)
- 30 (h) PPAKLQPR (SEQ ID NO: 53)



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- (i) AKLQPR(SEQ ID NO: 54)
- (j) GSSFLSPEHQ(SEQ ID NO: 55)
- (k) EHQRVQQRKE(SEQ ID NO: 56)
- (l) KLQPR (SEQ ID NO: 59)
- 5 (m) GSSFLSPEHQRVQ (SEQ ID NO: 60)
- (n) QRKESKKPPAKLQPR (SEQ ID NO: 61)
- (o) GSSFLSPEHQKLQ (SEQ ID NO: 62)
- (p) QRKESKKPPAKLQPR (SEQ ID NO: 63)
- (q) EHQRVQQRKES (SEQ ID NO: 111)
- 10 (r) EHQKAQQRKE (SEQ ID NO: 112)
- (s) EHQKAQQRKES (SEQ ID NO: 113)
- (t) EHQKLQQRKE (SEQ ID NO: 114)
- (u) EHQKLQQRKES (SEQ ID NO: 115)
- (v) LSPEHQRVQQ (SEQ ID NO: 116)
- 15 (w) LSPEHQKAQQ (SEQ ID NO: 117)
- (x) LSPEHQKLQQ (SEQ ID NO: 118), and
- (y) GSSFLSP (SEQ ID NO: 119).

29. The composition of any one of the preceding claims, wherein said  
20 ghrelin or said ghrelin peptide has an amino acid sequence selected from the group  
consisting of:

- (a) GSSFLSPEHQRVQQRKESKKPPAKLQPR (SEQ ID NO: 48)
- (b) GSSFLSPEHQRVQQRKESKKPPAKLQPR(SEQ ID NO: 31)
- (c) GSSFLSPEHQKLQQRKESKKPPAKLQPR(SEQ ID NO: 49)
- 25 (d) GSSFLSPEHQKLQRKESKKPPAKLQPR(SEQ ID NO: 50)
- (e) GSSFLSPEHQKAQQRKESKKPPAKLQPR(SEQ ID NO: 32)
- (f) GSSFLSPEHQKAQRKESKKPPAKLQPR(SEQ ID NO: 51)
- (g) KKPPAKLQPR(SEQ ID NO: 52)
- (h) PPAKLQPR(SEQ ID NO: 53)
- 30 (i) AKLQPR(SEQ ID NO: 54)

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- (j) GSSFLSPEHQ(SEQ ID NO: 55)  
(k) EHQRVQQRKE(SEQ ID NO: 56)  
(l) KLQPR (SEQ ID NO: 59)  
(m) GSSFLSPEHQRVQ (SEQ ID NO: 60)  
5 (n) QRKESKKPPAKLQPR (SEQ ID NO: 61)  
(o) GSSFLSPEHQKLQ (SEQ ID NO: 62)  
(p) QRKESKKPPAKLQPR (SEQ ID NO: 63)  
(q) EHQRVQQRKES (SEQ ID NO: 111)  
(r) EHQKAQQRKE (SEQ ID NO: 112)  
10 (s) EHQKAQQRKES (SEQ ID NO: 113)  
(t) EHQKLQQRKE (SEQ ID NO: 114)  
(u) EHQKLQQRKES (SEQ ID NO: 115)  
(v) LSPEHQRVQQ (SEQ ID NO: 116)  
(w) LSPEHQKAQQ (SEQ ID NO: 117)  
15 (x) LSPEHQKLQQ (SEQ ID NO: 118), and  
(y) GSSFLSP (SEQ ID NO: 119).

30. The composition of any one of the preceding claims, wherein said antigen or antigenic determinant is a ghrelin peptide comprising at least one  
20 antigenic site of a ghrelin.

31. The composition of any one of the preceding claims further comprising an amino acid linker, wherein said amino acid linker comprises, or alternatively consists of, said second attachment site.

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32. The composition of any one of the preceding claims, wherein said second attachment or said amino acid linker with said second attachment site is bound to said ghrelin or said ghrelin peptide at its C-terminus, or alternatively at its N-terminus.

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33. The composition of any one of the preceding claims, wherein said second attachment site or said amino acid linker with said second attachment site is selected from the group consisting of:

- (a) GGC;
- 5 (b) GGC-CONH<sub>2</sub>;
- (c) GC;
- (d) GC-CONH<sub>2</sub>;
- (e) C; and
- (f) C-CONH<sub>2</sub>.

10

34. The composition of any one of the preceding claims, wherein said ghrelin or said ghrelin peptide with said at least second attachment site comprising an amino acid sequence selected from the group consisting of:

- (a) CGSSFLSPEHQRVQRKESKKPPAKLQPR(SEQ ID NO: 64)

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- (b) CGSSFLSPEHQRVQQRKESKKPPAKLQPR(SEQ ID NO: 65)  
(c) CGSSFLSPEHQKLQQRKESKKPPAKLQPR(SEQ ID NO: 71)  
(d) CGSSFLSPEHQKLQRKESKKPPAKLQPR(SEQ ID NO: 72)  
(e) CGSSFLSPEHQKAQQRKESKKPPAKLQPR(SEQ ID NO: 77)  
5 (f) CGSSFLSPEHQKAQRKESKKPPAKLQPR(SEQ ID NO: 106)  
(g) GSSFLSPEHQRVQQRKESKKPPAKLQPRC(SEQ ID NO: 66)  
(h) GSSFLSPEHQRVQQRKESKKPPAKLQPRGC (SEQ ID NO: 120)  
(i) GSSFLSPEHQRVQQRKESKKPPAKLQPRC(SEQ ID NO: 67)  
(j) GSSFLSPEHQRVQQRKESKKPPAKLQPRGC (SEQ ID NO: 121)  
10 (k) GSSFLSPEHQKLQQRKESKKPPAKLQPRC(SEQ ID NO: 73)  
(l) GSSFLSPEHQKLQQRKESKKPPAKLQPRGC (SEQ ID NO: 123)  
(m) GSSFLSPEHQKLQRKESKKPPAKLQPRC(SEQ ID NO: 74)  
(n) GSSFLSPEHQKLQRKESKKPPAKLQPRGC (SEQ ID NO: 124)  
(o) GSSFLSPEHQKAQQRKESKKPPAKLQPRC(SEQ ID NO: 105)  
15 (p) GSSFLSPEHQKAQRKESKKPPAKLQPRC(SEQ ID NO: 107)  
(q) CKKPPAKLQPR(SEQ ID NO: 108)  
(r) CPPAKLQPR(SEQ ID NO: 70)  
(s) CAKLQPR(SEQ ID NO: 109)  
(t) GSSFLSPEHQC(SEQ ID NO: 110)  
20 (u) CEHQRVQQRKE(SEQ ID NO: 76)  
(v) GSSFLSPEHQRVQC (SEQ ID NO: 68)  
(w) GSSFLSPEHQRVQGC (SEQ ID NO: 122)  
(x) CQRKESKKPPAKLQPR (SEQ ID NO: 69)  
(y) GSSFLSPEHQKLQC (SEQ ID NO: 75)  
25 (z) GSSFLSPEHQKLQGC (SEQ ID NO: 125)  
(aa) GSSFLSPEHQKAQRKESKKPPAKLQPRC (SEQ ID NO: 126)  
(bb) GSSFLSPEHQKAQRKESKKPPAKLQPRGC (SEQ ID NO: 127)  
(cc) GSSFLSPEHQKAQQRKESKKPPAKLQPRC (SEQ ID NO: 128)  
(dd) GSSFLSPEHQKAQQRKESKKPPAKLQPRGC (SEQ ID NO: 129)  
30 (ee) GSSFLSPEHQKAQC (SEQ ID NO: 130)  
(ff) GSSFLSPEHQKAQGC (SEQ ID NO: 131)

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- (gg) GGSSFLSPEHQGC (SEQ ID NO: 132)  
 (hh) CKKPPAKLQPR (SEQ ID NO: 133)  
 (ii) CEHQKAQQRKE (SEQ ID NO: 134)  
 (jj) CEHQKAQQRKES (SEQ ID NO: 135)  
 5 (kk) CLSPEHQKAQQ (SEQ ID NO: 136)  
 (ll) CEHQRVQQRKES (SEQ ID NO: 137) and  
 (mm) CLSPEHQRVQQ (SEQ ID NO: 138).

35. The composition of any one of the preceding claims, wherein said  
 10 ghrelin or said ghrelin peptide with said at least second attachment site consisting of  
 an amino acid sequence selected from the group consisting of:

- (a) CGSSFLSPEHQRVQRKESKKPPAKLQPR(SEQ ID NO: 64)  
 (b) CGSSFLSPEHQRVQQRKESKKPPAKLQPR(SEQ ID NO: 65)  
 (c) CGSSFLSPEHQKLQQRKESKKPPAKLQPR(SEQ ID NO: 71)  
 15 (d) CGSSFLSPEHQKLQRKESKKPPAKLQPR(SEQ ID NO: 72)  
 (e) CGSSFLSPEHQKAQQRKESKKPPAKLQPR(SEQ ID NO: 77)  
 (f) CGSSFLSPEHQKAQRKESKKPPAKLQPR(SEQ ID NO: 106)  
 (g) GSSFLSPEHQRVQRKESKKPPAKLQPRC(SEQ ID NO: 66)  
 (h) GSSFLSPEHQRVQRKESKKPPAKLQPRGC (SEQ ID NO: 120)  
 20 (i) GSSFLSPEHQRVQQRKESKKPPAKLQPRC(SEQ ID NO: 67)  
 (j) GSSFLSPEHQRVQQRKESKKPPAKLQPRGC (SEQ ID NO: 121)  
 (k) GSSFLSPEHQKLQQRKESKKPPAKLQPRC(SEQ ID NO: 73)  
 (l) GSSFLSPEHQKLQQRKESKKPPAKLQPRGC (SEQ ID NO: 123)  
 (m) GSSFLSPEHQKLQRKESKKPPAKLQPRC(SEQ ID NO: 74)  
 25 (n) GSSFLSPEHQKLQRKESKKPPAKLQPRGC (SEQ ID NO: 124)  
 (o) GSSFLSPEHQKAQQRKESKKPPAKLQPRC(SEQ ID NO: 105)  
 (p) GSSFLSPEHQKAQRKESKKPPAKLQPRC(SEQ ID NO: 107)  
 (q) CKKPPAKLQPR(SEQ ID NO: 108)  
 (r) CPPAKLQPR(SEQ ID NO: 70)  
 30 (s) CAKLQPR(SEQ ID NO: 109)

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- (t) GSSFLSPEHQ(CSEQ ID NO: 110)
- (u) CEHQRVQQRKE(SEQ ID NO: 76)
- (v) GSSFLSPEHQRVQC (SEQ ID NO: 68)
- (w) GSSFLSPEHQRVQGC (SEQ ID NO: 122)
- 5 (x) CQRKESKKPPAKLQPR (SEQ ID NO: 69)
- (y) GSSFLSPEHQKLQC (SEQ ID NO: 75)
- (z) GSSFLSPEHQKLQGC (SEQ ID NO: 125)
- (aa) GSSFLSPEHQKAQRKESKKPPAKLQPRC (SEQ ID NO: 126)
- (bb) GSSFLSPEHQKAQRKESKKPPAKLQPRGC (SEQ ID NO: 127)
- 10 (cc) GSSFLSPEHQKAQQRKESKKPPAKLQPRC (SEQ ID NO: 128)
- (dd) GSSFLSPEHQKAQQRKESKKPPAKLQPRGC (SEQ ID NO: 129)
- (ee) GSSFLSPEHQKAQC (SEQ ID NO: 130)
- (ff) GSSFLSPEHQKAQGC (SEQ ID NO: 131)
- (gg) GGSSFLSPEHQGC (SEQ ID NO: 132)
- 15 (hh) CKKPPAKLQPR (SEQ ID NO: 133)
- (ii) CEHQKAQQRKE (SEQ ID NO: 134)
- (jj) CEHQKAQQRKES (SEQ ID NO: 135)
- (kk) CLSPEHQKAQQ (SEQ ID NO: 136)
- (ll) CEHQRVQQRKES (SEQ ID NO: 137) and
- 20 (mm) CLSPEHQRVQQ (SEQ ID NO: 138).

36. The composition of any one of the preceding claims, wherein said antigen or antigenic determinant is a biologically inactive form of ghrelin.

25 37. The composition of claim 36, wherein said antigen or antigenic determinant is a ghrelin peptide, not containing a n-octanoyl-modification.

38. A pharmaceutical composition comprising:

(a) the composition of claim 1; and

30 (b) an acceptable pharmaceutical carrier.

39. The pharmaceutical composition of claim 38 further comprising an adjuvant.

5 40. The pharmaceutical composition of any one of claims 38 or 39, wherein said vaccine composition is devoid of an adjuvant.

41. A vaccine composition comprising the composition of any one of the preceding claims.

10

42. The vaccine composition of claim 41, further comprising an adjuvant.

43. The vaccine composition of any one of claims 41 or 42, wherein said vaccine composition is devoid of an adjuvant.

15

44. The vaccine composition of any one of claims 41 to 43, wherein said virus-like particle comprises recombinant proteins or fragments thereof, of RNA-phage Q $\beta$ .

20

45. The vaccine composition of any one of claims 41 to 44, wherein said ghrelin or said ghrelin peptide being selected from the group consisting of:

(a) human ghrelin;

(b) bovine ghrelin;

(c) sheep ghrelin;

25

(d) dog ghrelin;

(e) cat ghrelin;

(f) mouse ghrelin;

(g) pig ghrelin;

(h) horse ghrelin and

30

(k) a peptide or fragment thereof of any ghrelin of (a)- (h).

