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(54) METHODS AND COMPOSITIONS FOR TARGETING PROTEINS ACROSS THE BLOOD BRAIN BARRIER

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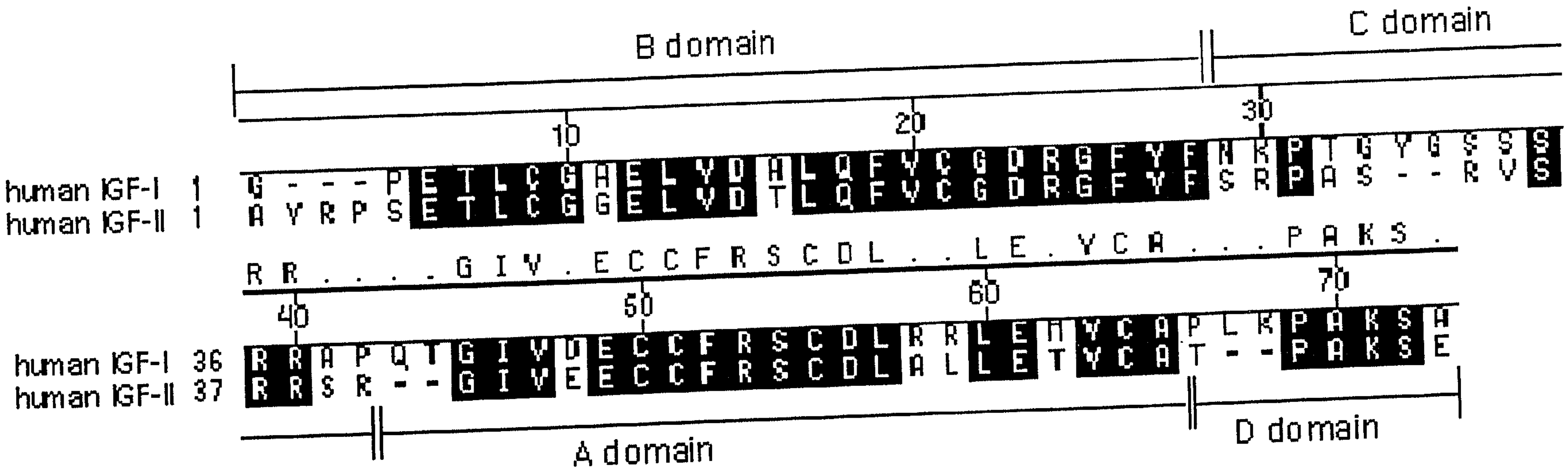
(57) **ABSTRACT**

Disclosed are methods and compositions for targeting therapeutic proteins to the brain. Methods and compositions of the invention involve associating an IGF moiety with a therapeutic protein in order to target the therapeutic protein to the brain. Soluble fusion proteins that include an IGF targeting moiety are transported to neural tissue in the brain from blood. Methods and compositions of the invention include therapeutic applications for treating lysosomal storage diseases. The invention also provides nucleic acids and cells for expressing IGF fusion proteins.



Alignment of human IGF-I and IGF-II mature proteins showing location of domains.

Figure 1

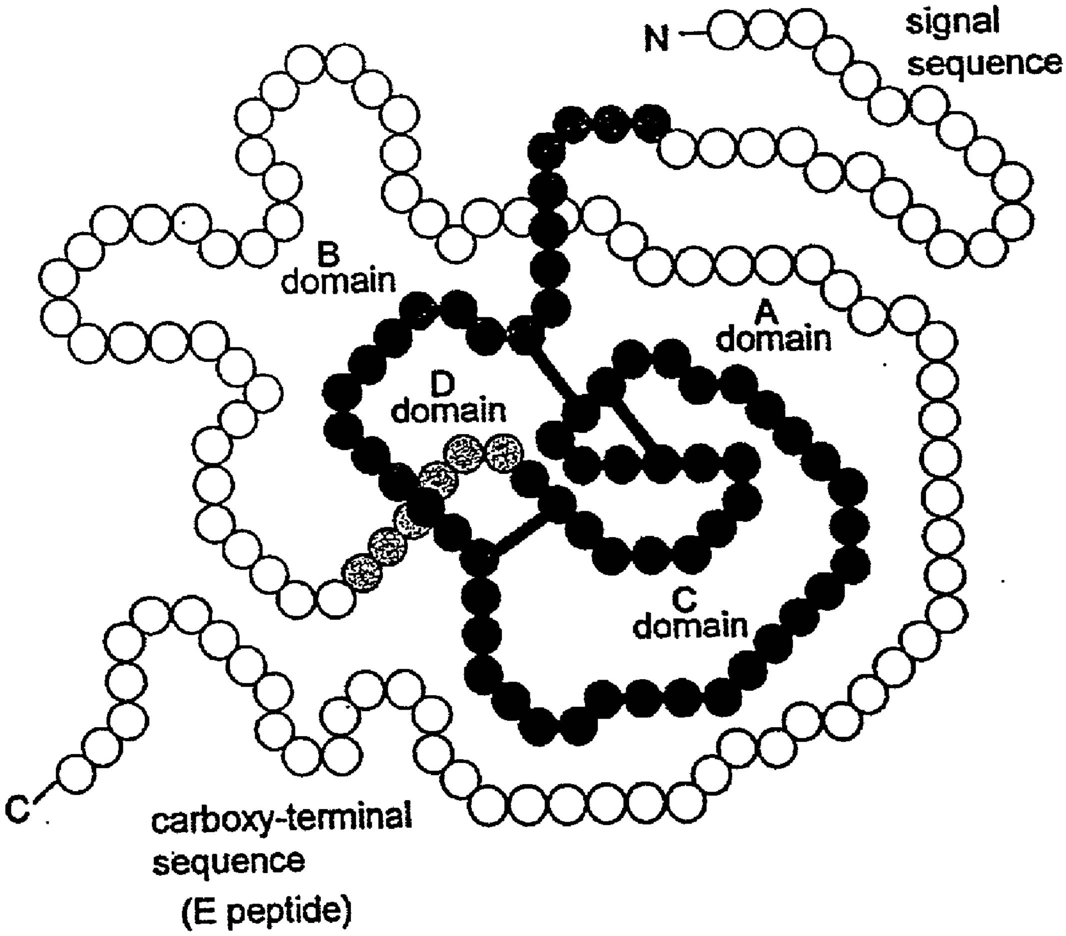

N
signal sequence
B domain
A domain
D domain
C domain
C
carboxy-terminal sequence (E peptide)

Figure 2

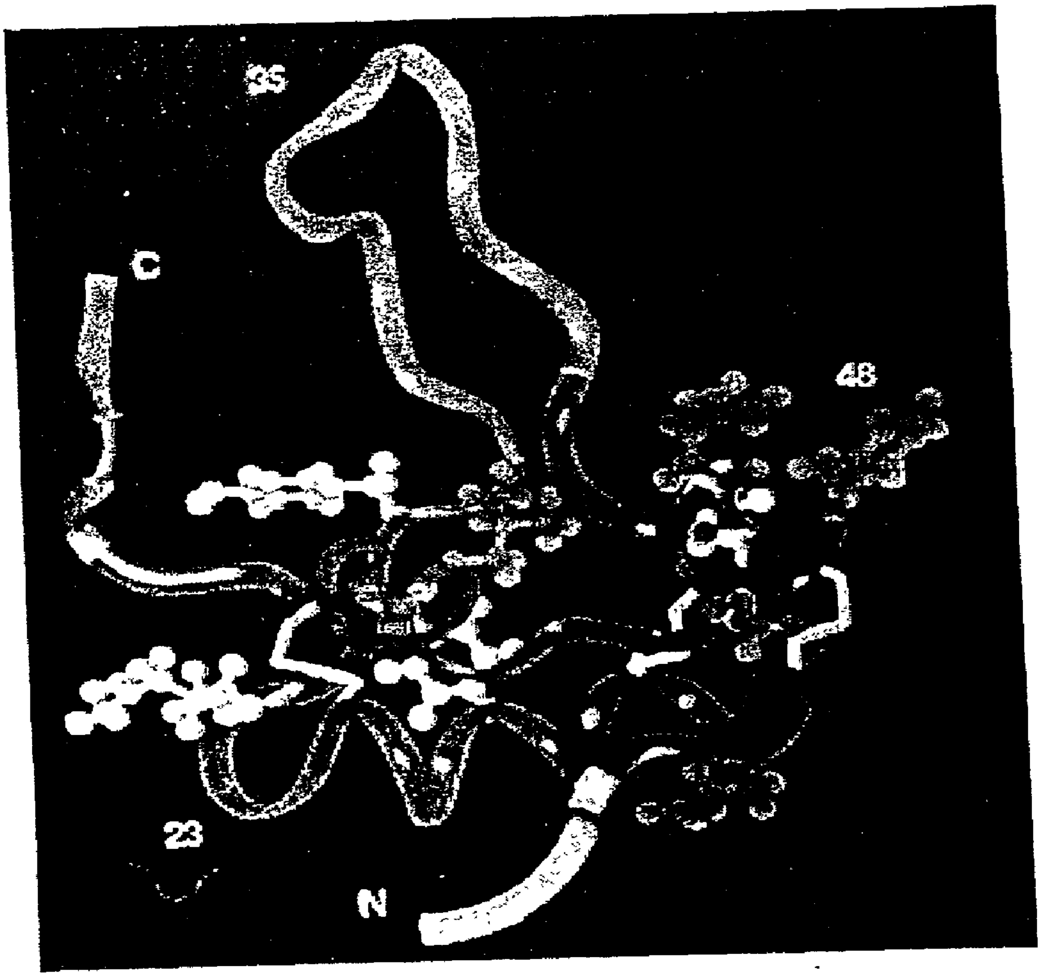


Figure 3

MGKISSLPTQLFKCCFCDFLKVKMHTMSSSHLFYLALCLLTFTS
SATAGPETLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRAPQTGIVDECCFRSCDL
RRLEMYCAPLKPAKSARSVRAQRHTDMPKTQKEVHLKNASRGSAGNKNYRM

