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(54) Title: THROMBIN RECEPTOR PEPTIDES AND USES THEREOF

(57) Abstract

The invention is based on the discovery that isolated, e.g., synthetic, peptides corresponding to all or a portion of the first 41 amino acids of the thrombin receptor are potent agonists for platelets. These new TR_{1-41} peptides can be used to activate platelets *in vitro* and *in vivo*, to prepare TR_{1-41} peptide antagonists and antibodies, and in various assays for determining a patient's level of platelet reactivity and thrombin inhibitor efficacy.

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THROMBIN RECEPTOR PEPTIDES AND USES THEREOF

Background of the Invention

The invention relates to isolated or synthetic thrombin receptor peptides and methods of using these peptides.

Thrombin is one of the most physiologically important platelet agonists, and is an essential component in the hemostatic, proliferative, and inflammatory responses to injury. The seven transmembrane domain G protein-linked thrombin receptor (proteinase-activated receptor-1) has been identified, and studies of this receptor revealed a novel proteolytic mechanism of activation (Vu et al., *Cell*, **64**:1057-1068, 1991). The receptor is activated when thrombin binds to a hirudin-like domain of the receptor via an anion binding exosite, and then cleaves the receptor between amino acids Arg-41 and Ser-42. This cut releases a thrombin receptor cleavage fragment and forms a new amino-terminal that acts as a so-called "tethered ligand." The tethered ligand then binds to another, as yet unknown, binding site of the thrombin receptor to activate the receptor (Vu et al., supra; Coughlin et al., *J. Clin. Invest.*, **89**:351-355, 1992; Vu et al., *Nature*, **353**:674-677, 1991).

The tethered ligand hypothesis has been confirmed by a number of studies, including experiments in which synthetic peptides that correspond to the amino acid sequence of the new amino terminus were tested as thrombin receptor agonists. Examples of these thrombin receptor agonist peptides (TRAPs) include TR_{42-47} (thrombin receptor peptide having amino acids 42 to 47 of the full receptor), TR_{42-48}, and TR_{42-55}. These synthetic peptides mimic the new amino terminus in sequence, and in many instances, in effect as well. For example, TRAPs

have been shown to activate the thrombin receptor and platelets to varying extents (Vu et al., *Cell*, **64**:1057-1068, 1991; Lau et al., *Biochem. J.*,**303**:91-400 1994), and to cause vascular contractions (Tesfamariam, *Circulat. Res.*, **74**:930-936, 1994).

On the other hand, a synthetic peptide mimicking a portion PESKATNATLDPRSFLL (TR_{29-45}, SEQ ID NO:11) of the thrombin receptor cleavage fragment and a portion the cleavage site, was found to be devoid of agonist activity for the wild-type thrombin receptor expressed in Xenopus oocytes (Vu et al., *Cell*, **64**:1057-1068, 1991).

Summary of the Invention

The invention is based on the discovery that isolated, e.g., synthetic, peptides corresponding to all or a portion of the first 41 amino acids of the thrombin receptor are potent agonists for platelets. These new peptides will be referred to collectively as TR_{1-41} peptides, although some do not include all 41 of the amino acids, and some include substituted amino acids (as described below). The isolated peptides can be used to activate platelets *in vitro* and *in vivo*, to prepare TR_{1-41} antibodies and antagonists, and in various assays for determining a patient's level of platelet reactivity, and thrombin inhibitor efficacy.

In general, the invention features isolated peptides including a sequence of at least 6, 10, 15, or 21 consecutive amino acids, and up to 41, selected from the sequence:

MGPRRLLLVAACFSLCGPLLSARTRARRPESKATNATLDPR (SEQ ID NO:1)

and including a core region of consecutive amino acids that are the same as, or substantially the same as, amino acids 17 to 22, 15 to 24, 12 to 26, or 10 to 30 of SEQ ID

NO:1. The peptides must include amino acids Lys-19 and Lys-20 of SEQ ID NO:1 without substitution. Thus, useful TR_{1-41} peptides include TR_{1-41}, TR_{17-22}, TR_{15-24}, TR_{12-26}, TR_{10-30}, and TR_{5-35}, which include peptides that have the identical sequence of the correspondingly numbered amino acids in SEQ ID NO:1, or are substantially identical to those sequences.

The isolated peptides can be synthetic peptides. One preferred peptide is TR_{1-41} having the amino acid sequence of SEQ ID NO:1.

In another aspect, the invention features a method of activating platelets by providing a TR_{1-41} peptide and exposing the platelets to an amount of the peptide effective to achieve platelet activation. The method can be carried out *in vitro*, *ex vivo*, and *in vivo*, e.g., to induce thrombosis. The method also can be carried out by additionally exposing the platelets to a TR_{42-55} peptide in an amount sufficient to achieve a synergistic platelet activation effect together with the TR_{1-41} peptide, either simultaneously or in succession with the TR_{1-41} peptide. An alternative *in vivo* method of activating platelets involves administering to a patient, e.g., a mammal such as a human, a nucleic acid (e.g., an expression vector) containing a sequence that encodes a TR_{1-41} peptide.