46. The vaccine composition of any one of claims 41 to 45, wherein said ghrelin or said ghrelin peptide comprises an amino acid sequence selected from the group consisting of:

- 5 (a) GSSFLSPEHQRVQRKESKKPPAKLQPR (SEQ ID NO: 48)  
(b) GSSFLSPEHQRVQQRKESKKPPAKLQPR(SEQ ID NO: 31)  
(c) GSSFLSPEHQKLQQRKESKKPPAKLQPR(SEQ ID NO: 49)  
(d) GSSFLSPEHQKLQRKESKKPPAKLQPR(SEQ ID NO: 50)  
(e) GSSFLSPEHQKAQQRKESKKPPAKLQPR(SEQ ID NO: 32)
- 10 (f) GSSFLSPEHQKAQRKESKKPPAKLQPR(SEQ ID NO: 51)  
(g) KKPPAKLQPR(SEQ ID NO: 52)  
(h) PPAKLQPR(SEQ ID NO: 53)  
(i) AKLQPR(SEQ ID NO: 54)  
(j) GSSFLSPEHQ(SEQ ID NO: 55)
- 15 (k) EHQRVQQRKE(SEQ ID NO: 56)  
(l) KLQPR (SEQ ID NO: 59)  
(m) GSSFLSPEHQRVQ (SEQ ID NO: 60)  
(n) QRKESKKPPAKLQPR (SEQ ID NO: 61)  
(o) GSSFLSPEHQKLQ (SEQ ID NO: 62)
- 20 (p) QRKESKKPPAKLQPR (SEQ ID NO: 63)  
(q) EHQRVQQRKES (SEQ ID NO: 111)  
(r) EHQKAQQRKE (SEQ ID NO: 112)  
(s) EHQKAQQRKES (SEQ ID NO: 113)  
(t) EHQKLQQRKE (SEQ ID NO: 114)
- 25 (u) EHQKLQQRKES (SEQ ID NO: 115)  
(v) LSPEHQRVQQ (SEQ ID NO: 116)  
(w) LSPEHQKAQQ (SEQ ID NO: 117)  
(x) LSPEHQKLQQ (SEQ ID NO: 118), and  
(y) GSSFLSP (SEQ ID NO: 119).
- 30



47. The vaccine composition of any one of claims 41 to 45, wherein said ghrelin or said ghrelin peptide has an amino acid sequence selected from the group consisting of:

- (a) GSSFLSPEHQRVQRKESKKPPAKLQPR (SEQ ID NO: 48)
- 5 (b) GSSFLSPEHQRVQQRKESKKPPAKLQPR(SEQ ID NO: 31)
- (c) GSSFLSPEHQKLQQRKESKKPPAKLQPR(SEQ ID NO: 49)
- (d) GSSFLSPEHQKLQRKESKKPPAKLQPR(SEQ ID NO: 50)
- (e) GSSFLSPEHQKAQQRKESKKPPAKLQPR(SEQ ID NO: 32)
- (f) GSSFLSPEHQKAQRKESKKPPAKLQPR(SEQ ID NO: 51)
- 10 (g) KKPPAKLQPR(SEQ ID NO: 52)
- (h) PPAKLQPR(SEQ ID NO: 53)
- (i) AKLQPR(SEQ ID NO: 54)
- (j) GSSFLSPEHQ(SEQ ID NO: 55)
- (k) EHQRVQQRKE(SEQ ID NO: 56)
- 15 (l) KLQPR (SEQ ID NO: 59)
- (m) GSSFLSPEHQRVQ (SEQ ID NO: 60)
- (n) QRKESKKPPAKLQPR (SEQ ID NO: 61)
- (o) GSSFLSPEHQKLQ (SEQ ID NO: 62)
- (p) QRKESKKPPAKLQPR (SEQ ID NO: 63)
- 20 (q) EHQRVQQRKES (SEQ ID NO: 111)
- (r) EHQKAQQRKE (SEQ ID NO: 112)
- (s) EHQKAQQRKES (SEQ ID NO: 113)
- (t) EHQKLQQRKE (SEQ ID NO: 114)
- (u) EHQKLQQRKES (SEQ ID NO: 115)
- 25 (v) LSPEHQRVQQ (SEQ ID NO: 116)
- (w) LSPEHQKAQQ (SEQ ID NO: 117)
- (x) LSPEHQKLQQ (SEQ ID NO: 118), and
- (y) GSSFLSP (SEQ ID NO: 119).

48. The vaccine composition of any one of claims 41 to 45, wherein said antigen or antigenic determinant is a ghrelin peptide comprising an amino acid sequence selected from the group consisting of:

- (a) GSSFLSPEHQRVQRKESKKPPAKLQPR (SEQ ID NO: 48)
- 5 (b) GSSFLSPEHQRVQQRKESKKPPAKLQPR (SEQ ID NO: 31)
- (c) GSSFLSPEHQKLQQRKESKKPPAKLQPR (SEQ ID NO: 49)
- (d) GSSFLSPEHQKLQRKESKKPPAKLQPR (SEQ ID NO: 50)
- (e) GSSFLSPEHQKAQQRKESKKPPAKLQPR (SEQ ID NO: 32)
- (f) GSSFLSPEHQKAQRKESKKPPAKLQPR (SEQ ID NO: 51)
- 10 (g) KKPPAKLQPR (SEQ ID NO: 52)
- (h) PPAKLQPR (SEQ ID NO: 53)
- (i) AKLQPR (SEQ ID NO: 54)
- (j) GSSFLSPEHQ (SEQ ID NO: 55)
- (k) EHQRVQQRKE (SEQ ID NO: 56)
- 15 (l) KLQPR (SEQ ID NO: 59)
- (m) GSSFLSPEHQRVQ (SEQ ID NO: 60)
- (n) QRKESKKPPAKLQPR (SEQ ID NO: 61)
- (o) GSSFLSPEHQKLQ (SEQ ID NO: 62)
- (p) QRKESKKPPAKLQPR (SEQ ID NO: 63)
- 20 (q) EHQRVQQRKES (SEQ ID NO: 111)
- (r) EHQKAQQRKE (SEQ ID NO: 112)
- (s) EHQKAQQRKES (SEQ ID NO: 113)
- (t) EHQKLQQRKE (SEQ ID NO: 114)
- (u) EHQKLQQRKES (SEQ ID NO: 115)
- 25 (v) LSPEHQRVQQ (SEQ ID NO: 116)
- (w) LSPEHQKAQQ (SEQ ID NO: 117)
- (x) LSPEHQKLQQ (SEQ ID NO: 118), and
- (y) GSSFLSP (SEQ ID NO: 119).

49. The vaccine composition of any one of claims 41 to 45, wherein said antigen or antigenic determinant is a ghrelin peptide consisting of an amino acid sequence selected from the group consisting of:

- (a) GSSFLSPEHQRVQRKESKKPPAKLQPR (SEQ ID NO: 48)
- 5 (b) GSSFLSPEHQRVQQRKESKKPPAKLQPR(SEQ ID NO: 31)
- (c) GSSFLSPEHQKLQQRKESKKPPAKLQPR(SEQ ID NO: 49)
- (d) GSSFLSPEHQKLQRKESKKPPAKLQPR(SEQ ID NO: 50)
- (e) GSSFLSPEHQKAQQRKESKKPPAKLQPR(SEQ ID NO: 32)
- (f) GSSFLSPEHQKAQRKESKKPPAKLQPR(SEQ ID NO: 51)
- 10 (g) KKPPAKLQPR(SEQ ID NO: 52)
- (h) PPAKLQPR(SEQ ID NO: 53)
- (i) AKLQPR(SEQ ID NO: 54)
- (j) GSSFLSPEHQ(SEQ ID NO: 55)
- (k) EHQRVQQRKE(SEQ ID NO: 56)
- 15 (l) KLQPR (SEQ ID NO: 59)
- (m) GSSFLSPEHQRVQ (SEQ ID NO: 60)
- (n) QRKESKKPPAKLQPR (SEQ ID NO: 61)
- (o) GSSFLSPEHQKLQ (SEQ ID NO: 62)
- (p) QRKESKKPPAKLQPR (SEQ ID NO: 63)
- 20 (q) EHQRVQQRKES (SEQ ID NO: 111)
- (r) EHQKAQQRKE (SEQ ID NO: 112)
- (s) EHQKAQQRKES (SEQ ID NO: 113)
- (t) EHQKLQQRKE (SEQ ID NO: 114)
- (u) EHQKLQQRKES (SEQ ID NO: 115)
- 25 (v) LSPEHQRVQQ (SEQ ID NO: 116)
- (w) LSPEHQKAQQ (SEQ ID NO: 117)
- (x) LSPEHQKLQQ (SEQ ID NO: 118), and
- (y) GSSFLSP (SEQ ID NO: 119).

50. The vaccine composition of any one of claims 41 to 49, wherein said antigen or antigenic determinant with said at least second attachment site comprising an amino acid sequence selected from the group consisting of:

- (a) CGSSFLSPEHQRVQRKESKKPPAKLQPR(SEQ ID NO: 64)
- 5 (b) CGSSFLSPEHQRVQQRKESKKPPAKLQPR(SEQ ID NO: 65)
- (c) CGSSFLSPEHQKLQQRKESKKPPAKLQPR(SEQ ID NO: 71)
- (d) CGSSFLSPEHQKLQRKESKKPPAKLQPR(SEQ ID NO: 72)
- (e) CGSSFLSPEHQKAQQRKESKKPPAKLQPR(SEQ ID NO: 77)
- (f) CGSSFLSPEHQKAQRKESKKPPAKLQPR(SEQ ID NO: 106)
- 10 (g) GSSFLSPEHQRVQRKESKKPPAKLQPRC(SEQ ID NO: 66)
- (h) GSSFLSPEHQRVQRKESKKPPAKLQPRGC (SEQ ID NO: 120)
- (i) GSSFLSPEHQRVQQRKESKKPPAKLQPRC(SEQ ID NO: 67)
- (j) GSSFLSPEHQRVQQRKESKKPPAKLQPRGC (SEQ ID NO: 121)
- (k) GSSFLSPEHQKLQQRKESKKPPAKLQPRC(SEQ ID NO: 73)
- 15 (l) GSSFLSPEHQKLQQRKESKKPPAKLQPRGC (SEQ ID NO: 123)
- (m) GSSFLSPEHQKLQRKESKKPPAKLQPRC(SEQ ID NO: 74)
- (n) GSSFLSPEHQKLQRKESKKPPAKLQPRGC (SEQ ID NO: 124)
- (o) GSSFLSPEHQKAQQRKESKKPPAKLQPRC(SEQ ID NO: 105)
- (p) GSSFLSPEHQKAQRKESKKPPAKLQPRC(SEQ ID NO: 107)
- 20 (q) CKKPPAKLQPR(SEQ ID NO: 108)
- (r) CPPAKLQPR(SEQ ID NO: 70)
- (s) CAKLQPR(SEQ ID NO: 109)
- (t) GSSFLSPEHQC(SEQ ID NO: 110)
- (u) CEHQRVQQRKE(SEQ ID NO: 76)
- 25 (v) GSSFLSPEHQRVQC (SEQ ID NO: 68)
- (w) GSSFLSPEHQRVQGC (SEQ ID NO: 122)
- (x) CQRKESKKPPAKLQPR (SEQ ID NO: 69)
- (y) GSSFLSPEHQKLQC (SEQ ID NO: 75)
- (z) GSSFLSPEHQKLQGC (SEQ ID NO: 125)
- 30 (aa) GSSFLSPEHQKAQRKESKKPPAKLQPRC (SEQ ID NO: 126)

- (bb) GSSFLSPEHQKAQRKESKKPPAKLQPRGC (SEQ ID NO: 127)  
(cc) GSSFLSPEHQKAQQRKESKKPPAKLQPRC (SEQ ID NO: 128)  
(dd) GSSFLSPEHQKAQQRKESKKPPAKLQPRGC (SEQ ID NO: 129)  
(ee) GSSFLSPEHQKAQC (SEQ ID NO: 130)  
5 (ff) GSSFLSPEHQKAQGC (SEQ ID NO: 131)  
(gg) GGSSFLSPEHQGC (SEQ ID NO: 132)  
(hh) CKKPPAKLQPR (SEQ ID NO: 133)  
(ii) CEHQKAQQRKE (SEQ ID NO: 134)  
(jj) CEHQKAQQRKES (SEQ ID NO: 135)  
10 (kk) CLSPEHQKAQQ (SEQ ID NO: 136)  
(ll) CEHQRVQQRKES (SEQ ID NO: 137) and  
(mm) CLSPEHQRVQQ (SEQ ID NO: 138).

51. A process for producing a composition of any one of the preceding  
15 claims comprising:
- (a) providing a core particle with at least one first attachment site;  
(b) providing at least one antigen or antigenic determinant with at  
least one second attachment site,  
wherein said antigen or antigenic determinant is a ghrelin or ghrelin  
20 peptide, and wherein said second attachment site being selected from  
the group consisting of:
- (i) an attachment site not naturally occurring with said  
antigen or antigenic determinant; and  
(ii) an attachment site naturally occurring with said antigen  
25 or antigenic determinant; and  
wherein said second attachment site is capable of association to said  
first attachment site; and
- (c) combining said core particle and said at least one antigen or  
antigenic determinant, wherein said antigen or antigenic

determinant and said core particle interact through said association to form an ordered and repetitive antigen array.

52. A composition of any one of the claims 1 to 50 for use in a method of immunization comprising administering said composition to an animal or human.

53. The composition of claim 52, wherein said antigen or antigenic determinant is a self-antigen.

54. The composition of any one of claims 52 to 53, wherein said animal is a human, and wherein said antigen or antigenic determinant is a human ghrelin or a human ghrelin peptide.

55. The composition of any one of claims 52 to 54, wherein said animal is of feline origin, and wherein said antigen or antigenic determinant is a ghrelin or ghrelin peptide.

56. The composition of any one of claims 52 to 55, wherein said animal is of canine origin, and wherein said antigen or antigenic determinant is a feline ghreline or ghreline peptide.

57. Use of a composition of any one of the claims 1 to 50 for the manufacture of a medicament for use in a method of immunization, wherein said composition is to be administered to an animal or human.

58. The use of claim 57, wherein said antigen or antigenic determinant is a self-antigen.

59. The use of any one of claims 57 to 58, wherein said animal is a human, and wherein said antigen or antigenic determinant is a human ghrelin or a human ghrelin peptide.

5 60. The use of any one of claims 57 to 59, wherein said animal is of feline origin, and wherein said antigen or antigenic determinant is a ghrelin or ghrelin peptide.

61. The use of any one of claims 57 to 60, wherein said animal is of  
10 canine origin, and wherein said antigen or antigenic determinant is a feline ghreline or ghreline peptide.

62. Composition of any one of claims 1 to 50 for use as a medicament.

15 63. Use of a composition of any one of claims 1 to 50 for the manufacture of a medicament for treatment of obesity.