FIG 4A

1 tcactgtcac tgctaaattc agagcagatt agagcctgcg caatggaata aagtcctcaa
61 aattgaaatg tgacattgct ctcaacatct cccatctctc tggatttcct tttgcttcat
121 tattcctgct aaccaattca ttttcagact ttgtacttca gaagcaatgg gaaaaatcag
181 cagtcttcca acccaattat ttaagtgctg cttttgtgat ttcttgaagg tgaagatgca
241 caccatgtcc tcctcgcatc tcttctacct ggcgctgtgc ctgctcacct tcaccagctc
301 tgccacggct ggaccggaga cgctctgcgg ggctgagctg gtggatgctc ttcagttcgt
361 gtgtggagac aggggctttt atttcaacaa gcccacaggg tatggctcca gcagtcggag
421 ggcgcctcag acaggcatcg tggatgagtg ctgcttccgg agctgtgatc taaggaggct
481 ggagatgtat tgcgcacccc tcaagcctgc caagtcagct cgctctgtcc gtgcccagcg
541 ccacaccgac atgcccaaga cccagaagga agtacatttg aagaacgcaa gtagagggag
601 tgcaggaaac aagaactaca ggatgtagga agaccctcct gaggagtgaa gagtgacatg
661 ccaccgcagg atcctttgct ctgcacgagt tacctgttaa actttggaac acctaccaaa
721 aaataagttt gataacattt aaaagatggg cgtttccccc aatgaaatac acaagtaaac
781 attccaacat tgtctttagg agtgatttgc accttgcaaa aatggtcctg gagttggtag
841 attgctgttg atcttttatc aataatgttc tatagaaaag aaaaaaaaat atatatatat
901 atatatctta gtccctgcct ctcaagagcc acaaatgcat gggtgttgta tagatccagt
961 tgcactaaat tcctctctga atcttggctg ctggagccat tcattcagca accttgtcta
1021 agtggtttat gaattgtttc cttatttgca cttctttcta cacaactcgg gctgtttgtt
1081 ttacagtgtc tgataatctt gttagtctat acccaccacc tcccttcata acctttatat
1141 ttgccgaatt tggcctcctc aaaagcagca gcaagtcgtc aagaagcaca ccaattctaa
1201 cccacaagat tccatctgtg gcatttgtac caaatataag ttggatgcat tttattttag
1261 acacaaagct ttatttttcc acatcatgct tacaaaaaag aataatgcaa atagttgcaa
1321 ctttgaggcc aatcattttt aggcatatgt tttaaacata gaaagtttct tcaactcaaa
1381 agagttcctt caaatgatga gttaatgtgc aacctaatta gtaactttcc tctttttatt
1441 ttttccatat agagcactat gtaaatttag catatcaatt atacaggata tatcaaacag
1501 tatgtaaaac tctgtttttt agtataatgg tgctattttg tagtttgtta tatgaaagag
1561 tctggccaaa acggtaatac gtgaaagcaa aacaataggg gaagcctgga gccaaagatg
1621 acacaagggg aagggtactg aaaacaccat ccatttggga aagaaggcaa agtcccccca
1681 gttatgcctt ccaagaggaa cttcagacac aaaagtccac tgatgcaaat tggactggcg
1741 agtccagaga ggaaactgtg gaatggaaaa agcagaaggc taggaatttt agcagtcctg
1801 gtttcttttt ctcatggaag aaatgaacat ctgccagctg tgtcatggac tcaccactgt
1861 gtgaccttgg gcaagtcact tcacctctct gtgcctcagt ttcctcatct gcaaaatggg
1921 ggcaatatgt catctaccta cctcaaaggg gtggtataag gtttaaaaag ataaagattc
1981 agattttttt accctgggtt gctgtaaggg tgcaacatca gggcgcttga gttgctgaga
2041 tgcaaggaat tctataaata acccattcat agcatagcta gagattggtg aattgaatgc
2101 tcctgacatc tcagttcttg tcagtgaagc tatccaaata actggccaac tagttgttaa
2161 aagctaacag ctcaatctct taaaacactt ttcaaaatat gtgggaagca tttgattttc
2221 aatttgattt tgaattctgc atttggtttt atgaatacaa agataagtga aaagagagaa
2281 aggaaaagaa aaaggagaaa aacaaagaga tttctaccag tgaaagggga attaattact
2341 ctttgttagc actcactgac tcttctatgc agttactaca tatctagtaa aaccttgttt
2401 aatactataa ataatattct attcattttg aaaaacacaa tgattccttc ttttctaggc
2461 aatataagga aagtgatcca aaatttgaaa tattaaaata atatctaata aaaagtcaca
2521 aagttatctt ctttaacaaa ctttactctt attcttagct gtatatacat ttttttaaaa
2581 agtttgttaa aatatgcttg actagagttt cagttgaaag gcaaaaactt ccatcacaac
2641 aagaaatttc ccatgcctgc tcagaagggt agcccctagc tctctgtgaa tgtgttttat
2701 ccattcaact gaaaattggt atcaagaaag tccactggtt agtgtactag tccatcatag
2761 cctagaaaat gatccctatc tgcagatcaa gattttctca ttagaacaat gaattatcca
2821 gcattcagat ctttctagtc accttagaac tttttggtta aaagtaccca ggcttgatta
2881 tttcatgcaa attctatatt ttacattctt ggaaagtcta tatgaaaaac aaaaataaca
2941 tcttcagttt ttctcccact gggtcacctc aaggatcaga ggccaggaaa aaaaaaaaag
3001 actccctgga tctctgaata tatgcaaaaa gaaggcccca tttagtggag ccagcaatcc
3061 tgttcagtca acaagtattt taactctcag tccaacatta tttgaattga gcacctcaag
3121 catgcttagc aatgttctaa tcactatgga cagatgtaaa agaaactata catcattttt
3181 gccctctgcc tgttttccag acatacaggt tctgtggaat aagatactgg actcctcttc
3241 ccaagatggc acttcttttt atttcttgtc cccagtgtgt accttttaaa attattccct