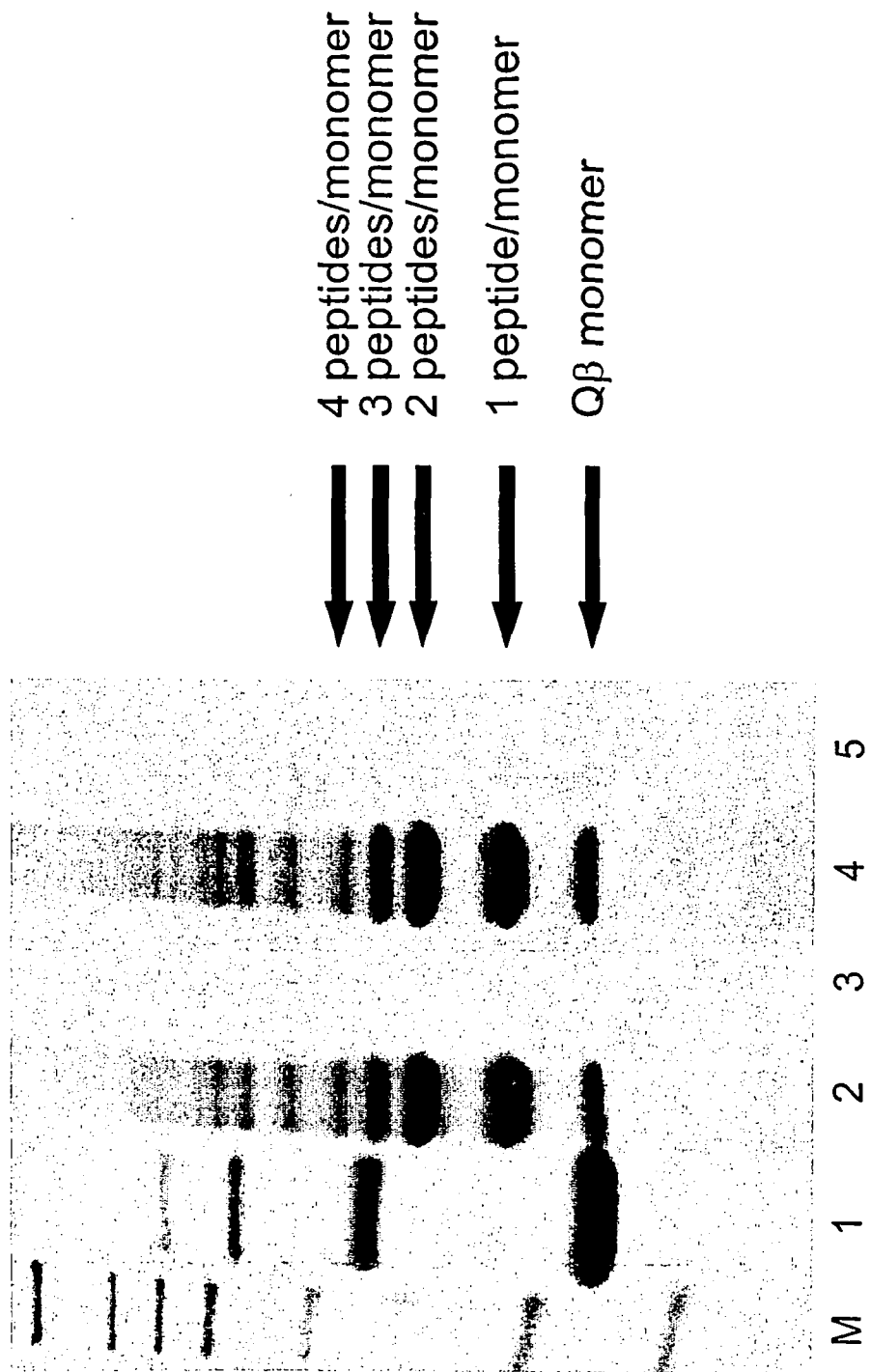


Fig. 1



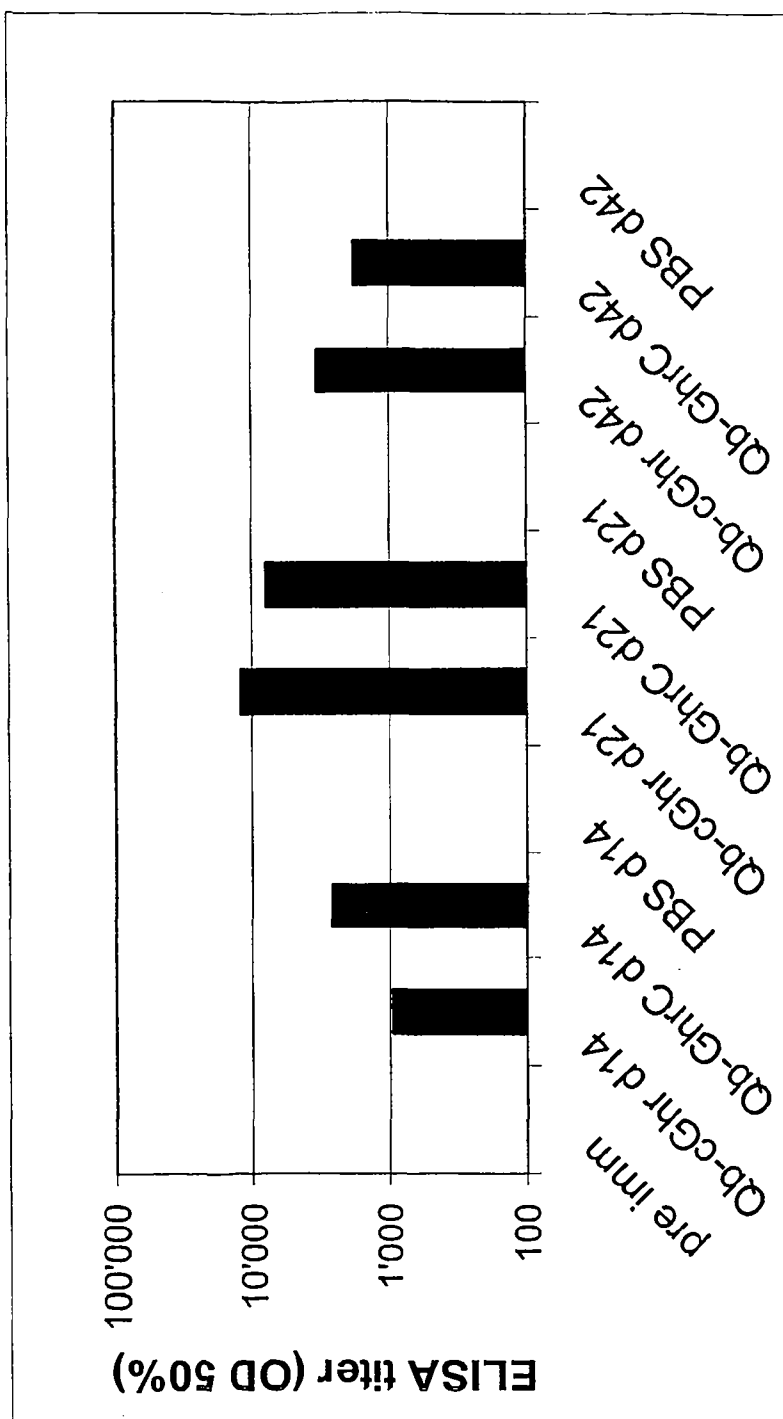
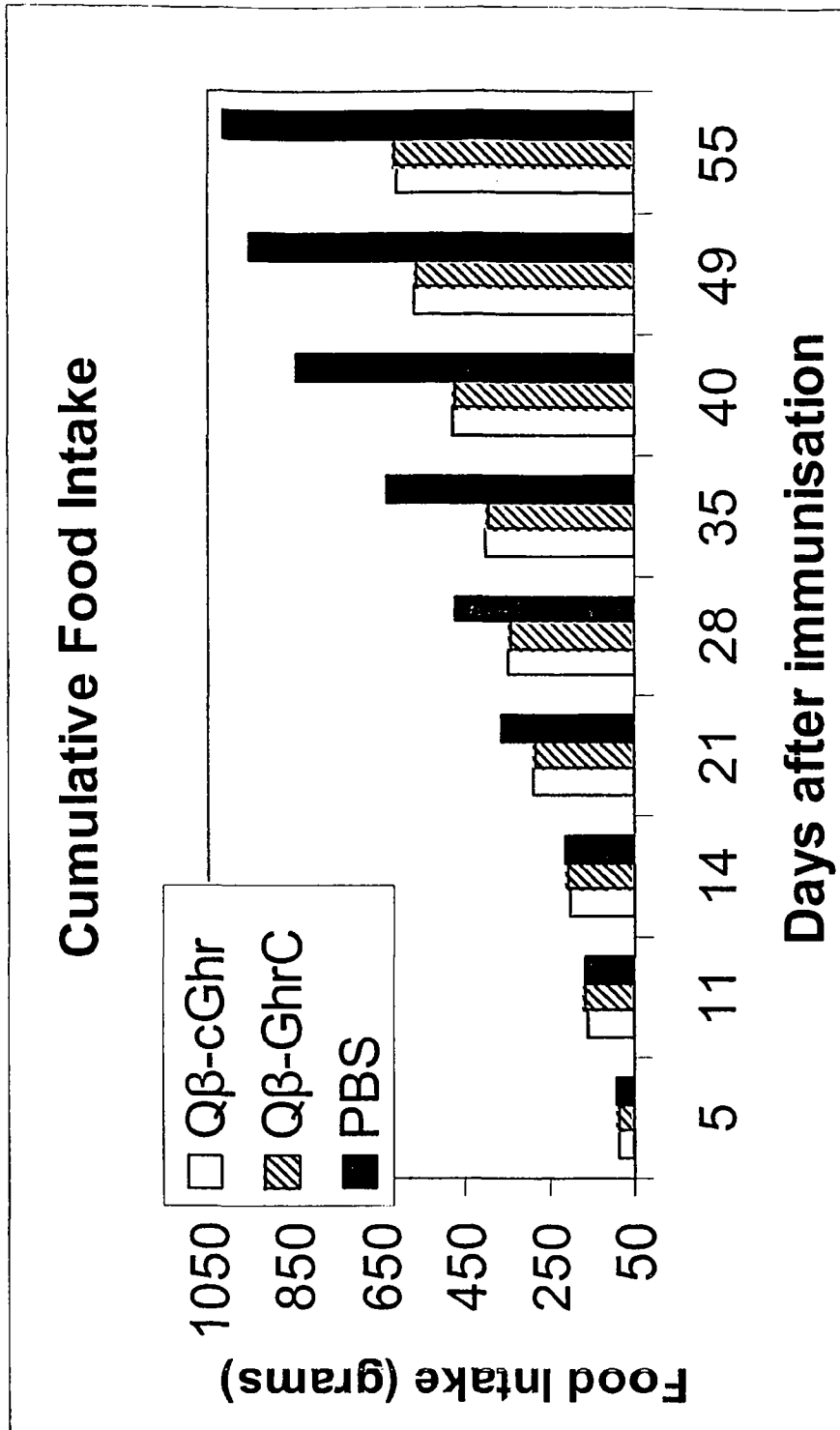


Fig. 2



**Fig. 3**

PA040WO\_sequence\_listing.ST25.txt  
SEQUENCE LISTING

<110> Cytos Biotechnology AG  
 Bachmann, Martin F  
 Fulurija, Alma

<120> Grehlin-Carrier Conjugates

<130> PA040WO

<150> US 60/396,638  
 <151> 2002-07-19

<160> 146

<170> PatentIn version 3.2

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 <211> 172  
 <212> PRT  
 <213> Escherichia coli

<400> 1

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Ser Ile Ser Gln Lys Ser Ala Asp Gln Ser Ile Asp Phe Gly Gln Leu  
 35 40 45

Ser Lys Ser Phe Leu Ala Asn Asp Gly Gln Ser Lys Pro Met Asn Leu  
 50 55 60

Asp Ile Glu Leu Val Asn Cys Asp Ile Thr Ala Phe Lys Asn Gly Asn  
 65 70 75 80

Ala Lys Thr Gly Ser Val Lys Leu Ala Phe Thr Gly Pro Thr Val Ser  
 85 90 95

Gly His Pro Ser Glu Leu Ala Thr Asn Gly Gly Pro Gly Thr Ala Ile  
 100 105 110

Met Ile Gln Ala Ala Gly Lys Asn Val Pro Phe Asp Gly Thr Glu Gly  
 115 120 125

Asp Pro Asn Leu Leu Lys Asp Gly Asp Asn Val Leu His Tyr Thr Thr  
 130 135 140

Val Gly Lys Lys Ser Ser Asp Gly Asn Ala Gln Ile Thr Glu Gly Ala  
 145 150 155 160

Phe Ser Gly Val Ala Thr Phe Asn Leu Ser Tyr Gln  
 165 170

PA040WO\_sequence\_listing.ST25.txt

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 <212> PRT  
 <213> Escherichia coli

<400> 2

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 1 5 10 15

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 20 25 30

Val His Phe Lys Gly Glu Val Val Asn Ala Ala Cys Ala Val Asp Ala  
 35 40 45

Gly Ser Val Asp Gln Thr Val Gln Leu Gly Gln Val Arg Thr Ala Ser  
 50 55 60

Leu Ala Gln Glu Gly Ala Thr Ser Ser Ala Val Gly Phe Asn Ile Gln  
 65 70 75 80

Leu Asn Asp Cys Asp Thr Asn Val Ala Ser Lys Ala Ala Val Ala Phe  
 85 90 95

Leu Gly Thr Ala Ile Asp Ala Gly His Thr Asn Val Leu Ala Leu Gln  
 100 105 110

Ser Ser Ala Ala Gly Ser Ala Thr Asn Val Gly Val Gln Ile Leu Asp  
 115 120 125

Arg Thr Gly Ala Ala Leu Thr Leu Asp Gly Ala Thr Phe Ser Ser Glu  
 130 135 140

Thr Thr Leu Asn Asn Gly Thr Asn Thr Ile Pro Phe Gln Ala Arg Tyr  
 145 150 155 160

Phe Ala Thr Gly Ala Ala Thr Pro Gly Ala Ala Asn Ala Asp Ala Thr  
 165 170 175

Phe Lys Val Gln Tyr Gln  
 180

<210> 3  
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 gtttgtccgc gatgctttac ctctatgagt caaaatggcc ccaatgtttc atcttttggg 180  
 ggaaactgtg cagtgttggc agtcaaactc gttgacaaac aaagtgtaca gaacgactgc 240

## PA040WO\_sequence\_listing.ST25.txt

ccatgtcgat ttagaaatag tttttgaaa ggaaagcagc atgaaaatta aaactctggc 300  
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 ggtaaatggt gggaccgttc actttaaagg ggaagttggt aacgccgctt gcgcagttga 420  
 tgcaggctct gttgatcaaa ccgttcagtt aggacagggt cgtaccgcat cgctggcaca 480  
 ggaaggagca accagttctg ctgtcggttt taacattcag ctgaatgatt gcgataccaa 540  
 tgttgcatct aaagccgctg ttgccttttt aggtacggcg attgatgcgg gtcataccaa 600  
 cgttctggct ctgcagagtt cagctgcggg tagcgcaaca aacgttggtg tgcagatcct 660  
 ggacagaacg ggtgctgcgc tgacgctgga tggcgcaaca tttagttcag aaacaaccct 720  
 gaataacgga accaatacca ttccgttcca ggcgcggttat tttgcaaccg gggccgcaac 780  
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 tcagggacgt tca 853

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 <213> Bacteriophage Q-beta

<400> 4

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 1 5 10 15

Gln Thr Leu Val Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly Val  
 20 25 30

Ala Ser Leu Ser Gln Ala Gly Ala Val Pro Ala Leu Glu Lys Arg Val  
 35 40 45

Thr Val Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys Val  
 50 55 60

Gln Val Lys Ile Gln Asn Pro Thr Ala Cys Thr Ala Asn Gly Ser Cys  
 65 70 75 80

Asp Pro Ser Val Thr Arg Gln Ala Tyr Ala Asp Val Thr Phe Ser Phe  
 85 90 95

Thr Gln Tyr Ser Thr Asp Glu Glu Arg Ala Phe Val Arg Thr Glu Leu  
 100 105 110

Ala Ala Leu Leu Ala Ser Pro Leu Leu Ile Asp Ala Ile Asp Gln Leu  
 115 120 125

Asn Pro Ala Tyr  
 130

<210> 5

## PA040W0\_sequence\_listing.ST25.txt

&lt;211&gt; 329

&lt;212&gt; PRT

&lt;213&gt; Bacteriophage Q-beta

&lt;400&gt; 5

Met Ala Lys Leu Glu Thr Val Thr Leu Gly Asn Ile Gly Lys Asp Gly  
1 5 10 15

Lys Gln Thr Leu Val Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly  
20 25 30

Val Ala Ser Leu Ser Gln Ala Gly Ala Val Pro Ala Leu Glu Lys Arg  
35 40 45

Val Thr Val Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys  
50 55 60

Val Gln Val Lys Ile Gln Asn Pro Thr Ala Cys Thr Ala Asn Gly Ser  
65 70 75 80

Cys Asp Pro Ser Val Thr Arg Gln Ala Tyr Ala Asp Val Thr Phe Ser  
85 90 95

Phe Thr Gln Tyr Ser Thr Asp Glu Glu Arg Ala Phe Val Arg Thr Glu  
100 105 110

Leu Ala Ala Leu Leu Ala Ser Pro Leu Leu Ile Asp Ala Ile Asp Gln  
115 120 125

Leu Asn Pro Ala Tyr Trp Thr Leu Leu Ile Ala Gly Gly Gly Ser Gly  
130 135 140

Ser Lys Pro Asp Pro Val Ile Pro Asp Pro Pro Ile Asp Pro Pro Pro  
145 150 155 160

Gly Thr Gly Lys Tyr Thr Cys Pro Phe Ala Ile Trp Ser Leu Glu Glu  
165 170 175

Val Tyr Glu Pro Pro Thr Lys Asn Arg Pro Trp Pro Ile Tyr Asn Ala  
180 185 190

Val Glu Leu Gln Pro Arg Glu Phe Asp Val Ala Leu Lys Asp Leu Leu  
195 200 205

Gly Asn Thr Lys Trp Arg Asp Trp Asp Ser Arg Leu Ser Tyr Thr Thr  
210 215 220

Phe Arg Gly Cys Arg Gly Asn Gly Tyr Ile Asp Leu Asp Ala Thr Tyr  
225 230 235 240

Leu Ala Thr Asp Gln Ala Met Arg Asp Gln Lys Tyr Asp Ile Arg Glu  
245 250 255

## PA040WO\_sequence\_listing.ST25.txt

Gly Lys Lys Pro Gly Ala Phe Gly Asn Ile Glu Arg Phe Ile Tyr Leu  
 260 265 270

Lys Ser Ile Asn Ala Tyr Cys Ser Leu Ser Asp Ile Ala Ala Tyr His  
 275 280 285

Ala Asp Gly Val Ile Val Gly Phe Trp Arg Asp Pro Ser Ser Gly Gly  
 290 295 300

Ala Ile Pro Phe Asp Phe Thr Lys Phe Asp Lys Thr Lys Cys Pro Ile  
 305 310 315 320

Gln Ala Val Ile Val Val Pro Arg Ala  
 325

<210> 6  
 <211> 129  
 <212> PRT  
 <213> Bacteriophage R17

<400> 6

Ala Ser Asn Phe Thr Gln Phe Val Leu Val Asn Asp Gly Gly Thr Gly  
 1 5 10 15

Asn Val Thr Val Ala Pro Ser Asn Phe Ala Asn Gly Val Ala Glu Trp  
 20 25 30

Ile Ser Ser Asn Ser Arg Ser Gln Ala Tyr Lys Val Thr Cys Ser Val  
 35 40 45

Arg Gln Ser Ser Ala Gln Asn Arg Lys Tyr Thr Ile Lys Val Glu Val  
 50 55 60

Pro Lys Val Ala Thr Gln Thr Val Gly Gly Val Glu Leu Pro Val Ala  
 65 70 75 80

Ala Trp Arg Ser Tyr Leu Asn Met Glu Leu Thr Ile Pro Ile Phe Ala  
 85 90 95

Thr Asn Ser Asp Cys Glu Leu Ile Val Lys Ala Met Gln Gly Leu Leu  
 100 105 110

Lys Asp Gly Asn Pro Ile Pro Ser Ala Ile Ala Ala Asn Ser Gly Ile  
 115 120 125

Tyr

<210> 7  
 <211> 130  
 <212> PRT

PA040WO\_sequence\_listing.ST25.txt

&lt;213&gt; Bacteriophage fr

&lt;400&gt; 7

Met Ala Ser Asn Phe Glu Glu Phe Val Leu Val Asp Asn Gly Gly Thr  
 1 5 10 15

Gly Asp Val Lys Val Ala Pro Ser Asn Phe Ala Asn Gly Val Ala Glu  
 20 25 30

Trp Ile Ser Ser Asn Ser Arg Ser Gln Ala Tyr Lys Val Thr Cys Ser  
 35 40 45

Val Arg Gln Ser Ser Ala Asn Asn Arg Lys Tyr Thr Val Lys Val Glu  
 50 55 60

Val Pro Lys Val Ala Thr Gln Val Gln Gly Gly Val Glu Leu Pro Val  
 65 70 75 80

Ala Ala Trp Arg Ser Tyr Met Asn Met Glu Leu Thr Ile Pro Val Phe  
 85 90 95

Ala Thr Asn Asp Asp Cys Ala Leu Ile Val Lys Ala Leu Gln Gly Thr  
 100 105 110

Phe Lys Thr Gly Asn Pro Ile Ala Thr Ala Ile Ala Ala Asn Ser Gly  
 115 120 125

Ile Tyr  
 130

&lt;210&gt; 8

&lt;211&gt; 130

&lt;212&gt; PRT

&lt;213&gt; Bacteriophage GA

&lt;400&gt; 8

Met Ala Thr Leu Arg Ser Phe Val Leu Val Asp Asn Gly Gly Thr Gly  
 1 5 10 15

Asn Val Thr Val Val Pro Val Ser Asn Ala Asn Gly Val Ala Glu Trp  
 20 25 30

Leu Ser Asn Asn Ser Arg Ser Gln Ala Tyr Arg Val Thr Ala Ser Tyr  
 35 40 45

Arg Ala Ser Gly Ala Asp Lys Arg Lys Tyr Ala Ile Lys Leu Glu Val  
 50 55 60

Pro Lys Ile Val Thr Gln Val Val Asn Gly Val Glu Leu Pro Gly Ser  
 65 70 75 80

Ala Trp Lys Ala Tyr Ala Ser Ile Asp Leu Thr Ile Pro Ile Phe Ala



PA040WO\_sequence\_listing.ST25.txt

85

90

95

Ala Thr Asp Asp Val Thr Val Ile Ser Lys Ser Leu Ala Gly Leu Phe  
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Lys Val Gly Asn Pro Ile Ala Glu Ala Ile Ser Ser Gln Ser Gly Phe  
 115 120 125

Tyr Ala  
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 <211> 132  
 <212> PRT  
 <213> Bacteriophage SP  
 <400> 9

Met Ala Lys Leu Asn Gln Val Thr Leu Ser Lys Ile Gly Lys Asn Gly  
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Asp Gln Thr Leu Thr Leu Thr Pro Arg Gly Val Asn Pro Thr Asn Gly  
 20 25 30

Val Ala Ser Leu Ser Glu Ala Gly Ala Val Pro Ala Leu Glu Lys Arg  
 35 40 45

Val Thr Val Ser Val Ala Gln Pro Ser Arg Asn Arg Lys Asn Phe Lys  
 50 55 60

Val Gln Ile Lys Leu Gln Asn Pro Thr Ala Cys Thr Arg Asp Ala Cys  
 65 70 75 80

Asp Pro Ser Val Thr Arg Ser Ala Phe Ala Asp Val Thr Leu Ser Phe  
 85 90 95

Thr Ser Tyr Ser Thr Asp Glu Glu Arg Ala Leu Ile Arg Thr Glu Leu  
 100 105 110

Ala Ala Leu Leu Ala Asp Pro Leu Ile Val Asp Ala Ile Asp Asn Leu  
 115 120 125

Asn Pro Ala Tyr  
 130

<210> 10  
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 <212> PRT  
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Ala Lys Leu Asn Gln Val Thr Leu Ser Lys Ile Gly Lys Asn Gly Asp  
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## PA040WO\_sequence\_listing.ST25.txt

Tyr Arg Ser Tyr Gly Met Val Ile Gly Phe Trp Thr Asp Ser Lys Ser  
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Pro Gln Leu Pro Thr Asp Phe Thr Gln Phe Asn Ser Ala Asn Cys Pro  
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Val Gln Thr Val Ile Ile Ile Pro Ser  
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<210> 11  
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 <212> PRT  
 <213> Bacteriophage MS2

<400> 11

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 1 5 10 15

Gly Asp Val Thr Val Ala Pro Ser Asn Phe Ala Asn Gly Val Ala Glu  
 20 25 30

Trp Ile Ser Ser Asn Ser Arg Ser Gln Ala Tyr Lys Val Thr Cys Ser  
 35 40 45

Val Arg Gln Ser Ser Ala Gln Asn Arg Lys Tyr Thr Ile Lys Val Glu  
 50 55 60

Val Pro Lys Val Ala Thr Gln Thr Val Gly Gly Val Glu Leu Pro Val  
 65 70 75 80

Ala Ala Trp Arg Ser Tyr Leu Asn Met Glu Leu Thr Ile Pro Ile Phe  
 85 90 95

Ala Thr Asn Ser Asp Cys Glu Leu Ile Val Lys Ala Met Gln Gly Leu  
 100 105 110

Leu Lys Asp Gly Asn Pro Ile Pro Ser Ala Ile Ala Ala Asn Ser Gly  
 115 120 125

Ile Tyr  
 130

<210> 12  
 <211> 133  
 <212> PRT  
 <213> Bacteriophage M11

<400> 12

Met Ala Lys Leu Gln Ala Ile Thr Leu Ser Gly Ile Gly Lys Lys Gly  
 1 5 10 15

Asp Val Thr Leu Asp Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly

PA040WO\_sequence\_listing.ST25.txt

20

25

30

Val Ala Ala Leu Ser Glu Ala Gly Ala Val Pro Ala Leu Glu Lys Arg  
 35 40 45

Val Thr Ile Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys  
 50 55 60

Val Gln Val Lys Ile Gln Asn Pro Thr Ser Cys Thr Ala Ser Gly Thr  
 65 70 75 80

Cys Asp Pro Ser Val Thr Arg Ser Ala Tyr Ser Asp Val Thr Phe Ser  
 85 90 95

Phe Thr Gln Tyr Ser Thr Val Glu Glu Arg Ala Leu Val Arg Thr Glu  
 100 105 110

Leu Gln Ala Leu Leu Ala Asp Pro Met Leu Val Asn Ala Ile Asp Asn  
 115 120 125

Leu Asn Pro Ala Tyr  
 130

<210> 13  
 <211> 133  
 <212> PRT  
 <213> Bacteriophage MX1

<400> 13

Met Ala Lys Leu Gln Ala Ile Thr Leu Ser Gly Ile Gly Lys Asn Gly  
 1 5 10 15

Asp Val Thr Leu Asn Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly  
 20 25 30

Val Ala Ala Leu Ser Glu Ala Gly Ala Val Pro Ala Leu Glu Lys Arg  
 35 40 45

Val Thr Ile Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys  
 50 55 60

Val Gln Val Lys Ile Gln Asn Pro Thr Ser Cys Thr Ala Ser Gly Thr  
 65 70 75 80

Cys Asp Pro Ser Val Thr Arg Ser Ala Tyr Ala Asp Val Thr Phe Ser  
 85 90 95

Phe Thr Gln Tyr Ser Thr Asp Glu Glu Arg Ala Leu Val Arg Thr Glu  
 100 105 110

Leu Lys Ala Leu Leu Ala Asp Pro Met Leu Ile Asp Ala Ile Asp Asn  
 115 120 125

## PA040WO\_sequence\_listing.ST25.txt

Leu Asn Pro Ala Tyr  
130

<210> 14  
<211> 330  
<212> PRT  
<213> Bacteriophage NL95

<400> 14

Met Ala Lys Leu Asn Lys Val Thr Leu Thr Gly Ile Gly Lys Ala Gly  
1 5 10 15

Asn Gln Thr Leu Thr Leu Thr Pro Arg Gly Val Asn Pro Thr Asn Gly  
20 25 30

Val Ala Ser Leu Ser Glu Ala Gly Ala Val Pro Ala Leu Glu Lys Arg  
35 40 45

Val Thr Val Ser Val Ala Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys  
50 55 60

Val Gln Ile Lys Leu Gln Asn Pro Thr Ala Cys Thr Lys Asp Ala Cys  
65 70 75 80

Asp Pro Ser Val Thr Arg Ser Gly Ser Arg Asp Val Thr Leu Ser Phe  
85 90 95

Thr Ser Tyr Ser Thr Glu Arg Glu Arg Ala Leu Ile Arg Thr Glu Leu  
100 105 110

Ala Ala Leu Leu Lys Asp Asp Leu Ile Val Asp Ala Ile Asp Asn Leu  
115 120 125

Asn Pro Ala Tyr Trp Ala Ala Leu Leu Ala Ala Ser Pro Gly Gly Gly  
130 135 140

Asn Asn Pro Tyr Pro Gly Val Pro Asp Ser Pro Asn Val Lys Pro Pro  
145 150 155 160