FIG 4B-1

3301 ctcaacaaaa ctttataggc agtcttctgc agacttaaca tgttttctgt catagttaga
3361 tgtgataatt ctaagagtgt ctatgactta tttccttcac ttaattctat ccacagtcaa
3421 aaatccccca aggaggaaag ctgaaagatg caactgccaa tattatcttt cttaactttt
3481 tccaacacat aatcctctcc aactggatta taaataaatt gaaaataact cattatacca
3541 attcactatt ttatttttta atgaattaaa actagaaaac aaattgatgc aaaccctgga
3601 agtcagttga ttactatata ctacagcaga atgactcaga tttcatagaa aggagcaacc
3661 aaaatgtcac aaccaaaact ttacaagctt tgcttcagaa ttagattgct ttataattct
3721 tgaatgaggc aatttcaaga tatttgtaaa agaacagtaa acattggtaa gaatgagctt
3781 tcaactcata ggcttatttc caatttaatt gaccatactg gatacttagg tcaaatttct
3841 gttctctctt gcccaaataa tattaaagta ttatttgaac tttttaagat gaggcagttc
3901 ccctgaaaaa gttaatgcag ctctccatca gaatccactc ttctagggat atgaaaatct
3961 cttaacaccc accctacata cacagacaca cacacacaca cacacacaca cacacacaca
4021 cacacattca ccctaaggat ccaatggaat actgaaaaga aatcacttcc ttgaaaattt
4081 tattaaaaaa caaacaaaca aacaaaaagc ctgtccaccc ttgagaatcc ttcctctcct
4141 tggaacgtca atgtttgtgt agatgaaacc atctcatgct ctgtggctcc agggtttctg
4201 ttactatttt atgcacttgg gagaaggctt agaataaaag atgtagcaca ttttgctttc
4261 ccatttattg tttggccagc tatgccaatg tggtgctatt gtttctttaa gaaagtactt
4321 gactaaaaaa aaaagaaaaa aagaaaaaaa agaaagcata gacatatttt tttaaagtat
4381 aaaaacaaca attctataga tagatggctt aataaaatag cattaggtct atctagccac
4441 caccaccttt caacttttta tcactcacaa gtagtgtact gttcaccaaa ttgtgaattt
4501 gggggtgcag gggcaggagt tggaaatttt ttaaagttag aaggctccat tgttttgttg
4561 gctctcaaac ttagcaaaat tagcaatata ttatccaatc ttctgaactt gatcaagagc
4621 atggagaata aacgcgggaa aaaagatctt ataggcaaat agaagaattt aaaagataag
4681 taagttcctt attgattttt gtgcactctg ctctaaaaca gatattcagc aagtggagaa
4741 aataagaaca aagagaaaaa atacatagat ttacctgcaa aaaatagctt ctgccaaatc
4801 ccccttgggt attctttggc atttactggt ttatagaaga cattctccct tcacccagac
4861 atctcaaaga gcagtagctc tcatgaaaag caatcactga tctcatttgg gaaatgttgg
4921 aaagtatttc cttatgagat gggggttatc tactgataaa gaaagaattt atgagaaatt
4981 gttgaaagag atggctaaca atctgtgaag atttttgttt tcttggtttt gttttttttt
5041 ttttttttac tttatacagt ctttatgaat ttcttaatgt tcaaaatgac ttggttcttt
5101 tcttcttttt tttatatcag aatgaggaat aataagttaa acccacatag actctttaaa
5161 actataggct agatagaaat gtatgtttga cttgttgaag ctataatcag actatttaaa
5221 atgttttgct atttttaatc ttaaaagatt gtgctaattt attagagcag aacctgtttg
5281 gctctcctca gaagaaagaa tctttccatt caaatcacat ggctttccac caatattttc
5341 aaaagataaa tctgatttat gcaatggcat catttatttt aaaacagaag aattgtgaaa
5401 gtttatgccc ctcccttgca aagaccataa agtccagatc tggtaggggg gcaacaacaa
5461 aaggaaaatg ttgttgattc ttggttttgg attttgtttt gttttcaatg ctagtgttta
5521 atcctgtagt acatatttgc ttattgctat tttaatattt tataagacct tcctgttagg
5581 tattagaaag tgatacatag atatcttttt tgtgtaattt ctatttaaaa aagagagaag
5641 actgtcagaa gctttaagtg catatggtac aggataaaga tatcaattta aataaccaat
5701 tcctatctgg aacaatgctt ttgtttttta aagaaacctc tcacagataa gacagaggcc
5761 caggggattt ttgaagctgt ctttattctg cccccatccc aacccagccc ttattatttt
5821 agtatctgcc tcagaatttt atagagggct gaccaagctg aaactctaga attaaaggaa
5881 cctcactgaa aacatatatt tcacgtgttc cctctctttt ttttcctttt tgtgagatgg
5941 ggtctcgcac tgtcccccag gctggagtgc agtggcatga tctcggctca ctgcaacctc
6001 cacctcctgg gtttaagcga ttctcctgcc tcagcctcct gagtagctgg gattacaggc
6061 acccaccact atgcccggct aattttttgg atttttaata gagacggggt tttaccatgt
6121 tggccaggtt ggactcaaac tcctgacctt gtgatttgcc cgcctcagcc tcccaaattg
6181 ctgggattac aggcatgagc caccacaccc tgcccatgtg ttccctctta atgtatgatt
6241 acatggatct taaacatgat ccttctctcc tcattcttca actatctttg atggggtctt
6301 tcaaggggaa aaaaatccaa gcttttttaa agtaaaaaaa aaaaaagaga ggacacaaaa
6361 ccaaatgtta ctgctcaact gaaatatgag ttaagatgga gacagagttt ctcctaataa
6421 ccggagctga attacctttc actttcaaaa acatgacctt ccacaatcct tagaatctgc
6481 ctttttttat attactgagg cctaaaagta aacattactc attttatttt gcccaaaatg
6541 cactgatgta aagtaggaaa aataaaaaca gagctctaaa atccctttca agccacccat
6601 tgaccccact caccaactca tagcaaagtc acttctgtta atcccttaat ctgattttgt

FIG 4B-2

6661 ttggatattt atcttgtacc cgctgctaaa cacactgcag gagggactct gaaacctcaa
6721 gctgtctact tacatctttt atctgtgtct gtgtatcatg aaaatgtcta ttcaaaatat
6781 caaaaccttt caaatatcac gcagcttata ttcagtttac ataaaggccc caaataccat
6841 gtcagatctt tttggtaaaa gagttaatga actatgagaa ttgggattac atcatgtatt
6901 ttgcctcatg tatttttatc acacttatag gccaagtgtg ataaataaac ttacagacac
6961 tgaattaatt tcccctgcta ctttgaaacc agaaaataat gactggccat tcgttacatc
7021 tgtcttagtt gaaaagcata tttttatta aattaattct gattgtattt gaaattatta
7081 ttcaattcac ttatggcaga ggaatatcaa tcctaatgac ttctaaaaat gtaactaatt
7141 gaatcattat cttacattta ctgtttaata agcatatttt gaaaatgtat ggctagagtg
7201 tcataataaa atggtatatc tttctttagt aattacaaaa aaaaaaaaaa aaaaaaaaaa //

FIG 4B-3

METHODS AND COMPOSITIONS FOR TARGETING PROTEINS ACROSS THE BLOOD BRAIN BARRIER

RELATED APPLICATIONS

[**0001**] This application claims the benefit of U.S. Ser. No. 60/329,650, filed Oct. 16, 2001, the entire disclosure of which is incorporated herein by reference.

FIELD OF THE INVENTION

[**0002**] This invention provides a means for specifically delivering proteins to the brain. The ability to target proteins to the brain is of great utility in the treatment of neurological diseases. Methods and compositions of the invention are useful to target proteins to cells across the blood brain barrier, and in particular, to target proteins to the lysosomes of cells in the CNS, including neuronal cells, macrophage cells, and other cell types. Accordingly, the invention provides methods and compositions to deliver therapeutically useful proteins to treat lysosomal storage diseases that affect the CNS.

BACKGROUND

[**0003**] The blood-brain barrier maintains a homeostatic environment in the central nervous system (CNS). The capillaries that supply the blood to the brain have tight junctions which block passage of most molecules through the capillary endothelial membranes. While the membranes do allow passage of lipid soluble materials, water soluble materials such as glucose, proteins and amino acids do not pass through the blood brain barrier. Mediated transport mechanisms exist to transport glucose and essential amino acids across the blood brain barrier. Active transport mechanisms remove molecules which become in excess, such as potassium, from the brain. However, the blood brain barrier impedes the delivery of drugs to the CNS.

[**0004**] Many neurological diseases result from cellular defects in the CNS. In particular, many lysosomal storage diseases affect cells of the CNS and result in mild to serious neurological symptoms. Accordingly, the ability to deliver therapeutic compositions to the CNS is an important aspect of an effective treatment for many diseases, including many lysosomal storage diseases.

[**0005**] Methods have been designed to deliver needed drugs to the CNS such as direct delivery within the CNS by intrathecal delivery. However, methods are not available in the art to efficiently deliver drugs, and particularly protein-based drugs, from the blood stream to the CNS through the blood brain barrier.

[**0006**] Therefore, there is a need in the art for methods to deliver proteins to the brain parenchyma on the CNS side of the blood brain barrier, and in particular to deliver proteins to the lysosomes of cells in the CNS.

SUMMARY OF THE INVENTION

[**0007**] The present invention provides general methods and compositions for targeting compositions from the blood stream to the brain or CNS. According to the invention, an IGF moiety is used to target a molecule from the blood stream to the brain parenchyma on the other side of the blood brain barrier. Preferred molecules are therapeutic polypeptides.

[**0008**] Accordingly, the invention relates in one aspect to a protein including a therapeutic agent attached to an insulin-like growth factor (IGF) moiety or tag. In one embodiment, the protein is expressed as a fusion protein along with the IGF tag. In a preferred embodiment, the fusion protein also includes a lysosomal targeting portion sufficiently duplicative of IGF-II such that the targeting portion binds the cation independent mannose-6-phosphate/IGF-II receptor to mediate uptake by a lysosome. In another embodiment, the fusion protein also comprises mannose-6-phosphate in order to target the protein to the lysosomes.

[**0009**] Preferred IGF moieties or tags are IGF-I or IGF-II tags. Most preferred IGF tags are IGF-I tags. In one aspect, the IGF tag is an intact IGF-I or IGF-II protein. Alternatively, an IGF tag is a portion of an IGF-I or IGF-II protein that is sufficient for targeting through the blood brain barrier. Preferred portions comprise at least one of the A, B, C, or D domains, or the C-terminal region or a portion thereof, of either IGF-I or IGF-II. In one embodiment, an IGF tag includes both an A and a B domain. According to the invention, the A and B domains provide core structural features of a preferred IGF moiety. The A and B domains may be linked by a linker peptide. Alternatively, the A and B domains may be provided as separate peptides that dimerize to form an IGF tag. Preferably, A and B domains from the same IGF protein are used. However, an A domain from IGF-I can be associated with a B domain from IGF-II. Similarly, an A domain from IGF-II can be associated with a B domain from IGF-I. Accordingly, composition of the invention include chimeric IGF-I/IGF-II molecules. For example, an A domain from one IGF protein can be joined to the C and B domains of another IGF protein. Alternative combinations of A, B, and C domains are also useful. In further embodiments, the A domain of one IGF protein can be joined directly to the domain of another IGF protein, for example by using an amino acid bridge such as a two amino acid bridge.