Gly Gly Thr Gly Thr Tyr Arg Cys Pro Phe Ala Cys Tyr Arg Arg Gly  
165 170 175

Glu Leu Ile Thr Glu Ala Lys Asp Gly Ala Cys Ala Leu Tyr Ala Cys  
180 185 190

Gly Ser Glu Ala Leu Val Glu Phe Glu Tyr Ala Leu Glu Asp Phe Leu  
195 200 205

Gly Asn Glu phe Trp Arg Asn Trp Asp Gly Arg Leu Ser Lys Tyr Asp  
210 215 220

## PA040WO\_sequence\_listing.ST25.txt

Ile Glu Thr His Arg Arg Cys Arg Gly Asn Gly Tyr Val Asp Leu Asp  
 225 230 235 240  
 Ala Ser Val Met Gln Ser Asp Glu Tyr Val Leu Ser Gly Ala Tyr Asp  
 245 250 255  
 Val Val Lys Met Gln Pro Pro Gly Thr Phe Asp Ser Pro Arg Tyr Tyr  
 260 265 270  
 Leu His Leu Met Asp Gly Ile Tyr Val Asp Leu Ala Glu Val Thr Ala  
 275 280 285  
 Tyr Arg Ser Tyr Gly Met Val Ile Gly Phe Trp Thr Asp Ser Lys Ser  
 290 295 300  
 Pro Gln Leu Pro Thr Asp Phe Thr Arg Phe Asn Arg His Asn Cys Pro  
 305 310 315 320  
 Val Gln Thr Val Ile Val Ile Pro Ser Leu  
 325 330  
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 <211> 129  
 <212> PRT  
 <213> Bacteriophage f2  
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 20 25 30  
 Ile Ser Ser Asn Ser Arg Ser Gln Ala Tyr Lys Val Thr Cys Ser Val  
 35 40 45  
 Arg Gln Ser Ser Ala Gln Asn Arg Lys Tyr Thr Ile Lys Val Glu Val  
 50 55 60  
 Pro Lys Val Ala Thr Gln Thr Val Gly Gly Val Glu Leu Pro Val Ala  
 65 70 75 80  
 Ala Trp Arg Ser Tyr Leu Asn Leu Glu Leu Thr Ile Pro Ile Phe Ala  
 85 90 95  
 Thr Asn Ser Asp Cys Glu Leu Ile Val Lys Ala Met Gln Gly Leu Leu  
 100 105 110  
 Lys Asp Gly Asn Pro Ile Pro Ser Ala Ile Ala Ala Asn Ser Gly Ile  
 115 120 125

## PA040WO\_sequence\_listing.ST25.txt

Tyr

<210> 16  
 <211> 128  
 <212> PRT  
 <213> Bacteriophage PP7

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 1 5 10 15

Thr Glu Ile Gln Ser Thr Ala Asp Arg Gln Ile Phe Glu Glu Lys Val  
 20 25 30

Gly Pro Leu Val Gly Arg Leu Arg Leu Thr Ala Ser Leu Arg Gln Asn  
 35 40 45

Gly Ala Lys Thr Ala Tyr Arg Val Asn Leu Lys Leu Asp Gln Ala Asp  
 50 55 60

Val Val Asp Cys Ser Thr Ser Val Cys Gly Glu Leu Pro Lys Val Arg  
 65 70 75 80

Tyr Thr Gln Val Trp Ser His Asp Val Thr Ile Val Ala Asn Ser Thr  
 85 90 95

Glu Ala Ser Arg Lys Ser Leu Tyr Asp Leu Thr Lys Ser Leu Val Ala  
 100 105 110

Thr Ser Gln Val Glu Asp Leu Val Val Asn Leu Val Pro Leu Gly Arg  
 115 120 125

<210> 17  
 <211> 132  
 <212> PRT  
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<220>  
 <223> Bacteriophage Qbeta 240 mutant

&lt;400&gt; 17

Ala Lys Leu Glu Thr Val Thr Leu Gly Asn Ile Gly Arg Asp Gly Lys  
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Gln Thr Leu Val Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly Val  
 20 25 30

Ala Ser Leu Ser Gln Ala Gly Ala Val Pro Ala Leu Glu Lys Arg Val  
 35 40 45

Thr Val Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys Val  
 50 55 60

## PA040WO\_sequence\_listing.ST25.txt

Gln Val Lys Ile Gln Asn Pro Thr Ala Cys Thr Ala Asn Gly Ser Cys  
65 70 75 80

Asp Pro Ser Val Thr Arg Gln Lys Tyr Ala Asp Val Thr Phe Ser Phe  
85 90 95

Thr Gln Tyr Ser Thr Asp Glu Glu Arg Ala Phe Val Arg Thr Glu Leu  
100 105 110

Ala Ala Leu Leu Ala Ser Pro Leu Leu Ile Asp Ala Ile Asp Gln Leu  
115 120 125

Asn Pro Ala Tyr  
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<210> 18  
<211> 132  
<212> PRT  
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<220>  
<223> Bacteriophage Q-beta 243 mutant  
<400> 18

Ala Lys Leu Glu Thr Val Thr Leu Gly Lys Ile Gly Lys Asp Gly Lys  
1 5 10 15

Gln Thr Leu Val Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly Val  
20 25 30

Ala Ser Leu Ser Gln Ala Gly Ala Val Pro Ala Leu Glu Lys Arg Val  
35 40 45

Thr Val Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys Val  
50 55 60

Gln Val Lys Ile Gln Asn Pro Thr Ala Cys Thr Ala Asn Gly Ser Cys  
65 70 75 80

Asp Pro Ser Val Thr Arg Gln Lys Tyr Ala Asp Val Thr Phe Ser Phe  
85 90 95

Thr Gln Tyr Ser Thr Asp Glu Glu Arg Ala Phe Val Arg Thr Glu Leu  
100 105 110

Ala Ala Leu Leu Ala Ser Pro Leu Leu Ile Asp Ala Ile Asp Gln Leu  
115 120 125

Asn Pro Ala Tyr  
130

<210> 19



## PA040WO\_sequence\_listing.ST25.txt

&lt;211&gt; 132

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Bacteriophage Q-beta 250 mutant

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Ala Arg Leu Glu Thr Val Thr Leu Gly Asn Ile Gly Arg Asp Gly Lys  
 1 5 10 15

Gln Thr Leu Val Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly Val  
 20 25 30

Ala Ser Leu Ser Gln Ala Gly Ala Val Pro Ala Leu Glu Lys Arg Val  
 35 40 45

Thr Val Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys Val  
 50 55 60

Gln Val Lys Ile Gln Asn Pro Thr Ala Cys Thr Ala Asn Gly Ser Cys  
 65 70 75 80

Asp Pro Ser Val Thr Arg Gln Lys Tyr Ala Asp Val Thr Phe Ser Phe  
 85 90 95

Thr Gln Tyr Ser Thr Asp Glu Glu Arg Ala Phe Val Arg Thr Glu Leu  
 100 105 110

Ala Ala Leu Leu Ala Ser Pro Leu Leu Ile Asp Ala Ile Asp Gln Leu  
 115 120 125

Asn Pro Ala Tyr  
 130

&lt;210&gt; 20

&lt;211&gt; 132

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Bacteriophage Q-beta 251 mutant

&lt;400&gt; 20

Ala Lys Leu Glu Thr Val Thr Leu Gly Asn Ile Gly Lys Asp Gly Arg  
 1 5 10 15

Gln Thr Leu Val Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly Val  
 20 25 30

Ala Ser Leu Ser Gln Ala Gly Ala Val Pro Ala Leu Glu Lys Arg Val  
 35 40 45

Thr Val Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys Val

50 PA040WO\_sequence\_listing.ST25.txt  
55 60

Gln Val Lys Ile Gln Asn Pro Thr Ala Cys Thr Ala Asn Gly Ser Cys  
65 70 75 80

Asp Pro Ser Val Thr Arg Gln Lys Tyr Ala Asp Val Thr Phe Ser Phe  
85 90 95

Thr Gln Tyr Ser Thr Asp Glu Glu Arg Ala Phe Val Arg Thr Glu Leu  
100 105 110

Ala Ala Leu Leu Ala Ser Pro Leu Leu Ile Asp Ala Ile Asp Gln Leu  
115 120 125

Asn Pro Ala Tyr  
130

<210> 21  
<211> 132  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Bacteriophage Q-beta 259 mutant  
<400> 21

Ala Arg Leu Glu Thr Val Thr Leu Gly Asn Ile Gly Lys Asp Gly Arg  
1 5 10 15

Gln Thr Leu Val Leu Asn Pro Arg Gly Val Asn Pro Thr Asn Gly Val  
20 25 30

Ala Ser Leu Ser Gln Ala Gly Ala Val Pro Ala Leu Glu Lys Arg Val  
35 40 45

Thr Val Ser Val Ser Gln Pro Ser Arg Asn Arg Lys Asn Tyr Lys Val  
50 55 60

Gln Val Lys Ile Gln Asn Pro Thr Ala Cys Thr Ala Asn Gly Ser Cys  
65 70 75 80

Asp Pro Ser Val Thr Arg Gln Lys Tyr Ala Asp Val Thr Phe Ser Phe  
85 90 95

Thr Gln Tyr Ser Thr Asp Glu Glu Arg Ala Phe Val Arg Thr Glu Leu  
100 105 110

Ala Ala Leu Leu Ala Ser Pro Leu Leu Ile Asp Ala Ile Asp Gln Leu  
115 120 125

Asn Pro Ala Tyr  
130

## PA040W0\_sequence\_listing.ST25.txt

<210> 22  
 <211> 185  
 <212> PRT  
 <213> Hepatitis B virus

<400> 22

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
 1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
 20 25 30

Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys  
 35 40 45

Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
 50 55 60

Leu Met Thr Leu Ala Thr Trp Val Gly Asn Asn Leu Glu Asp Pro Ala  
 65 70 75 80

Ser Arg Asp Leu Val Val Asn Tyr Val Asn Thr Asn Met Gly Leu Lys  
 85 90 95

Ile Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
 100 105 110

Glu Thr Val Leu Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
 115 120 125

Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
 130 135 140

Glu Thr Thr Val Val Arg Arg Arg Asp Arg Gly Arg Ser Pro Arg Arg  
 145 150 155 160

Arg Thr Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg  
 165 170 175

Arg Ser Gln Ser Arg Glu Ser Gln Cys  
 180 185

<210> 23  
 <211> 212  
 <212> PRT  
 <213> Hepatitis B virus

<400> 23

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
 1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile

PA040WO\_sequence\_listing.ST25.txt  
25 30

20

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp Leu Met Asn  
85 90 95

Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Val Ser Arg Asp  
100 105 110

Leu Val Val Gly Tyr Val Asn Thr Thr Val Gly Leu Lys Phe Arg Gln  
115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
195 200 205

Glu Ser Gln Cys  
210

- <210> 24
- <211> 188
- <212> PRT
- <213> Hepatitis B virus

<400> 24

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ser Ser Tyr Gln Leu Leu  
1 5 10 15

Asn Phe Leu Pro Leu Asp Phe Phe Pro Asp Leu Asn Ala Leu Val Asp  
20 25 30

Thr Ala Thr Ala Leu Tyr Glu Glu Glu Leu Thr Gly Arg Glu His Cys  
35 40 45

PA040WO\_sequence\_listing.ST25.txt

Ser Pro His His Thr Ala Ile Arg Gln Ala Leu Val Cys Trp Asp Glu  
 50 55 60

Leu Thr Lys Leu Ile Ala Trp Met Ser Ser Asn Ile Thr Ser Glu Gln  
 65 70 75 80

Val Arg Thr Ile Ile Val Asn His Val Asn Asp Thr Trp Gly Leu Lys  
 85 90 95

Val Arg Gln Ser Leu Trp Phe His Leu Ser Cys Leu Thr Phe Gly Gln  
 100 105 110

His Thr Val Gln Glu Phe Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
 115 120 125

Pro Ala Pro Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
 130 135 140

Glu His Thr Val Ile Arg Arg Arg Gly Gly Ala Arg Ala Ser Arg Ser  
 145 150 155 160

Pro Arg Arg Arg Thr Pro Ser Pro Arg Arg Arg Ser Gln Ser Pro  
 165 170 175

Arg Arg Arg Ser Gln Ser Pro Ser Thr Asn Cys  
 180 185

<210> 25  
 <211> 185  
 <212> PRT  
 <213> Hepatitis B virus

<400> 25

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
 1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
 20 25 30

Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys  
 35 40 45

Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
 50 55 60

Leu Met Thr Leu Ala Thr Trp Val Gly Asn Asn Leu Glu Asp Pro Ala  
 65 70 75 80

Ser Arg Asp Leu Val Val Asn Tyr Val Asn Thr Asn Met Gly Leu Lys  
 85 90 95

## PA040WO\_sequence\_listing.ST25.txt

Ile Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
 100 105 110

Glu Thr Val Leu Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
 115 120 125

Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
 130 135 140

Glu Thr Thr Val Val Arg Arg Arg Asp Arg Gly Arg Ser Pro Arg Arg  
 145 150 155 160

Arg Thr Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg  
 165 170 175

Arg Ser Gln Ser Arg Glu Ser Gln Cys  
 180 185

<210> 26  
 <211> 152  
 <212> PRT  
 <213> Hepatitis B virus

<400> 26

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
 1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
 20 25 30

Thr Ala Ala Ala Leu Tyr Arg Asp Ala Leu Glu Ser Pro Glu His Cys  
 35 40 45

Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp  
 50 55 60

Leu Met Thr Leu Ala Thr Trp Val Gly Thr Asn Leu Glu Asp Gly Gly  
 65 70 75 80

Lys Gly Gly Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Val  
 85 90 95

Gly Leu Lys Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr  
 100 105 110

Phe Gly Arg Glu Thr Val Leu Glu Tyr Leu Val Ser Phe Gly Val Trp  
 115 120 125

Ile Arg Thr Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser  
 130 135 140

PA040wo\_sequence\_listing.ST25.txt

Thr Leu Pro Glu Thr Thr Val Val  
 145 150

<210> 27  
 <211> 3635  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> plasmid pAP283-58

<400> 27  
 cgagctcgcc cctggcttat cgaaattaat acgactcact atagggagac cggaattcga 60  
 gctcgcgccg ggatcctcta gaattttctg cgcacccatc ccgggtggcg cccaaagtga 120  
 ggaaaatcac atggcaaata agccaatgca accgatcaca tctacagcaa ataaaattgt 180  
 gtggtcggat ccaactcgtt tatcaactac attttcagca agtctgttac gccaacgtgt 240  
 taaagttggt atagccgaac tgaataatgt ttcagggtcaa tatgtatctg tttataagcg 300  
 tcctgcacct aaaccggaag gttgtgcaga tgcctgtgtc attatgccga atgaaaacca 360  
 atccattcgc acagtgattt cagggtcagc cgaaaacttg gctaccttaa aagcagaatg 420  
 ggaaactcac aaacgtaacg ttgacacact cttcgcgagc ggcaacgccg gtttgggttt 480  
 ccttgaccct actgcggcta tcgtatcgtc tgatactact gcttaagctt gtattctata 540  
 gtgtcaccta aatcgtatgt gtatgataca taaggttatg tattaattgt agccgcgttc 600  
 taacgacaat atgtacaagc ctaattgtgt agcatctggc ttactgaagc agaccctatc 660  
 atctctctcg taaactgccg tcagagtcgg tttggttga cgaaccttct gagtttctgg 720  
 taacgccggt ccgcaccccc gaaatggtca ccgaaccaat cagcagggtc atcgctagcc 780  
 agatcctcta cgccggacgc atcgtggccg gcatcaccgg cgcacacagt gcggttgctg 840  
 gcgcctatat cgccgacatc accgatgggg aagatcgggc tcgccacttc gggctcatga 900  
 gcgcttggtt cggcgtgggt atggtggcag gccccgtggc cgggggactg ttgggcgcca 960  
 tctccttgca tgcaccattc cttgcggcgg cgggtcttca acggcctcaa cctactactg 1020  
 ggctgcttcc taatgcagga gtcgcataag ggagagcgtc gatatggtgc actctcagta 1080  
 caatctgctc tgatgccgca tagttaagcc aactccgcta tcgctacgtg actgggtcat 1140  
 ggctgcgccc cgacacccgc caacacccgc tgacgcgccc tgacgggctt gtctgtctcc 1200  
 ggcattccgct tacagacaag ctgtgaccgt ctccgggagc tgcatgtgtc agaggttttc 1260  
 accgtcatca ccgaaacgcg cgaggcagct tgaagacgaa agggcctcgt gatacgccta 1320  
 tttttatagg ttaatgtcat gataataatg gtttcttaga cgtcagggtg cacttttctg 1380  
 ggaaatgtgc gcggaacccc tatttgttta tttttctaaa tacattcaaa tatgtatccg 1440  
 ctcatgagac aataaccctg ataaatgctt caataatatt gaaaaaggaa gagtatgagt 1500  
 attcaacatt tccgtgtcgc ccttattccc tttttgctg cattttgcct tcctgttttt 1560  
 gctcaccag aaacgctggg gaaagtaaaa gatgctgaag atcagttggg tgcacgagtg 1620  
 ggttacatcg aactggatct caacagcggg aagatccttg agagttttcg ccccgaagaa 1680

## PA040WO\_sequence\_listing.ST25.txt

cgttttccaa tgatgagcac ttttaaagtt ctgctatgtg gcgcggtatt atccccgtatt 1740  
gacgccgggc aagagcaact cggtcgccgc atacactatt ctcagaatga cttggttgag 1800  
tactcaccag tcacagaaaa gcatcttacg gatggcatga cagtaagaga attatgcagt 1860  
gctgccataa ccatgagtga taactctgcg gccaaacttac ttctgacaac gatcggagga 1920  
ccgaaggagc taaccgcttt tttgcacaac atgggggatc atgtaactcg ccttgatcgt 1980  
tgggaaccgg agctgaatga agccatacca aacgacgagc gtgacaccac gatgcctgta 2040  
gcaatggcaa caacgttgcg caaactatta actggcgaac tacttactct agcttcccgg 2100  
caacaattaa tagactggat ggaggcggat aaagttgacg gaccacttct gcgctcggcc 2160  
cttccggctg gctggtttat tgctgataaa tctggagccg gtgagcgtgg gtctcgcggt 2220  
atcattgcag cactggggcc agatggtaag cctcccgtat tcgtagttat ctacacgacg 2280  
gggagtcagg caactatgga tgaacgaaat agacagatcg ctgagatagg tgcctcactg 2340  
attaagcatt ggtaactgtc agaccaagtt tactcatata tacttttagat tgatttaaaa 2400  
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atccctaac gtgagttttc gttccactga gcgtcagacc ccgtagaaaa gatcaaagga 2520  
tcttcttgag atcctttttt tctgcgcgta atctgctgct tgcaaacaaa aaaaccaccg 2580  
ctaccagcgg tggtttgttt gccggatcaa gagctaccaa ctctttttcc gaaggtaact 2640  
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cacttcaaga actctgtagc accgcctaca tacctcgtct tgctaatacct gttaccagtg 2760  
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gctcgccgca gccgaacgac gagcgcagcg agtcagtgag cgaggaagcg gaagagcgcc 3300  
caatacgcaa accgcctctc cccgcgcggt ggccgattca ttaatgcagc tgtggtgtca 3360  
tggtcgggtga tcgccagggt gccgacgcgc atctcgactg catggtgcac caatgcttct 3420  
ggcgtcaggc agccatcggg agctgtggta tggccgtgca ggtcgtaaact cactgcataa 3480  
ttcgtgtcgc tcaaggcga ctcccgttct ggataatgtt ttttgcgccg acatcataac 3540  
ggttctggca aatattctga aatgagctgt tgacaattaa tcatcgaact agttaactag 3600  
tacgcaagtt cacgtaaaaa gggtatcgcg gaatt 3635



## PA040WO\_sequence\_listing.ST25.txt

<210> 28  
 <211> 131  
 <212> PRT  
 <213> Bacteriophage AP205  
 <400> 28  
 Met Ala Asn Lys Pro Met Gln Pro Ile Thr Ser Thr Ala Asn Lys Ile  
 1 5 10 15  
 Val Trp Ser Asp Pro Thr Arg Leu Ser Thr Thr Phe Ser Ala Ser Leu  
 20 25 30  
 Leu Arg Gln Arg Val Lys Val Gly Ile Ala Glu Leu Asn Asn Val Ser  
 35 40 45  
 Gly Gln Tyr Val Ser Val Tyr Lys Arg Pro Ala Pro Lys Pro Glu Gly  
 50 55 60  
 Cys Ala Asp Ala Cys Val Ile Met Pro Asn Glu Asn Gln Ser Ile Arg  
 65 70 75 80  
 Thr Val Ile Ser Gly Ser Ala Glu Asn Leu Ala Thr Leu Lys Ala Glu  
 85 90 95  
 Trp Glu Thr His Lys Arg Asn Val Asp Thr Leu Phe Ala Ser Gly Asn  
 100 105 110  
 Ala Gly Leu Gly Phe Leu Asp Pro Thr Ala Ala Ile Val Ser Ser Asp  
 115 120 125  
 Thr Thr Ala  
 130

<210> 29  
 <211> 131  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> AP205 coat protein

<400> 29  
 Met Ala Asn Lys Thr Met Gln Pro Ile Thr Ser Thr Ala Asn Lys Ile  
 1 5 10 15  
 Val Trp Ser Asp Pro Thr Arg Leu Ser Thr Thr Phe Ser Ala Ser Leu  
 20 25 30  
 Leu Arg Gln Arg Val Lys Val Gly Ile Ala Glu Leu Asn Asn Val Ser  
 35 40 45  
 Gly Gln Tyr Val Ser Val Tyr Lys Arg Pro Ala Pro Lys Pro Glu Gly  
 50 55 60

PA040WO\_sequence\_listing.ST25.txt

Cys Ala Asp Ala Cys Val Ile Met Pro Asn Glu Asn Gln Ser Ile Arg  
 65 70 75 80

Thr Val Ile Ser Gly Ser Ala Glu Asn Leu Ala Thr Leu Lys Ala Glu  
 85 90 95

Trp Glu Thr His Lys Arg Asn Val Asp Thr Leu Phe Ala Ser Gly Asn  
 100 105 110

Ala Gly Leu Gly Phe Leu Asp Pro Thr Ala Ala Ile Val Ser Ser Asp  
 115 120 125

Thr Thr Ala  
 130

<210> 30  
 <211> 3607  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> plasmid pAP281-32

<400> 30  
 cgagctcgcc cctggcttat cgaaattaat acgactcact atagggagac cggaattcga 60  
 gctcgcccgg ggatcctcta gattaaccca acgcgtagga gtcaggccat ggcaaataag 120  
 acaatgcaac cgatcacatc tacagcaaat aaaattgtgt ggtcggatcc aactcgttta 180  
 tcaactacat tttcagcaag tctgttacgc caacgtgta aagttggtat agccgaactg 240  
 aataatgttt caggtcaata tgtatctggt tataagcgtc ctgcacctaa accgaaggtc 300  
 agatgcctgt gtcattatgc cgaatgaaaa ccaatccatt cgcacagtga tttcagggtc 360  
 agccgaaaac ttggctacct taaaagcaga atgggaaact cacaaacgta acgttgacac 420  
 actcttcgcg agcggcaacg ccggtttggg tttccttgac cctactgagg ctatcgatc 480  
 gtctgatact actgcttaag cttgtattct atagtgtcac ctaaactgta tgtgtatgat 540  
 acataagggt atgtattaat ggtagccgcg ttctaacgac aatatgtaca agcctaattg 600  
 tgtagcatct ggcttactga agcagaccct atcatctctc tcgtaaactg ccgtcagagt 660  
 cggttggggt ggacagacct ctgagtttct ggtaacgccg ttccgcaccc cggaaatggt 720  
 caccgaacca ttcagcaggg tcatcgctag ccagatctc tacgccggac gcatcgtggc 780  
 ccgcatcacc ggcgccacag gtgctggtct ggcgcctata tcgccgacat caccgatggg 840  
 gaagatcggg ctgcccactt cgggctcatg atcgtctggt tccgcctggg tatggtggca 900  
 ggccccgtgg cccggggggac tgttgggcgc catctccttg catgcacat tccttgccgc 960  
 ggcggtgctc aacggcctca acctactact gggctgctc ctaatgcagg agtcgcataa 1020  
 gggagagcgt cgatatggtg cactctcagt acaatctgct ctgatgccg atagttaagc 1080  
 caactccgct atcgtacgt gactgggtca tggctgcgcc ccgacacccg ccaacacccg 1140

## PA040W0\_sequence\_listing.ST25.txt

ctgacgcgcc	ctgacgggct	tgtctgcttc	cggcatccgc	ttacagacaa	gctgtgaccg	1200
tctccgggag	ctgcatgtgt	cagaggtttt	caccgtcatc	accgaaacgc	gcgaggcagc	1260
ttgaagacga	aagggcctcg	tgatacgctt	atTTTTatag	gttaatgtca	tgataataat	1320
ggtttcttag	acgtcagggtg	gcacttttctg	gggaaatgtg	cgcggaaccc	ctattggttt	1380
atTTTTctaa	atacattcaa	atatgtatcc	gctcatgaga	caataaccct	gataaatgct	1440
tcaataatat	tgaaaaagga	agagtatgag	tattcaacat	ttccgtgtcg	cccttattcc	1500
ctTTTTtgcg	gcattttgcc	ttcctgtttt	tgctcaccga	gaaacgctgg	tgaaagtaaa	1560
agatgctgaa	gatcagttgg	gtgcacgagt	gggttacatc	gaactggatc	tcaacagcgg	1620
taagatcctt	gagagttttc	gccccgaaga	acgtttttca	atgatgagca	cttttaaagt	1680
tctgctatgt	gtcgcgggat	tatcccgtat	tgacgccggg	caagagcaac	tcggtcgccg	1740
catacactat	tctcagaatg	acttggtggt	acctaccagt	cacagaaaag	catcttacgg	1800
atggcatgac	agtaagagaa	ttatgcagtg	ctgccataac	catgagtgat	aacctgcgg	1860
ccaacttact	tctgacaacg	atcggaggac	cgaaggagct	aaccgctttt	ttgcacaaca	1920
tgggggatca	tgtaactcgc	cttgatcgtt	gggaaccgga	gctgaatgaa	gccataccaa	1980
acgacgagcg	tgacaccacg	atgcctgtac	gaacggcaac	aacgttgccg	aaactattaa	2040
ctggcgaact	acttactcta	gcttcccggc	aacaattaat	agactggatg	gaggcggata	2100
aagttgcagg	accacttctg	cgctcggccc	ttccggctgg	ctggtttatt	gctgataaat	2160
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gacagatcgc	tgagataggt	gcctcactga	ttaagcattg	gtaactgtca	gaccaagttt	2340
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cggtcagacc	ccgtagaaaag	atcaaaggat	cttcttgaga	tccttttttt	ctgvcgctaa	2520
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acctcgctct	gctaatacctg	ttaccagtgg	ctgctgccag	tgccgataag	tcgtgtctta	2760
ccgggttggg	ctcaagacga	taggtaccgg	ataaggcgca	gcggtcgggc	tgaacggggg	2820
gttcgtgcac	acagcccagc	ttggagcgaa	cgacctacac	cgaactgaga	tacctacagc	2880
gcgagcattg	agaaagcgcc	acgcttcccg	aaggagaaaa	ggcggacagg	tatccggtaa	2940
gcggcagggg	cggaacaaga	gagcgcacga	gggagcttcc	agggggaaac	gcctggtatc	3000
tttatagtcc	tgctcgggtt	cgccacctct	gacttgagcg	tcgatttttg	tgatgctcgt	3060
cagggggggc	gagcctatgg	aaaaacgcca	gcaacgcggc	ctttttacgg	ttcctggcct	3120
ttggctggcc	ttttgctcac	atgttctttc	ctgcgttatc	ccctgattct	gtggataacc	3180

PA040WO\_sequence\_listing.ST25.txt

gtattaccgc ctttgagtga gctgataccg ctcgccgcag ccgaacgacc gacggcgag 3240  
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 ttggccgatt cattaatgca gctgtggtgt catggctcggg gatcgccagg gtgccgacgc 3360  
 gcatctcgac tgcattggtgc accaatgctt ctggcgtagc gcagccatcg gaagctgtgg 3420  
 tatggccgtg caggctgtaa atcactgcat aattcgtgtc gctcaaggcg cactcccgtt 3480  
 ctggataatg ttttttgcgg cgacatcata acggttctgg caaatattct gaaatgagct 3540  
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 cggaatt 3607

<210> 31  
 <211> 28  
 <212> PRT  
 <213> Homo sapiens

<400> 31

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Arg Val Gln Gln Arg Lys  
 1 5 10 15

Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg  
 20 25

<210> 32  
 <211> 28  
 <212> PRT  
 <213> Mus musculus

<400> 32

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Ala Gln Gln Arg Lys  
 1 5 10 15

Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg  
 20 25

<210> 33  
 <211> 5  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Linker

<400> 33

Gly Gly Lys Gly Gly  
 1 5

<210> 34  
 <211> 3  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> N-terminal glycine linker

## PA040WO\_sequence\_listing.ST25.txt

<220>  
 <221> REPEAT  
 <222> (1)..(1)  
 <223> Glycine can be repeated from zero to five times

<220>  
 <221> REPEAT  
 <222> (3)..(3)  
 <223> Glycine can be repeated from zero to twelve times

<400> 34

Gly Cys Gly  
 1

<210> 35  
 <211> 9  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> N terminal glycine serine linkers

<220>  
 <221> REPEAT  
 <222> (1)..(1)  
 <223> Glycine can be repeated from zero to five times

<220>  
 <221> REPEAT  
 <222> (3)..(3)  
 <223> Glycine can be repeated from zero to ten times

<220>  
 <221> REPEAT  
 <222> (4)..(4)  
 <223> Serine can be repeated from zero to two times

<220>  
 <221> REPEAT  
 <222> (5)..(9)  
 <223> These residues can be repeated from zero to three times as a group

<400> 35

Gly Cys Gly ser Gly Gly Gly Gly ser  
 1 5

<210> 36  
 <211> 3  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> C-terminal glycine linker

<220>  
 <221> REPEAT  
 <222> (1)..(1)  
 <223> Glycine can be repeated from zero to twelve times

PA040WO\_sequence\_listing.ST25.txt

&lt;220&gt;

&lt;221&gt; REPEAT

&lt;222&gt; (3)..(3)

&lt;223&gt; Glycine can be repeated from zero to five times

&lt;400&gt; 36

Gly Cys Gly

1

&lt;210&gt; 37

&lt;211&gt; 10

&lt;212&gt; PRT

&lt;213&gt; Artificial sequence

&lt;220&gt;

&lt;223&gt; C terminal glycine serine linkers

&lt;220&gt;

&lt;221&gt; REPEAT

&lt;222&gt; (1)..(1)

&lt;223&gt; Glycine can be repeated from zero to ten times

&lt;220&gt;

&lt;221&gt; REPEAT

&lt;222&gt; (2)..(2)

&lt;223&gt; Serine can be repeated from zero to two times

&lt;220&gt;

&lt;221&gt; REPEAT

&lt;222&gt; (3)..(7)

&lt;223&gt; These residues can be repeated from zero to three times as a group

&lt;220&gt;

&lt;221&gt; REPEAT

&lt;222&gt; (8)..(8)

&lt;223&gt; Glycine can be repeated from zero to eight times

&lt;220&gt;

&lt;221&gt; REPEAT

&lt;222&gt; (10)..(10)

&lt;223&gt; Glycine can be repeated from zero to five times

&lt;400&gt; 37

Gly ser Gly Gly Gly Gly Ser Gly Cys Gly

1

5

10

&lt;210&gt; 38

&lt;211&gt; 5

&lt;212&gt; PRT

&lt;213&gt; Artificial sequence

&lt;220&gt;

&lt;223&gt; Glycine serine linker

&lt;220&gt;

&lt;221&gt; REPEAT

&lt;222&gt; (1)..(5)

&lt;223&gt; These residues can be repeated any times as a group

&lt;400&gt; 38

PA040WO\_sequence\_listing.ST25.txt

Gly Gly Gly Gly Ser  
1 5

<210> 39  
<211> 10  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> N-terminal gamma1  
<400> 39

Cys Gly Asp Lys Thr His Thr Ser Pro Pro  
1 5 10

<210> 40  
<211> 10  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> C-terminal gamma 1  
<400> 40

Asp Lys Thr His Thr Ser Pro Pro Cys Gly  
1 5 10

<210> 41  
<211> 17  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> N-terminal gamma 3  
<400> 41

Cys Gly Gly Pro Lys Pro Ser Thr Pro Pro Gly Ser Ser Gly Gly Ala  
1 5 10 15

Pro

<210> 42  
<211> 18  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> C-terminal gamma 3  
<400> 42

Pro Lys Pro Ser Thr Pro Pro Gly Ser Ser Gly Gly Ala Pro Gly Gly  
1 5 10 15

Cys Gly

PA040WO\_sequence\_listing.ST25.txt

<210> 43  
<211> 6  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> N-terminal glycine linker

<400> 43

Gly Cys Gly Gly Gly Gly  
1 5

<210> 44  
<211> 6  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> C-terminal glycine linker

<400> 44

Gly Gly Gly Gly Cys Gly  
1 5

<210> 45  
<211> 6  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> C-terminal glycine-lysine linker

<400> 45

Gly Gly Lys Lys Gly Cys  
1 5

<210> 46  
<211> 6  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> N-terminal glycine-lysine linker

<400> 46

Cys Gly Lys Lys Gly Gly  
1 5

<210> 47  
<211> 4  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> C-terminal linker

<400> 47

Gly Gly Cys Gly  
1



## PA040WO\_sequence\_listing.ST25.txt

<210> 48  
 <211> 27  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Ghrelin precursor mutant

<400> 48

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Arg Val Gln Arg Lys Glu  
 1 5 10 15

Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg  
 20 25

<210> 49  
 <211> 28  
 <212> PRT  
 <213> Canis familiaris

<400> 49

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Leu Gln Gln Arg Lys  
 1 5 10 15

Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg  
 20 25

<210> 50  
 <211> 27  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Canis familiaris ghrelin mutant

<400> 50

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Leu Gln Arg Lys Glu  
 1 5 10 15

Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg  
 20 25

<210> 51  
 <211> 27  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Mus musculus ghrelin mutant

<400> 51

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Ala Gln Arg Lys Glu  
 1 5 10 15

Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg

20

PA040WO\_sequence\_listing.ST25.txt  
25

<210> 52  
 <211> 10  
 <212> PRT  
 <213> Homo sapiens

<400> 52

Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg  
 1 5 10

<210> 53  
 <211> 8  
 <212> PRT  
 <213> Homo sapiens

<400> 53

Pro Pro Ala Lys Leu Gln Pro Arg  
 1 5

<210> 54  
 <211> 6  
 <212> PRT  
 <213> Homo sapiens

<400> 54

Ala Lys Leu Gln Pro Arg  
 1 5

<210> 55  
 <211> 10  
 <212> PRT  
 <213> Homo sapiens

<400> 55

Gly Ser Ser Phe Leu Ser Pro Glu His Gln  
 1 5 10

<210> 56  
 <211> 10  
 <212> PRT  
 <213> Homo sapiens

<400> 56

Glu His Gln Arg Val Gln Gln Arg Lys Glu  
 1 5 10

<210> 57  
 <211> 57  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> AP205 ribosomal binding site

<400> 57  
 tctagaattt tctgcgacc catcccggt ggcgccc aaa gtgaggaaaa tcacatg

57

## PA040WO\_sequence\_listing.ST25.txt

<210> 58  
 <211> 35  
 <212> DNA  
 <213> Artificial sequence

<220>  
 <223> Shine-Dalgarno sequence of vector pQb185

<400> 58  
 tctagattaa cccaacgcgt aggagtcagg ccatg 35

<210> 59  
 <211> 5  
 <212> PRT  
 <213> Homo sapiens

<400> 59  
 Lys Leu Gln Pro Arg  
 1 5

<210> 60  
 <211> 13  
 <212> PRT  
 <213> Homo sapiens

<400> 60  
 Gly Ser Ser Phe Leu Ser Pro Glu His Gln Arg Val Gln  
 1 5 10

<210> 61  
 <211> 15  
 <212> PRT  
 <213> Homo sapiens

<400> 61  
 Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg  
 1 5 10 15

<210> 62  
 <211> 13  
 <212> PRT  
 <213> Canis familiaris

<400> 62  
 Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Leu Gln  
 1 5 10

<210> 63  
 <211> 15  
 <212> PRT  
 <213> Canis familiaris

<400> 63  
 Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg  
 1 5 10 15

## PA040WO\_sequence\_listing.ST25.txt

<210> 64  
 <211> 28  
 <212> PRT  
 <213> Artificial Sequence  
  
 <220>  
 <223> human ghrelin peptide mutant  
  
 <400> 64  
 Cys Gly Ser Ser Phe Leu Ser Pro Glu His Gln Arg Val Gln Arg Lys  
 1 5 10 15

Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg  
 20 25

<210> 65  
 <211> 29  
 <212> PRT  
 <213> Artificial Sequence  
  
 <220>  
 <223> human ghrelin peptide 24-51 mutant  
  
 <400> 65  
 Cys Gly Ser Ser Phe Leu Ser Pro Glu His Gln Arg Val Gln Gln Arg  
 1 5 10 15

Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg  
 20 25

<210> 66  
 <211> 28  
 <212> PRT  
 <213> Artificial Sequence  
  
 <220>  
 <223> human ghrelin peptide mutant  
  
 <400> 66  
 Gly Ser Ser Phe Leu Ser Pro Glu His Gln Arg Val Gln Arg Lys Glu  
 1 5 10 15

Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg Cys  
 20 25

<210> 67  
 <211> 29  
 <212> PRT  
 <213> Artificial Sequence  
  
 <220>  
 <223> human ghrelin peptide 24-51 mutant  
  
 <400> 67  
 Gly Ser Ser Phe Leu Ser Pro Glu His Gln Arg Val Gln Gln Arg Lys  
 1 5 10 15

## PA040WO\_sequence\_listing.ST25.txt

Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg Cys  
                   20                                  25

<210> 68  
 <211> 14  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> human ghrelin peptide 24-36 mutant  
 <400> 68

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Arg Val Gln Cys  
 1                  5                                  10

<210> 69  
 <211> 16  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> human ghrelin peptide 37-51 mutant  
 <400> 69

Cys Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg  
 1                  5                                  10                                  15

<210> 70  
 <211> 9  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> human ghrelin peptide 44-51 mutant  
 <400> 70

Cys Pro Pro Ala Lys Leu Gln Pro Arg  
 1                  5

<210> 71  
 <211> 29  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> dog ghrelin peptide 24-51 mutant  
 <400> 71

Cys Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Leu Gln Gln Arg  
 1                  5                                  10                                  15

Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg  
                   20                                  25

<210> 72  
 <211> 28  
 <212> PRT

PA040WO\_sequence\_listing.ST25.txt

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; dog ghrelin peptide mutant

&lt;400&gt; 72

Cys Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Leu Gln Arg Lys  
 1 5 10 15

Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg  
 20 25

&lt;210&gt; 73

&lt;211&gt; 29

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; dog ghrelin peptide24-51 mutant

&lt;400&gt; 73

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Leu Gln Gln Arg Lys  
 1 5 10 15

Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg Cys  
 20 25

&lt;210&gt; 74

&lt;211&gt; 28

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; dog ghrelin mutant

&lt;400&gt; 74

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Leu Gln Arg Lys Glu  
 1 5 10 15

Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg Cys  
 20 25

&lt;210&gt; 75

&lt;211&gt; 14

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; dog ghrelin peptide 24-36 mutant

&lt;400&gt; 75

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Leu Gln Cys  
 1 5 10

&lt;210&gt; 76

&lt;211&gt; 11

&lt;212&gt; PRT

PA040WO\_sequence\_listing.ST25.txt

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; human ghrelin peptide 31-40 mutant

&lt;400&gt; 76

Cys Glu His Gln Arg Val Gln Gln Arg Lys Glu  
 1 5 10

&lt;210&gt; 77

&lt;211&gt; 29

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; mouse ghrelin peptide 24-51 mutant

&lt;400&gt; 77

Cys Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Ala Gln Gln Arg  
 1 5 10 15

Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg  
 20 25

&lt;210&gt; 78

&lt;211&gt; 37

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; oligonucleotide primer

&lt;400&gt; 78

ggtaacatcg gtcgagatgg aaaacaaact ctggtcc

37

&lt;210&gt; 79

&lt;211&gt; 37

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; oligonucleotide primer

&lt;400&gt; 79

ggaccagagt ttgttttcca tctcgaccga tgttacc

37

&lt;210&gt; 80

&lt;211&gt; 22

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; oligonucleotide primer

&lt;400&gt; 80

agctcgcccg gggatcctct ag

22

&lt;210&gt; 81

&lt;211&gt; 40

&lt;212&gt; DNA

PA040WO\_sequence\_listing.ST25.txt

<213> Artificial Sequence  
 <220>  
 <223> Oligonucleotide primer  
 <400> 81  
 cgatgcattt catccttagt tatcaatacg ctgggttcag 40

<210> 82  
 <211> 36  
 <212> DNA  
 <213> Artificial Sequence  
 <220>  
 <223> Oligonucleotide primer  
 <400> 82  
 ggcaaaatta gagactgta ctttaggtaa gatcgg 36

<210> 83  
 <211> 36  
 <212> DNA  
 <213> Artificial Sequence  
 <220>  
 <223> Oligonucleotide primer  
 <400> 83  
 ccgatcttac ctaaagtaac agtctctaatt tttgcc 36

<210> 84  
 <211> 33  
 <212> DNA  
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 <223> Oligonucleotide primer  
 <400> 84  
 ggccatggca cgactcgaga ctgttacttt agg 33

<210> 85  
 <211> 19  
 <212> DNA  
 <213> Artificial Sequence  
 <220>  
 <223> Oligonucleotide primer  
 <400> 85  
 gatttaggtg aactatag 19

<210> 86  
 <211> 37  
 <212> DNA  
 <213> Artificial Sequence  
 <220>  
 <223> Oligonucleotide primer  
 <400> 86  
 gatggacgtc aaactctggt cctcaatccg cgtgggg 37



## PA040WO\_sequence\_listing.ST25.txt

<210> 87  
<211> 37  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> oligonucleotide primer  
  
<400> 87  
ccccacgcg attgaggacc agagtttgac gtccatc 37

<210> 88  
<211> 31  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> EcoRIHBCAg(s) primer  
  
<400> 88  
ccggaattca tggacattga cccttataaa g 31

<210> 89  
<211> 51  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Lys-HBCAg(as) primer  
  
<400> 89  
cctagagcca cctttgccac catcttctaa attagtagcc acccaggtag c 51

<210> 90  
<211> 48  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Lys-HBCAg(s) primer  
  
<400> 90  
gaagatggtg gcaaaggtgg ctctagggac ctagtagtca gttatgtc 48

<210> 91  
<211> 38  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> HBcAg(1-149)Hind(as) primer  
  