[**0010**] A most preferred IGF moiety comprises an IGF-I portion selected from the group consisting of IGF-I fragments from about residue 1 to about residue 25, IGF-I fragments from about residue 25 to about residue 40, IGF-I fragments from about residue 40 to about residue 65, and IGF-I fragments from about residue 65 to about residue 70 of the IGF-I sequence shown in **FIG. 1**. Alternative preferred regions of IGF-I and IGF-II comprise regions of homology between IGF-I and IGF-II such as those shown in **FIG. 1** for human IGF-I and IGF-II. The sequences shown in **FIG. 1** relate to mature IGF-I and IGF-1l proteins. Specific IGF variants described herein refer to the mature amino acid sequence numbering shown in **FIG. 1**. In a further embodiment, an IGF tag comprises the C-terminal fragment of an IGF protein, for example the region C-terminal to the D domain shown in **FIG. 2**. A preferred IGF tag includes an IGF-I C-terminal fragment. In addition, according to the invention, IGF tags include peptide tags with a sequence that is sufficiently duplicative of the IGF tags described herein to effectively target compositions of the invention to the brain parenchyma across the blood brain barrier. In some embodiments, an IGF tag includes at least one peptide sequence from an IGF-I protein and one from an IGF-II protein.

[**0011**] Most preferred IGF tags are based on human IGF proteins. However, IGF tags based on IGF proteins from other mammals, such as mouse, rabbit, monkey, and pig IGF proteins, are also useful according to the invention. Preferred IGF tags such as the IGF fragments, peptides, or domains described herein are between 1 and 100 amino acids long, more preferably between 10 and 50 amino acids long, and even more preferably about 25 amino acids long, and are sufficient for targeting associated peptides to the brain. Preferred IGF fragments, peptides, or domains are based on the mature IGF-I and IGF-II sequences.

[**0012**] IGF tags of the invention can be fused to a therapeutic peptide at its N-terminus, C-terminus, within the body of the therapeutic peptide, or a combination of the above. When an IGF moiety is fused to the N-terminus of a therapeutic protein, an IGF signal peptide is preferably included in the expression construct, However, an IGF signal peptide can also be included at the N-terminus when the IGF targeting moiety is located at the C-terminus or within the body of the therapeutic protein. In a preferred embodiment, the IGF tag is fused to the C-terminal end of a peptide. In one embodiment, a first domain of an IGF tag is fused to a therapeutic peptide, and a second domain of the IGF tag is provided in a form that dimerizes with the first domain resulting in a protein that is targeted to the brain. For example, the therapeutic peptide can be fused to the A domain of an IGF protein, and dimerized with a B domain that is provided separately. Alternatively, the therapeutic peptide can be fused to the B domain of an IGF protein, and dimerized with an A domain that is provided separately.

[**0013**] The invention also relates to methods for identifying IGF-based peptide fragments that can reach neuronal tissue from blood and are useful to target an associated protein to the brain or CNS. According to the invention, the effectiveness of IGF-based tags can be assayed using methods described herein, such as localization assays based on radioactive labels or histochemical staining.

[**0014**] The invention also relates to a nucleic acid encoding an IGF tag or a protein fused to an IGF tag, and to a cell (e.g., a cell cultured in vitro including a mammalian cell culture such as a CHO cell culture, and/or a unicellular organism such as *E. coli* or Leishmania) containing such a nucleic acid.

[**0015**] In another aspect, the invention relates to a method of producing a therapeutic agent for targeting across the blood brain barrier, and in particular to the lysosomes of cells in the CNS. The agent is produced by culturing a cell expressing a nucleic acid encoding a protein containing both a therapeutic agent and an IGF tag effective to target the protein across the blood brain barrier. The protein is then harvested (e.g. from the milieu about the cell, or by lysing the cell). The invention also relates to protein compositions described herein.

[**0016**] The invention also relates to methods of treating a patient (e.g. a patient with a disorder in the CNS, and preferably a CNS disorder resulting from a lysosomal storage disorder) by administering, for example, a protein including a therapeutic agent effective in the mammalian CNS and an IGF tag to target the protein to the CNS. Preferably, the protein also comprises a lysosomal targeting portion such as those described in attorney docket number SYM-007 entitled "Methods and Compositions for Lysosomal Targeting" filed on Apr. 30, 2002, or mannose-6-phosphate to target the protein to the lysosomes of deficient cells in the CNS. Similarly, the invention relates to methods of treating a patient by administering a nucleic acid encoding such a protein and/or by administering a cell (e.g. a human cell, or an organism such as Leishmania) containing a nucleic acid encoding such a protein.

BRIEF DESCRIPTION OF THE DRAWINGS

[**0017**] **FIG. 1** shows a sequence alignment of mature human IGF-I and IGF-II, indicating regions of homology and the A, B, C, and D domains.

[**0018**] **FIG. 2** is a two-dimensional representation of an IGF protein showing the signal sequence, the A, B, C, and D domains, and the C terminal sequence.

[**0019**] **FIG. 3** is a depiction the 3 dimensional structure of an IGF protein.

[**0020**] **FIG. 4** shows protein (**FIG. 4A**) and nucleic acid (**FIG. 4B**) sequences for human IGF-I mRNA.

DETAILED DESCRIPTION OF THE INVENTION

[**0021**] CNS Targeting Portion

[**0022**] According to the invention, an IGF moiety is useful for targeting a composition, preferably a protein composition, to the CNS, across the blood brain barrier. Preferably, an IGF tag is used to target a composition to the brain parenchyma. According to the invention, a composition may enter the CNS or brain parenchyma either directly across the blood-brain barrier (the BBB) or indirectly across the blood-cerebrospinal fluid barrier (the BCB). The BBB is formed by capillary endothelial cells and the BCB is formed by epithelial cells of the choroid plexus. Transport across either barrier typically involves transcytosis. According to the invention, a composition that is targeted across the BCB to the CSF can subsequently reach the brain parenchyma. The CSF and brain parenchyma are separated by the ependyma, and diffusion or bulk flow can transport substances between these two compartments.

[**0023**] The invention exploits, in part, the recognition that [125I] IGF-I and IGF-II can be detected in the brain when infused into the carotid artery, and that IGF-I and analogs administered subcutaneously can be found in the cerebrospinal fluid. According to the invention, this suggests that both can traverse the BBB or BCB. According to the invention, the observed saturation of the transport process suggests that the process is carrier mediated. However, experimental analysis using a series of IGF-I analogs suggests that the IGF-I receptor, the IGF-II receptor, and IGF binding proteins-1,-3,-4, or 5 do not play a role in the blood brain barrier transport.

[**0024**] According to one aspect of the invention, preferred therapeutic compositions include a therapeutic peptide fused to an IGF tag. Preferred therapeutic composition include IGF-I tags that will direct LSD (lysosomal storage disease) proteins to which they are fused across the blood brain barrier. In this instance, the tag will not necessarily direct the protein to the lysosome of multiple cell types. However, by expressing such fusion proteins in mammalian cell culture systems, the invention exploits the endogenous M6P signal for lysosomal localization and uses the IGF-I tag to traverse the blood brain barrier. In preferred embodiments of the invention, a human IGF-I tag is used. In alternative embodiments, methods and compositions of the invention involve using allelic, species or other sequence variants of an IGF-I tag. Preferred sequence variants include mutations that lessen binding of the IGF tag to the IGF-I receptor and/or IGF binding proteins such as Leu^{60}-IGF-I, or Leu^{24}IGF-I which have diminished binding to the IGF-I receptor or Δ1-3 IGF-I which has diminished binding to IGF-binding proteins. Additional useful sequence variants include IGF-I variants with amino acid replacements of Arg^{55} and Arg^{56}. Other mutant IGF protein tags with similar properties are also useful.

[0025] IGF-II based tags are also useful to target proteins to the brain. IGF-II has been reported to be transported across the blood brain barrier via transcytosis (Bickel et al. (2001) *Adv. Drug Deliv. Rev.* 46(1-3):247-79). According to the invention, preferred IGF-II-based tags target proteins to the brain and also target proteins to the lysosome via receptor binding in order to treat neurological symptoms associated with lysosomal storage diseases. Preferred variants of IGF-II have an amino acid replacement at LeU^{24}.

[0026] In another aspect of the invention, chimeric tags are used that include fragments of IGF-I and IGF-II, conferring preferred functional properties of each protein. In one embodiment, the retained portion of IGF-II includes regions of IGF-II known to be critical for binding to the IGF-II M6P receptor while the remainder of IGF-II would be substituted for the corresponding regions of IGF-I. This embodiment, is particularly useful where IGF-I is more active as a tag for traversing the blood brain barrier. In this embodiment, the tag has optimized activity for lysosomal targeting in addition to brain targeting. A recombinant form of this embodiment could be made in any expression system.

[0027] In a further aspect of the invention, a useful recombinant LSD protein includes any one of the different IGF-based lysosomal targeting tags described in attorney docket number SYM-007 entitled "Methods and Compositions for Lysosomal Targeting" filed on Apr. 30, 2002.

[0028] In preferred embodiments, recombinant proteins of the invention including IGF-II tags are expressed in a mammalian expression system such as a CHO cell expression system. According to the invention, the endogenous M6P signal added in the mammalian cell culture enhances the lysosomal targeting that may be provided by an IGF-II tag.

[0029] According to the invention, useful minimal IGF tags and variant IGF tags can be identified based on known IGF-I and IGF-II sequences by testing minimal or variant IGF fragments in a CNS localization assay such as one described herein.

[0030] A preferred IGF tag is sufficiently duplicative of IGF-I to be targeted to the brain, but has reduced binding affinity for the IGF-I receptor thereby removing the mitogenic properties of IGF-I. However, a preferred IGF tag does bind to the IGF-II receptor in order to be targeted to lysosomes. Accordingly, in one embodiment, an IGF tag is based on the IGF-I sequence but includes two hydrophobic IGF-II residues at positions 54 and 55 instead of the IGF-I Arg residues at these positions.

[0031] Structure of IGF-II

[0032] NMR structures of IGF-II have been solved by two groups (see, e.g., Protein Data Bank record 1IGL). The general features of the IGF-II structure are similar to IGF-I and insulin. The A and B domains of IGF-II correspond to the A and B chains of insulin. Secondary structural features include an alpha helix from residues 11-21 of the B region connected by a reverse turn in residues 22-25 to a short beta strand in residues 26-28. Residues 25-27 appear to form a small antiparallel beta sheet; residues 59-61 and residues 26-28 may also participate in intermolecular beta-sheet formation. In the A domain of IGF-II, alpha helices spanning residues 42-49 and 53-59 are arranged in an antiparallel configuration perpendicular to the B-domain helix. Hydrophobic clusters formed by two of the three disulfide bridges and conserved hydrophobic residues stabilize these secondary structure features. The N and C termini remain poorly defined as is the region between residues 31-40.

[0033] IGF-II binds to the IGF-II/M6P and IGF-I receptors with relatively high affinity and binds with lower affinity to the insulin receptor. IGF-II also interacts with a number if serum IGFBPs.

[0034] Binding to the IGF-II/M6P Receptor

[0035] Substitution of IGF-II residues 48-50 (Phe Arg Ser) with the corresponding residues from insulin, (Thr Ser Ile), or substitution of residues 54-55 (Ala Leu) with the corresponding residues from IGF-I (Arg Arg) result in loss of binding to the IGF-II/M6P receptor but retention of binding to the IGF-I and insulin receptors.

[0036] IGF-I and IGF-II share identical sequences and structures in the region of residues 48-50 yet have a 1000-fold difference in affinity for the IGF-II receptor. The NMR structure reveals a structural difference between IGF-I and IGF-II in the region of IGF-II residues 53-58 (IGF-I residues 54-59): the alpha-helix is better defined in IGF-II than in IGF-I and, unlike IGF-I, there is no bend in the backbone around residues 53 and 54. This structural difference correlates with the substitution of Ala 54 and Leu 55 in IGF-II with Arg 55 and Arg 56 in IGF-I. It is possible either that binding to the IGF-II receptor is disrupted directly by the presence of charged residues in this region or that changes in the structure engendered by the charged residues yield the changes in binding for the IGF-II receptor. In any case, substitution of uncharged residues for the two Arg residues in IGF-I resulted in higher affinities for the IGF-II receptor. Thus the presence of positively charged residues in these positions correlates with loss of binding to the IGF-II receptor.

[0037] IGF-II binds to repeat 11 of the cation-independent M6P receptor. Indeed, a minireceptor in which only repeat 11 is fused to the transmembrane and cytoplasmic domains of the cation-independent M6P receptor is capable of binding IGF-II (with an affinity approximately one tenth the affinity of the full length receptor) and mediating internalization of IGF-II and its delivery to lysosomes (Grimme et al. (2000) *J. Biol. Chem.* 275(43):33697-33703). The structure of domain 11 of the M6P receptor is known (Protein Data Base entries 1GP0 and 1GP3; Brown et al. (2002) *EMBO J.* 21(5):1054-1062). The putative IGF-II binding site is a hydrophobic pocket believed to interact with hydrophobic amino acids of IGF-II; candidate amino acids of IGF-II include leucine 8, phenylalanine 48, alanine 54, and leucine 55. Although repeat 11 is sufficient for IGF-II binding, constructs including larger portions of the cation-independent M6P receptor (e.g. repeats 10-13, or 1-15) generally bind IGF-II with greater affinity and with increased pH dependence (see, for example, Linnell et al. (2001) *J. Biol. Chem.* 276(26):23986-23991).

[0038] Binding to the IGF-I Receptor

[0039] Substitution of IGF-II residues Tyr 27 with Leu, Leu 43 with Val or Ser 26 with Phe diminishes the affinity of IGF-II for the IGF-I receptor by 94-, 56-, and 4-fold respectively. Deletion of residues 1-7 of human IGF-II resulted in a 30-fold decrease in affinity for the human IGF-I receptor and a concomitant 12 fold increase in affinity for the rat IGF-II receptor. The NMR structure of IGF-II shows that Thr 7 is located near residues 48 Phe and 50 Ser as well as near the 9 Cys-47 Cys disulfide bridge. It is thought that interaction of Thr 7 with these residues can stabilize the flexible N-terminal hexapeptide required for IGF-I receptor binding. At the same time this interaction can modulate binding to the IGF-II receptor. Truncation of the C-terminus of IGF-II (residues 62-67) also appear to lower the affinity of IGF-II for the IGF-I receptor by 5 fold.

[0040] Deletion Mutants of IGF-II

[0041] The binding surfaces for the IGF-I and cation-independent M6P receptors are on separate faces of IGF-II. Based on structural and mutational data, functional cation-independent M6P binding domains can be constructed that are substantially smaller than human IGF-II. For example, the amino terminal amino acids 1-7 and/or the carboxy terminal residues 62-67 can be deleted or replaced. Additionally, amino acids 29-40 can likely be eliminated or replaced without altering the folding of the remainder of the polypeptide or binding to the cation-independent M6P receptor. Thus, a targeting moiety including amino acids 8-28 and 41-61 can be constructed. These stretches of amino acids could perhaps be joined directly or separated by a linker. Alternatively, amino acids 8-28 and 41-61 can be provided on separate polypeptide chains. Comparable domains of insulin, which is homologous to IGF-II and has a tertiary structure closely related to the structure of IGF-II, have sufficient structural information to permit proper refolding into the appropriate tertiary structure, even when present in separate polypeptide chains (Wang et al. (1991) *Trends Biochem. Sci.* 279-281). Thus, for example, amino acids 8-28, or a conservative substitution variant thereof, could be fused to a therapeutic agent; the resulting fusion protein could be admixed with amino acids 41-61, or a conservative substitution variant thereof, and administered to a patient.

[0042] Binding to IGF Binding Proteins

[0043] IGF-II and related constructs can be modified to diminish their affinity for IGFBPs, thereby increasing the bioavailability of the tagged proteins.

[0044] Substitution of IGF-II residue phenylalanine 26 with serine reduces binding to IGFBPs 1-5 by 5-75 fold. Replacement of IGF-II residues 48-50 with threonine-serine-isoleucine reduces binding by more than 100 fold to most of the IGFBPs; these residues are, however, also important for binding to the cation-independent mannose-6-phosphate receptor. The Y27L substitution that disrupts binding to the IGF-I receptor interferes with formation of the ternary complex with IGFBP3 and acid labile subunit; this ternary complex accounts for most of the IGF-II in the circulation. Deletion of the first six residues of IGF-II also interferes with IGFBP binding.

[0045] Studies on IGF-I interaction with IGFBPs revealed additionally that substitution of serine for phenylalanine 16 did not effect secondary structure but decreased IGFBP binding by between 40 and 300 fold. Changing glutamate 9 to lysine also resulted in a significant decrease in IGFBP binding. Furthermore, the double mutant lysine 9/serine 16 exhibited the lowest affinity for IGFBPs. Although these mutations have not previously been tested in IGF-II, the conservation of sequence between this region of IGF-I and IGF-II suggests that a similar effect will be observed when the analogous mutations are made in IGF-II (glutamate 12 lysine/phenylalanine 19 serine).

[0046] IGF Homologs

[0047] The amino acid sequence of human IGF-I, IGF-II, or a portion thereof affecting transport into the brain, may be used as a reference sequence to determine whether a candidate sequence possesses sufficient amino acid similarity to have a reasonable expectation of success in the methods of the present invention. Preferably, variant sequences are at least 70% similar or 60% identical, more preferably at least 75% similar or 65% identical, and most preferably 80% similar or 70% identical to human IGF-I or IGF-II.

[0048] To determine whether a candidate peptide region has the requisite percentage similarity or identity to human IGF-I or IGF-II, the candidate amino acid sequence and human IGF-I or IGF-II are first aligned using the dynamic programming algorithm described in Smith and Waterman (1981) *J. Mol. Biol.* 147:195-197, in combination with the BLOSUM62 substitution matrix described in **FIG. 2** of Henikoff and Henikoff (1992) *PNAS* 89:10915-10919. For the present invention, an appropriate value for the gap insertion penalty is −12, and an appropriate value for the gap extension penalty is −4. Computer programs performing alignments using the algorithm of Smith-Waterman and the BLOSUM62 matrix, such as the GCG program suite (Oxford Molecular Group, Oxford, England), are commercially available and widely used by those skilled in the art.