<400> 91  
cgcgtcccaa gcttctaaac aacagtagtc tccggaag 38

<210> 92  
<211> 37  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> 48as primer

## PA040WO\_sequence\_listing.ST25.txt

<400> 92  
gtgcagtatg gtgaggtgag gaatgctcag gagactc 37

<210> 93  
<211> 37  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> 48s primer

<400> 93  
gagtctcctg agcattcctc acctcaccat actgcac 37

<210> 94  
<211> 33  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> 107as primer

<400> 94  
cttccaaaag tgaggaaga aatgtgaaac cac 33

<210> 95  
<211> 47  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> HBcAg149hind-as

<400> 95  
cgcgccccaa gcttctaaac aacagtagtc tccggaagcg ttgatag 47

<210> 96  
<211> 33  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> 107s primer

<400> 96  
gtggtttcac atttcttccc tcacttttg aag 33

<210> 97  
<211> 38  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> HBcAgwthindIIII primer

<400> 97  
cgcgccccaa gcttctaaca ttgagattcc cgagattg 38

<210> 98  
<211> 10  
<212> PRT

PA040WO\_sequence\_listing.ST25.txt

<213> Artificial sequence

<220>

<223> epitope CeH3

<400> 98

Val Asn Leu Thr Trp Ser Arg Ala Ser Gly  
 1 5 10

<210> 99

<211> 51

<212> DNA

<213> Artificial sequence

<220>

<223> CeH3fwd primer

<220>

<221> CDS

<222> (1)..(51)

<400> 99

ggt aac ttg acc tgg tct cgt gct tct ggt gca tcc agg gat cta gta 48  
 Val Asn Leu Thr Trp Ser Arg Ala Ser Gly Ala Ser Arg Asp Leu Val  
 1 5 10 15

gtc 51  
 Val

<210> 100

<211> 17

<212> PRT

<213> Artificial sequence

<220>

<223> CeH3fwd primer

<400> 100

Val Asn Leu Thr Trp Ser Arg Ala Ser Gly Ala Ser Arg Asp Leu Val  
 1 5 10 15

Val

<210> 101

<211> 51

<212> DNA

<213> Artificial sequence

<220>

<223> CeH3rev primer

<400> 101

accagaagca cgagaccagg tcaagttaac atcttccaaa ttattacca c 51

<210> 102

<211> 7

<212> PRT

<213> Artificial sequence

## PA040WO\_sequence\_listing.ST25.txt

&lt;220&gt;

&lt;223&gt; CeH3rev primer peptide

&lt;400&gt; 102

Asp Glu Leu Asn Asn Gly Val  
 1 5

&lt;210&gt; 103

&lt;211&gt; 31

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; HBcAg-wt EcoRI fwd primer

&lt;400&gt; 103

ccggaattca tggacattga cccttataaa g

31

&lt;210&gt; 104

&lt;211&gt; 38

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; HBcAg-wt Hind III rev primer

&lt;400&gt; 104

cgcggtcccaa gcttctaaca ttgagattcc cgagattg

38

&lt;210&gt; 105

&lt;211&gt; 30

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Ghre1C mutant

&lt;400&gt; 105

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Ala Gln Gln Arg Lys  
 1 5 10 15

Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg Gly Cys  
 20 25 30

&lt;210&gt; 106

&lt;211&gt; 28

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; cGhrQ14 mutant

&lt;400&gt; 106

Cys Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Ala Gln Arg Lys  
 1 5 10 15

Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg  
 20 25

## PA040WO\_sequence\_listing.ST25.txt

<210> 107  
 <211> 29  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> GhrQ14C mutant

<400> 107

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Ala Gln Arg Lys Glu  
 1 5 10 15

Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg Gly Cys  
 20 25

<210> 108  
 <211> 11  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Ghrelin peptide mutant

<400> 108

Cys Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg  
 1 5 10

<210> 109  
 <211> 7  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Ghrelin peptide mutant

<400> 109

Cys Ala Lys Leu Gln Pro Arg  
 1 5

<210> 110  
 <211> 11  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Ghrelin peptide mutant

<400> 110

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Cys  
 1 5 10

<210> 111  
 <211> 11  
 <212> PRT  
 <213> Homo sapiens

<400> 111

## PA040W0\_sequence\_listing.ST25.txt

Glu His Gln Arg Val Gln Gln Arg Lys Glu Ser  
 1 5 10

<210> 112  
 <211> 10  
 <212> PRT  
 <213> Mus musculus

<400> 112

Glu His Gln Lys Ala Gln Gln Arg Lys Glu  
 1 5 10

<210> 113  
 <211> 11  
 <212> PRT  
 <213> Mus musculus

<400> 113

Glu His Gln Lys Ala Gln Gln Arg Lys Glu Ser  
 1 5 10

<210> 114  
 <211> 10  
 <212> PRT  
 <213> Canis familiaris

<400> 114

Glu His Gln Lys Leu Gln Gln Arg Lys Glu  
 1 5 10

<210> 115  
 <211> 11  
 <212> PRT  
 <213> Canis familiaris

<400> 115

Glu His Gln Lys Leu Gln Gln Arg Lys Glu Ser  
 1 5 10

<210> 116  
 <211> 10  
 <212> PRT  
 <213> Homo sapiens

<400> 116

Leu Ser Pro Glu His Gln Arg Val Gln Gln  
 1 5 10

<210> 117  
 <211> 10  
 <212> PRT  
 <213> Mus musculus

<400> 117

Leu Ser Pro Glu His Gln Lys Ala Gln Gln

1 5 PA040WO\_sequence\_listing.ST25.txt  
10

<210> 118  
<211> 10  
<212> PRT  
<213> Canis familiaris

<400> 118

Leu Ser Pro Glu His Gln Lys Leu Gln Gln  
1 5 10

<210> 119  
<211> 7  
<212> PRT  
<213> Homo sapiens

<400> 119

Gly Ser Ser Phe Leu Ser Pro  
1 5

<210> 120  
<211> 29  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> ghrelin peptide mutant

<400> 120

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Arg Val Gln Arg Lys Glu  
1 5 10 15

Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg Gly Cys  
20 25

<210> 121  
<211> 30  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> ghrelin peptide mutant

<400> 121

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Arg Val Gln Gln Arg Lys  
1 5 10 15

Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg Gly Cys  
20 25 30

<210> 122  
<211> 15  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> ghrelin peptide mutant

## PA040WO\_sequence\_listing.ST25.txt

&lt;400&gt; 122

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Arg Val Gln Gly Cys  
1 5 10 15

&lt;210&gt; 123

&lt;211&gt; 30

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; ghrelin peptide mutant

&lt;400&gt; 123

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Leu Gln Gln Arg Lys  
1 5 10 15

Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg Gly Cys  
20 25 30

&lt;210&gt; 124

&lt;211&gt; 29

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; ghrelin peptide mutant

&lt;400&gt; 124

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Leu Gln Arg Lys Glu  
1 5 10 15

Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg Gly Cys  
20 25

&lt;210&gt; 125

&lt;211&gt; 15

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; ghrelin peptide mutant

&lt;400&gt; 125

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Leu Gln Gly Cys  
1 5 10 15

&lt;210&gt; 126

&lt;211&gt; 28

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; mouse ghrelin peptide mutant

&lt;400&gt; 126

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Ala Gln Arg Lys Glu



1 5 PA040wo\_sequence\_listing.ST25.txt 15  
10

Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg Cys  
20 25

<210> 127  
<211> 29  
<212> PRT  
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<220>  
<223> mouse ghrelin peptide mutant

<400> 127

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Ala Gln Arg Lys Glu  
1 5 10 15

Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg Gly Cys  
20 25

<210> 128  
<211> 29  
<212> PRT  
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<220>  
<223> mouse ghrelin peptide mutant

<400> 128

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Ala Gln Gln Arg Lys  
1 5 10 15

Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg Cys  
20 25

<210> 129  
<211> 30  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> mouse ghrelin peptide mutant

<400> 129

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Ala Gln Gln Arg Lys  
1 5 10 15

Glu Ser Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg Gly Cys  
20 25 30

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<211> 14  
<212> PRT  
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<220>  
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&lt;400&gt; 130

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Ala Gln Cys  
 1 5 10

&lt;210&gt; 131

&lt;211&gt; 15

&lt;212&gt; PRT

&lt;213&gt; Artificial sequence

&lt;220&gt;

&lt;223&gt; mouse ghrelin peptide mutant

&lt;400&gt; 131

Gly Ser Ser Phe Leu Ser Pro Glu His Gln Lys Ala Gln Gly Cys  
 1 5 10 15

&lt;210&gt; 132

&lt;211&gt; 13

&lt;212&gt; PRT

&lt;213&gt; Artificial sequence

&lt;220&gt;

&lt;223&gt; Ghrel24-33C

&lt;400&gt; 132

Gly Gly Ser Ser Phe Leu Ser Pro Glu His Gln Gly Cys  
 1 5 10

&lt;210&gt; 133

&lt;211&gt; 11

&lt;212&gt; PRT

&lt;213&gt; Artificial sequence

&lt;220&gt;

&lt;223&gt; cGhrel42-51

&lt;400&gt; 133

Cys Lys Lys Pro Pro Ala Lys Leu Gln Pro Arg  
 1 5 10

&lt;210&gt; 134

&lt;211&gt; 11

&lt;212&gt; PRT

&lt;213&gt; Artificial sequence

&lt;220&gt;

&lt;223&gt; cGhrel31-40

&lt;400&gt; 134

Cys Glu His Gln Lys Ala Gln Gln Arg Lys Glu  
 1 5 10

&lt;210&gt; 135

&lt;211&gt; 12

&lt;212&gt; PRT

&lt;213&gt; Artificial sequence

## PA040wo\_sequence\_listing.ST25.txt

&lt;220&gt;

&lt;223&gt; cGhrel31-41

&lt;400&gt; 135

Cys Glu His Gln Lys Ala Gln Gln Arg Lys Glu Ser  
 1 5 10

&lt;210&gt; 136

&lt;211&gt; 11

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; cGhrel28-37

&lt;400&gt; 136

Cys Leu Ser Pro Glu His Gln Lys Ala Gln Gln  
 1 5 10

&lt;210&gt; 137

&lt;211&gt; 12

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; human ghrelin peptide mutant

&lt;400&gt; 137

Cys Glu His Gln Arg Val Gln Gln Arg Lys Glu Ser  
 1 5 10

&lt;210&gt; 138

&lt;211&gt; 11

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; human ghrelin peptide mutant

&lt;400&gt; 138

Cys Leu Ser Pro Glu His Gln Arg Val Gln Gln  
 1 5 10

&lt;210&gt; 139

&lt;211&gt; 25

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; primer p1.44

&lt;400&gt; 139

aaccatggca aataagccaa tgcaa

25

&lt;210&gt; 140

&lt;211&gt; 30

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

PA040WO\_sequence\_listing.ST25.txt

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<220>
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<400> 140
aatctagaat tttctgcgca cccatcccgg          30

<210> 141
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> primer p1.46

<400> 141
aaaagcttaa gcagtagtat cagacgatac g          31

<210> 142
<211> 43
<212> DNA
<213> Artificial Sequence

<220>
<223> primer p1.47

<400> 142
gagtgatcca actcgtttat caactacatt ttcagcaagt ctg          43

<210> 143
<211> 43
<212> DNA
<213> Artificial Sequence

<220>
<223> primer p1.48

<400> 143
cagacttgct gaaaatgtag ttgataaacg agttggatca ctc          43

<210> 144
<211> 117
<212> PRT
<213> homo sapiens

<400> 144
Met Pro Ser Pro Gly Thr Val Cys Ser Leu Leu Leu Leu Gly Met Leu
1          5          10
Trp Leu Asp Leu Ala Met Ala Gly Ser Ser Phe Leu Ser Pro Glu His
          20          25          30
Gln Arg Val Gln Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu
          35          40          45
Gln Pro Arg Ala Leu Ala Gly Trp Leu Arg Pro Glu Asp Gly Gly Gln
          50          55          60
Ala Glu Gly Ala Glu Asp Glu Leu Glu Val Arg Phe Asn Ala Pro Phe
65          70          75          80

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## PA040WO\_sequence\_listing.ST25.txt

Asp Val Gly Ile Lys Leu Ser Gly Val Gln Tyr Gln Gln His Ser Gln  
85 90 95

Ala Leu Gly Lys Phe Leu Gln Asp Ile Leu Trp Glu Glu Ala Lys Glu  
100 105 110

Ala Pro Ala Asp Lys  
115

<210> 145  
<211> 117  
<212> PRT  
<213> Canis familiaris  
<400> 145

Met Pro Ser Pro Gly Thr Val Cys Ser Leu Leu Leu Leu Gly Met Leu  
1 5 10 15

Trp Leu Asp Leu Ala Met Ala Gly Ser Ser Phe Leu Ser Pro Glu His  
20 25 30

Gln Lys Leu Gln Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu  
35 40 45

Gln Pro Arg Ala Leu Ala Gly Trp Leu Arg Pro Glu Asp Gly Gly Gln  
50 55 60

Ala Glu Gly Ala Glu Asp Glu Leu Glu Val Arg Phe Asn Ala Pro Phe  
65 70 75 80

Asp Val Gly Ile Lys Leu Ser Gly Val Gln Tyr Gln Gln His Ser Gln  
85 90 95

Ala Leu Gly Lys Phe Leu Gln Asp Ile Leu Trp Glu Glu Ala Lys Glu  
100 105 110

Ala Pro Ala Asp Lys  
115

<210> 146  
<211> 117  
<212> PRT  
<213> Mus musculus  
<400> 146

Met Pro Ser Pro Gly Thr Val Cys Ser Leu Leu Leu Leu Gly Met Leu  
1 5 10 15

Trp Leu Asp Leu Ala Met Ala Gly Ser Ser Phe Leu Ser Pro Glu His  
20 25 30

## PA040WO\_sequence\_listing.ST25.txt

Gln Lys Ala Gln Gln Arg Lys Glu Ser Lys Lys Pro Pro Ala Lys Leu  
 35 40 45

Gln Pro Arg Ala Leu Ala Gly Trp Leu Arg Pro Glu Asp Gly Gly Gln  
 50 55 60

Ala Glu Gly Ala Glu Asp Glu Leu Glu Val Arg Phe Asn Ala Pro Phe  
 65 70 75 80

Asp Val Gly Ile Lys Leu Ser Gly Val Gln Tyr Gln Gln His Ser Gln  
 85 90 95

Ala Leu Gly Lys Phe Leu Gln Asp Ile Leu Trp Glu Glu Ala Lys Glu  
 100 105 110

Ala Pro Ala Asp Lys  
 115