[0049] Once the alignment between the candidate and reference sequence is made, a percent similarity score may be calculated. The individual amino acids of each sequence are compared sequentially according to their similarity to each other. If the value in the BLOSUM62 matrix corresponding to the two aligned amino acids is zero or a negative number, the pairwise similarity score is zero; otherwise the pairwise similarity score is 1.0. The raw similarity score is the sum of the pairwise similarity scores of the aligned amino acids. The raw score is then normalized by dividing it by the number of amino acids in the smaller of the candidate or reference sequences. The normalized raw score is the percent similarity. Alternatively, to calculate a percent identity, the aligned amino acids of each sequence are again compared sequentially. If the amino acids are non-identical, the pairwise identity score is zero; otherwise the pairwise identity score is 1.0. The raw identity score is the sum of the identical aligned amino acids. The raw score is then normalized by dividing it by the number of amino acids in the smaller of the candidate or reference sequences. The normalized raw score is the percent identity. Insertions and deletions are ignored for the purposes of calculating percent similarity and identity. Accordingly, gap penalties are not used in this calculation, although they are used in the initial alignment.

[0050] IGF Structural Analogs

[0051] The known structures of human IGF proteins permit the design of IGF analogs using computer-assisted design principles such as those discussed in U.S. Pat. Nos. 6,226,603 and 6,273,598. For example, the known atomic coordinates of IGF-II can be provided to a computer equipped with a conventional computer modeling program, such as INSIGHTII, DISCOVER, or DELPHI, commercially available from Biosym, Technologies Inc., or QUANTA, or CHARMM, commercially available from Molecular Simulations, Inc. These and other software programs allow analysis of molecular structures and simulations that predict the effect of molecular changes on structure and on intermolecular interactions. For example, the software can be used to identify modified analogs with the ability to form additional intermolecular hydrogen or ionic bonds, improving the affinity of the analog for the target receptor.

[0052] The software also permits the design of peptides and organic molecules with structural and chemical features that mimic the same features displayed on at least part of an IGF surface that is sufficient for targeting to the CNS. A preferred embodiment of the present invention relates to designing and producing a synthetic organic molecule having a framework that carries chemically interactive moieties in a spatial relationship that mimics the spatial relationship of the chemical moieties disposed on the amino acid sidechains which are identified as associated with CNS targeting as described herein.

[0053] For example, upon identification of relevant chemical groups, the skilled artisan using a conventional computer program can design a small molecule having appropriate chemical moieties disposed upon a suitable carrier framework. Useful computer programs are described in, for example, Dixon (1992) Tibtech 10: 357-363; Tschinke et al. (1993) J. Med. Chem 36: 3863-3870; and Eisen el al. (1994) Proteins: Structure, Function, and Genetics 19: 199-221, the disclosures of which are incorporated herein by reference.

[0054] One particular computer program entitled "CAVEAT" searches a database, for example, the Cambridge Structural Database, for structures which have desired spatial orientations of chemical moieties (Bartlett et al. (1989) in "Molecular Recognition: Chemical and Biological Problems" (Roberts, S. M., ed) pp 182-196). The CAVEAT program has been used to design analogs of tendamistat, a 74 residue inhibitor of .alpha.-amylase, based on the orientation of selected amino acid side chains in the three-dimensional structure of tendamistat (Bartlett et al. (1989) supra).

[0055] Alternatively, upon identification of a series of analogs which target transport to the CNS, the skilled artisan may use a variety of computer programs which assist the skilled artisan to develop quantitative structure activity relationships (QSAR) and further to assist in the de novo design of additional analogs. Other useful computer programs are described in, for example, Connolly-Martin (1991) Methods in Enzymology 203:587-613; Dixon (1992) supra; and Waszkowycz et al. (1994) J. Med. Chenm. 37: 3994-4002.

[0056] Therapeutic Agent

[0057] While methods and compositions of the invention are useful for producing and delivering any therapeutic agent to the CNS, the invention is particularly useful for gene products that overcome enzymatic defects associated with lysosomal storage diseases.

[0058] Preferred LSD genes are shown in Table 1. In a preferred embodiment, a wild-type LSD gene product is delivered to a patient suffering from a defect in the same LSD gene. In alternative embodiments, a functional sequence or species variant of the LSD gene is used. In further embodiments, a gene coding for a different enzyme that can rescue an LSD gene defect is used according to methods of the invention.

TABLE 1

Lysosomal Storage Diseases and associated enzyme defects

Disease Name	Enzyme Defect	Substance Stored
A. Glycogenosis Disorders		
Pompe Disease	Acid-al, 4-Glucosidase	Glycogen α 1–4 linked Oligosaccharides
B. Glycolipidosis Disorders		
GM1 Gangliodsidosis	β-Galactosidase	GM_1 Ganliosides
Tay-Sachs Disease	β-Hexosaminidase A	GM_2 Ganglioside
GM2 Gangliosidosis: AB Variant	GM_2 Activator Protein	GM_2 Ganglioside
Sandhoff Disease	β-Hexosaminidase A&B	GM_2 Ganglioside
Fabry Disease	α-Galactosidase A	Globosides
Gaucher Disease	Glucocerebrosidase	Glucosylceramide
Metachromatic Leukodystrophy	Arylsulfatase A	Sulphatides
Krabbe Disease	Galactosylceramidase	Galactocerebroside
Niemann-Pick, Types A and B	Acid Sphingomyelinase	Sphingomyelin
Niemann-Pick, Type C	Cholesterol Esterification Defect	Sphingomyelin
Nieman-Pick, Type D	Unknown	Sphingomyelin
Farber Disease	Acid Ceramidase	Ceramide
Wolman Disease	Acid Lipase	Cholesteryl Esters
C. Mucopolysaccharide Disorders		
Hurler Syndrome (MPS IH)	α-L-Iduronidase	Heparan & Dermatan Sulfates
Scheie Syndrome (MPS IS)	α-L-Iduronidase	Heparan & Dermatan, Sulfates
Hurler-Scheie (MPS IH/S)	α-L-Iduronidase	Heparan & Dermatan Sulfates
Hunter Syndrome (MPS II)	Iduronate Sulfatase	Heparan & Dermatan Sulfates
Sanfilippo A (MPS IIIA)	Heparan N-Sulfatase	Heparan Sulfate
Sanfilippo B (MPS IIIB)	α-N-Acetylglucosaminidase	Heparan Sulfate
Sanfilippo C (MPS IIIC)	Acetyl-CoA-Glucosaminide Acetyltransferase	Heparan Sulfate
Sanfilippo D (MPS IIID)	N-Acetylglucosamine-6-Sulfatase	Heparan Sulfate
Morquio A (MPS IVA)	Galactosamine-6-Sulfatase	Keratan Sulfate

TABLE 1-continued

Lysosomal Storage Diseases and associated enzyme defects

Disease Name	Enzyme Defect	Substance Stored
Morquio B (MPS IVB)	β-Galactosidase	Keratan Sulfate
Maroteaux-Lamy (MPS VI)	Arylsulfatase B	Dermatan Sulfate
Sly Syndrome (MPS VII)	β-Glucuronidase	
D. Oligosaccharide/Glycoprotein Disorders		
α-Mannosidosis	α-Mannosidase	Mannose/ Oligosaccharides
β-Mannosidosis	β-Mannosidase	Mannose/ Oligosaccharides
Fucosidosis	α-L-Fucosidase	Fucosyl Oligosaccharides
Asparylglucosaminuria	N-Aspartyl-β-Glucosaminidase	Asparylglucosamine Asparagines
Sialidosis (Mucolipidosis I)	α-Neuraminidase	Sialyloligosaccharides
Galactosialidosis (Goldberg Syndrome)	Lysosomal Protective Protein Deficiency	Sialyloligosaccharides
Schindler Disease	α-N-Acetyl-Galactosaminidase	
E. Lysosomal Enzyme Transport Disorders		
Mucolipidosis II (I-Cell Disease)	N-Acetylglucosamine-1-Phosphotransferase	Heparan Sulfate
Mucolipidosis III (Pseudo-Hurler Polydystrophy)	Same as ML II	
F. Lysosomal Membrane Transport Disorders		
Cystinosis	Cystine Transport Protein	Free Cystine
Salla Disease	Sialic Acid Transport Protein	Free Sialic Acid and Glucuronic Acid
Infantile Sialic Acid Storage Disease	Sialic Acid Transport Protein	Free Sialic Acid and Glucuronic Acid
G. Other		
Batten Disease (Juvenile Neuronal Ceroid Lipofuscinosis)	Unknown	Lipofuscins
Infantile Neuronal Ceroid Lipofuscinosis	Palmitoyl-Protein Thioesterase	Lipofuscins
Mucolipidosis IV	Unknown	Gangliosides & Hyaluronic Acid
Prosaposin	Saposins A, B, C or D	

[0059] In one embodiment, the therapeutic agent is glucocerebrosidase, currently manufactured by Genzyme as an effective enzyme replacement therapy for Gaucher Disease. Currently, the enzyme is prepared with exposed mannose residues, which targets the protein specifically to cells of the macrophage lineage. Although the primary pathology in type 1 Gaucher patients are due to macrophage accumulating glucocerebroside, there may be therapeutic advantage to delivering glucocerebrosidase to other cell types. Targeting glucocerebrosidase to lysosomes using the present invention would target the agent to multiple cell types and may have a therapeutic advantage compared to other preparations.

[0060] Association Between Targeting Portion and Therapeutic Portion

[0061] The therapeutic portion and the targeting portion of compositions of the invention are necessarily associated, directly or indirectly. In one embodiment, the therapeutic portion and the targeting portion are non-covalently associated. For example, the targeting portion could be biotinylated and bind an avidin moiety associated with the therapeutic portion. Alternatively, the targeting portion and the therapeutic portion could each be associated (e.g. as fusion proteins) with different subunits of a multimeric protein. In another embodiment, the targeting portion and the therapeutic portion are crosslinked to each other (e.g. using a chemical crosslinking agent).

[0062] In a preferred embodiment, the therapeutic portion is fused to the targeting portion as a fusion protein. The targeting portion may be at the amino-terminus of the fusion protein, the carboxy-terminus, or may be inserted within the sequence of the therapeutic portion at a position where the presence of the targeting portion does not unduly interfere with the therapeutic activity of the therapeutic portion.

[0063] Where the therapeutically active moiety is a heteromeric protein, one or more of the subunits may be associated with a targeting portion. Hexosaminidase A, for example, a lysosomal protein affected in Tay-Sachs disease, includes an alpha subunit and a beta subunit. Either the alpha subunit, or the beta subunit, or both may be associated with a targeting portion in accordance with the present invention. If, for example, the alpha subunit is associated with a targeting portion and is coexpressed with the beta subunit, an active complex is formed and targeted to the lysosome.

[0064] Methods

[0065] Methods and compositions of the invention are useful in the context of many different expression systems. For example, a protein of the invention can be targeted to the CNS, and preferably taken up by lysosomes, whether it is expressed and isolated from Leishmania, baculovirus, yeast or bacteria. Thus, the invention permits great flexibility in protein production. For example, if a protein to be produced includes one or more disulfide bonds, an appropriate expression system can be selected and modified, if appropriate, to further improve yield of properly folded protein. For example, one useful IGF targeting portion has three intramolecular disulfide bonds. Fusion proteins of the invention expressed in *E. coli* may be constructed to direct the protein to the periplasmic space. IGF tags, when fused to the C-terminus of another protein, can be secreted in an active form in the periplasm of *E. coli* (Wadensten, Ekebacke et al. 1991). To facilitate optimal folding of the IGF moiety, appropriate concentrations of reduced and oxidized glutathione are preferably added to the cellular milieu to promote disulfide bond formation. In the event that a fusion protein with disulfide bonds is incompletely soluble, any insoluble material is preferably treated with a chaotropic agent such as urea to solubilize denatured protein and refolded in a buffer having appropriate concentrations of reduced and oxidized glutathione, or other oxidizing and reducing agents, to facilitate formation of appropriate disulfide bonds (Smith, Cook et al. 1989). For example, IGF-I has been refolded using 6M guanidine-HCl and 0.1 M tris(2-carboxyethyl)phosphine reducing agent for denaturation and reduction of IGF-II (Yang, Wu et al. 1999). Refolding of proteins was accomplished in 0.1M Tris-HCl buffer (pH8.7) containing 1 mM oxidized glutathione, 10 mM reduced glutathione, 0.2M KCl and 1 mM EDTA.

[0066] Methods of the invention are also useful to target a protein directly to the CNS of a mammal without requiring a purification step. In one embodiment, an IGF fusion protein is expressed in a symbiotic or parasitic organism that is administered to a host. The expressed IGF fusion protein is secreted by the organism into the blood stream and delivered across the blood brain barrier.

[**0067**] In some embodiments of the invention, CNS targeted proteins are delivered in situ via live Leishmania secreting the proteins into the lysosomes of infected macrophage. From this organelle, it leaves the cell and may be delivered across the blood brain barrier. Thus, the IGF tag and the therapeutic agent necessarily remain intact while the protein resides in the macrophage lysosome. Accordingly, when proteins designed for delivery to lysosomes in the CNS are expressed in situ, they are preferably modified to ensure compatibility with the lysosomal environment. In alternative embodiments, therapeutic proteins of the invention can be delivered by expression in *T. brucei* which can penetrate the BCB.

EXAMPLES

Example 1

[**0068**] Fusion Protein Expressing Constructs.

[**0069**] Nucleic acid constructs for expressing therapeutic protein fusions of the invention can be made recombinantly according to methods known in the art. For example, oligonucleotides complementary to genes encoding the different components described herein can be used to make synthetic genes or to amplify the natural genes and construct gene fusions. In preferred embodiments, proteins of the invention are expressed from a recombinant gene comprising a signal sequence. Examples of useful nucleic acids include nucleic acids that encode IGF targeting moieties of the invention. Such nucleic acids can be based on the sequences of IGF-I shown in **FIG. 4**.

Example 2

[**0070**] Expression and Purification Methods.

[**0071**] Expression product can also be isolated from serum free media using other protozoa, including other Leishmania species. In general, the expression strain is grown in medium with serum, diluted into serum free medium, and allowed to grow for several generations, preferably 2-5 generations, before the expression product is isolated. For example, production of secreted recombinant LSD proteins can be isolated from *Leishmania mexicana* promastigotes that are cultured initially in 50 mL 1×M199 medium in a 75 cm2 flask at 27° C. When the cell density reaches 1-3×10^7/mL, the culture is used to inoculate 1.2 L of M199 media. When the density of this culture reaches about 5×10^6/mL, the cells were harvested by centrifugation, resuspended in 180 mL of the supernatant and used to inoculate 12 L of "Zima" medium in a 16 L spinner flask. The initial cell density of this culture is typically about 5×10^5/mL. This culture is expanded to a cell density of about 1.0-1.7×10e^7 cells/mL. When this cell density is reached, the cells are separated from the culture medium by centrifugation and the supernatant is filtered at 4° C. through a 0.2μ filter to remove residual promastigotes. The filtered media was concentrated from 12.0 L to 500 mL using a tangential flow filtration device (MILLIPORE Prep/Scale-TFF cartridge).

[**0072**] Preferred growth media for this method are M199 and "Zima" growth media. However, other serum containing and serum free media are also useful. M199 growth media is as follows: (1 L batch)=200 mL 5×M199 (with phenol pH indicator) mixed at 5×+637 mL H$_2$O, 50.0 mL FBS, 50.0 mL EF, 20.0 mL of 50 g/mL SAT, 2.0 mL of 0.25% hemin in 50% triethanolamine, 10 mL of 10 mM adenine in 50 mM Hepes pH 7.5, 40.0 mL of 1M Hepes pH 7.5, 1 mL of 0.1 % biotin in 95% ethanol, 10.0 mL of penicillin/streptomycin. All serums used are inactivated by heat. The final volume=1 L and is filter sterilized. "Zima" modified M199 media is as follows: (20.0 L batch)=217.8 g M199 powder (−)phenol red+7.0 g sodium bicarbonate, 200.0 mL of 10 mM adenine in 50 mM Hepes pH 7.5, 800.0 mL 0f Hepes free acid pH 7.5, 20.0 mL 0.1% biotin in 95% ethanol, 200.0 mL penicillin/streptomycin, 2780.0 mL H20 Final volume=20.0 L and is filter sterilized.

[**0073**] According to one aspect of the invention, LSD proteins secreted from Leishmania and containing carbohydrate with terminal mannose residues can be purified as follows. For example, recombinant β-glucuronidase from *Leishmania mexicana* containing plasmsid pXSAP0-GUS was grown in M199 culture medium with a small amount of serum proteins. When the culture reached a density of >1.0×10^7 promastigotes/mL the *L. mexicana* were removed by centrifugation, 10 min at 500×g. The harvested culture medium was passed through a 0.2 μm filter to remove particulates before being loaded directly onto a Concanavalin A (ConA)-agarose column (4% cross-linked beaded agarose, Sigma). The ConA-agarose column was pretreated with 1 M NaCl, 20 mM Tris pH 7.4, 5 mM each of CaCl$_2$, MgCl$_2$ and MnCl$_2$ and then equilibrated with 5 volumes of column buffer (20 mM Tris pH 7.4, 1 mM CaCl$_2$, and 1 mM MnCl$_2$). A total of 179,800 units (nmol/hr) of GUS activity (in 2 L) in culture medium was loaded onto a 22 mL ConA agarose column. No activity was detectable in the flow through or wash. The GUS activity was eluted with column buffer containing 200 mM methyl mannopyranoside. Eluted fractions containing the activity peak were pooled and concentrated: 143900 units of GUS activity were recovered from the column (80% recovery of activity loaded onto the column). This demonstrates that the recombinant β-GUS secreted from *L. mexicana* possesses carbohydrate with terminal mannose residues and further points out the potential for using the interaction of mannose with ConA as the basis for an affinity purification step. Accordingly, the presence of high mannose carbohydrate can serve as the basis of an affinity step in the purification of recombinant LSD proteins using lectin affinity chromatography.

Example 3

[**0074**] Assays for Crossing the Blood Brain Barrier.

[**0075**] According to the invention, a useful model system to determine whether a protein, particularly an LSD protein, tagged with an IGF tag crosses the blood-brain barrier, is the MPSVII mouse model (Wolfe and Sands (1996) *Protocols for Gene Transfer in Neuroscience: Towards Gene Therapy of Neurological Disorders* Chapter 20: 263-273). For example, recombinant human β-glucuronidase fused to an IGF tag can be produced in any convenient expression system such as Leishmania, yeast, mammalian, baculovirus and other expression systems. *L. mexicana* expressing and secreting β-GUS is grown at 26° C. in 100 ml Standard

Promastigote medium (M199 with 40 mM HEPES, pH 7.5, 0.1 mM adenine, 0.0005% hemin, 0.0001% biotin, 5% fetal bovine serum, 5% embryonic fluid, 50 units/ml penicillin, 50 μg/ml streptomycin and 50 μg/ml nourseothricin). After reaching a density of approximately 5×10^6 promastigotes/ml, the promastigotes is collected by centrifugation for 10 min. at 1000×g at room temperature; these promastigotes were used to inoculate 1 liter of low protein medium (M199 supplemented with 0.1 mM adenine, 0.0001% biotin, 50 units/ml penicillin and 50 μg/ml streptomycin) at room temperature. The 1 liter cultures are contained in 2 liter capped flasks with a sterile stir bar so that the cultures could be incubated at 26° C. with gentle stirring. The 1 liter cultures are aerated twice a day by moving them into a laminar flow hood, removing the caps and swirling vigorously before replacing the caps. When the cultures reach a density of $2\text{-}3\times10^7$ promastigotes/ml, the cultures are centrifuged as previously described except the promastigote pellet is discarded and the media decanted into sterile flasks. The addition of 434 g $(NH_4)_2SO_4$ per liter precipitates active GUS protein from the medium; the salted out medium is stored at 4° C. overnight. Precipitated proteins are harvested either by centrifugation at 10,500×g for 30 min. or filtration through Gelman Supor-800 membrane; the proteins are resuspended in 10 mM Tris pH 8, 1 mM $CaCl_2$ and stored at −80° C. until dialysis. The crude preparations from several liters of medium are thawed, pooled, placed in dialysis tubing (Spectra/Por-7, MWCO 25,000), and dialyzed overnight against two 1 liter volumes of DMEM with bicarbonate (Dulbecco's Modified Eagle's Medium). The ammonium sulfate fraction is further purified on a ConA column.

[0076] GUS minus mice generated by heterozygous matings of B6.C-H-2^{bml}/ByBIR-gus^{mps}/+ mice are used to assess the effectiveness of GUS-IGF fusion proteins or derivatives in enzyme replacement therapy. Two formats are used. In one format, 3-4 animals are given a single injection of 20,000 U of enzyme in 100 μl enzyme dilution buffer (150 mM NaCl, 10 mM Tris, pH7.5). Mice are killed 72-96 hours later to assess the efficacy of the therapy. In a second format, mice are given weekly injections of 20,000 units over 3-4 weeks and are killed 1 week after the final injection. Histochemical and histopathologic analysis of liver, spleen and brain are carried out by published methods. In the absence of therapy, cells (e.g. macrophages and Kupffer cells) of GUS minus mice develop large intracellular storage compartments resulting from the buildup of waste products in the lysosomes. It is anticipated that in cells in mice treated with GUS fusion constructs of the invention, the size of these compartments will be visibly reduced or the compartments will shrink until they are no longer visible with a light microscope.

[0077] According to the invention, newborn mice do not possess a complete blood brain barrier. However, by day 15 the blood brain barrier is formed to the point that B glucuronidase no longer can be detected in the brain. Accordingly, the above experiments are preferably performed on mice that are at day 15 or greater.

[0078] According to one embodiment of the invention, experiments first assess the ability of complete IGF-I and IGF-II tags to direct proteins across the blood brain barrier. Next, specific mutant versions of the proteins that disrupt receptor or IGF binding protein binding are assayed. For domain swaps, the B domain of IGF-II (residues 1-28 of the mature protein) contains only two differences from IGF-I that could conceivably alter transport across the blood brain barrier G11 and T16. Altering these residues in IGF-II would is essentially a domain B swap. Another swap of regions between residues 28 and 41 of IGF-II and the corresponding region of IGF-I can also be tested. This essentially swaps the C domains of the two proteins which contains the most divergent regions of the two proteins. An alternative swap switches the C-terninal 15 residues with the corresponding region of IGF-I. These three chimeras provide an essentially complete picture of how any differences in uptake across the blood brain barrier between IGF-I and IGF-II correlate with sequence/structural differences between the two proteins.

Example 4

[0079] Assays for Protein Accumulation in the Brain or CNS.

[0080] Radioactive assays can be used to monitor the accumulation of protein product in the brain. For example, the uptake and accumulation of a radioactively labeled protein in the brain parenchyma can be assayed as disclosed in Reinhardt and Bondy (1994) *Endocrinology* 135:1753-1761.

[0081] Enzyme assays can also be used to monitor the accumulation of protein product in the brain. Enzyme assays are particularly useful when the therapeutic protein moiety is an enzyme for which there is an assay that is applicable for histochemical staining. Useful enzyme assays for lysosomal storage disease proteins include assays disclosed in Sly at al. (2001) *P.N.A.S.* 98(5): 2205-2210, and in Wolfe and Sands (1996) *Protocols for Gene Transfer in Neuroscience: Towards Gene Therapy of Neurological Disorders* Chapter 20: 263-273.

Example 5

[0082] In vivo Therapy.

[0083] GUS minus mice generated by heterozygous matings of B6.C-H-2^{bml}/ByBIR-gus^{mps}/+ mice (Birkenmeier, Davisson et al. 1989) are used to assess the effectiveness of compositions of the invention in enzyme replacement therapy. Two formats are used. In one format, 3-4 animals are given a single injection of 20,000 U of enzyme in 100 μl enzyme dilution buffer (150 mM NaCl, 10 mM Tris, pH7.5). Mice are killed 72-96 hours later to assess the efficacy of the therapy. In a second format, mice are given weekly injections of 20,000 units over 3-4 weeks and are killed 1 week after the final injection. Histochemical and histopathologic analysis of liver, spleen and brain are carried out by published methods (Birkenmeier, Barker et al. 1991; Sands, Vogler et al. 1994; Daly, Vogler et al. 1999). In the absence of therapy, cells (e.g. macrophages and Kupffer cells) of GUS minus mice develop large intracellular storage compartments resulting from the buildup of waste products in the lysosomes. It is anticipated that in cells in mice treated with compositions of the invention, the size of these compartments will be visibly reduced or the compartments will shrink until they are no longer visible with a light microscope.

[0084] Similarly, humans with lysosomal storage diseases will be treated using constructs targeting an appropriate therapeutic portion to their CNS and in particular to lysosomes within the CNS. In some instances, treatment will take the form of regular (e.g. weekly) injections of a fusion protein of the invention. In other instances, treatment will be achieved through administration of a nucleic acid to permit persistent in vivo expression of the fusion protein, or through administration of a cell (e.g. a human cell, or a unicellular organism) expressing the fusion protein in the patient. For example, a protein the invention may be expressed in situ using a Leishmania vector as described in U.S. Pat. No. 6,020,144, issued Feb. 1, 2000; and PCT Serial No. PCT/US01/44935, filed Nov. 30, 2001.

Equivalents

[0085] The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting on the invention described herein. Scope of the invention is thus indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are intended to be embraced therein.

INCORPORATION BY REFERENCE

[0086] The disclosure of each of the patent documents and scientific publications disclosed herein, and U.S. Ser. No. 60/250,446 filed Nov. 30, 2000; U.S. Ser. No. 60/250,444 filed Nov. 30, 2000; U.S. Ser. No. 60/290,281 filed May 11, 2001; U.S. Ser. No. 60/287,531, filed Apr. 30, 2001; U.S. Ser. No. 60/304,609, filed Jul. 10, 2001; U.S. Ser. No. 60/329,461, filed Oct. 15, 2001, a U.S. Ser. No. 60/351,276, filed Jan. 23, 2002; and attorney docket number SYM-007 entitled "Methods and Compositions for Lysosomal Targeting" filed on Apr. 30, 2002; PCT Serial No. PCT/US01/44935, filed Nov. 30, 2001; are incorporated by reference into this application in their entirety.

1. A method for targeting a polypeptide to the brain, the method comprising the step of providing a polypeptide in association with an IGF moiety to a mammal, thereby resulting in accumulation of the polypeptide in the brain of said mammal.

2. A method for producing a CNS-targeted polypeptide, the method comprising the step of expressing a polypeptide in association with an IGF moiety, thereby to produce a polypeptide with a CNS-targeting tag.

3. A composition comprising a polypeptide in association with an IGF moiety, wherein said IGF moiety targets the composition to the brain.

4. The composition of claim 3 wherein said polypeptide and IGF moiety are expressed as a fusion protein.

5. The method of claim 1 or **2**, wherein said IGF moiety is an IGF-I targeting moiety.

6. The composition of claim 3 or **4**, wherein said IGF moiety is an IGF-I targeting moiety.

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