In another aspect, the invention features a method of assaying platelet reactivity by obtaining a biological sample containing platelets, e.g., blood, platelet-rich-plasma, or washed platelets; incubating the sample with a TR_{1-41} peptide, e.g., in an amount effective to activate normal platelets; and detecting platelet activation as a measure of platelet reactivity. Platelet activation can be detected by labeling the sample with an activation-dependent label, e.g., fluorescein isothiocyanate-fibrinogen, or an antibody, e.g., PAC1, F26, S12 and

others; and detecting any labeled platelets, wherein the presence of labeled platelets indicates the reactivity of the platelets in the sample.

The platelet assay can also be carried out to detect platelet activation by labeling the sample with an activation-dependent label, detecting any labeled platelets, and comparing the percentage of labeling with control values of known activated platelets as an indication of the degree of reactivity of the platelets in the sample. Platelet activation also can be detected by measuring platelet aggregation, e.g., optically (for platelet-rich plasma or washed platelet samples) or electrically (for whole blood and other platelet samples), or by measuring a change in calcium mobilization in the platelets.

In this method, the TR_{1-41} peptide can be added to separate samples in a range of different concentrations to determine the degree of reactivity of the platelets in the sample. Moreover, platelets can be considered "activated" when the percentage of labeling compared to a known non-activated platelet control value is greater than two standard deviations from the control value.

The platelet reactivity assay can be carried out by further incubating the sample with a TR_{42-55} peptide.

In another aspect, the invention features antagonist compounds, e.g., peptides or antibodies, that antagonize the action of the TR_{1-41} peptides, e.g., TR_{1-41} peptide-induced platelet activation. These compounds are antithrombotics, and include TR_{1-41} peptide mutant analogs that specifically bind to TR_{1-41} target cells, e.g., platelets, yet do not activate these cells. These compounds also include antibodies that bind specifically to naturally occurring TR_{1-41} peptides so as to diminish the levels of TR_{1-41} peptides capable of binding to their target cells. These compounds thus diminish or prevent

TR_{1-41} peptide-mediated events such as platelet aggregation.

The invention also features a method of inhibiting TR_{1-41} peptide-induced platelet activation in a patient by administering to the patient an amount of an antagonist of TR_{1-41} peptide effective to prevent TR_{1-41} peptide-induced platelet activation. This method can be used, for example, in patients that have undergone surgery or angioplasty, or who have had deep venous thrombosis, venous stasis, a pulmonary embolism, angina, coronary artery disease, unstable angina, myocardial infarction, congestive heart failure, cardiomyopathy, ventricular or atrial arrhythmias, or a stroke. An alternative method of inhibiting TR_{1-41} induced platelet activation in a patient involves administering to the patient a nucleic acid (e.g., an expression vector) containing a sequence that encodes an antagonist.

The "thrombin receptor" is the seven transmembrane domain G protein-linked thrombin receptor (proteinase activated receptor-1).

A "TR_{1-41} peptide" is any peptide that has an amino acid sequence that is the same as, or substantially identical to, at least amino acids 17 to 22 within the first 41 amino acids of the naturally occurring thrombin receptor protein (SEQ ID NO:1), and that activates platelets to the same extent as, or at least to 50%, as the full length TR_{1-41} peptide (in the flow cytometry and aggregation assays described below). The term includes "TR_{1-41} peptide analogs," i.e., mutant analogs of TR_{1-41} peptides, as well as peptide fragments of the full length TR_{1-41} peptide and mutant analogs, as long as these peptide fragments and analogs include at least a sequence that is the same as, or substantially the same as, amino acids 17 to 22 within SEQ ID NO:1, and activate platelets to at least 50% as well as the full length TR_{1-41} peptide.

A "mutant analog of a TR_{1-41} peptide" is a peptide that includes any change in the amino acid sequence when compared to the wild-type sequence of the first 41 amino acids of the thrombin receptor (SEQ ID NO:1). Mutations include, e.g., amino acid changes, deletions, insertions, inversions, translocations, or duplications. Preferred mutations are conservative amino acid substitutions in one or more locations in the peptide. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

By "substantially identical" or "substantially the same as" is meant that a peptide exhibits at least 80% (e.g., 90 or 95%) identity to a reference amino acid sequence. For TR_{1-41} peptides, the comparison sequences are the corresponding portions of the first 41 amino acids of the thrombin receptor (SEQ ID NO:1). Identity is typically measured visually or by using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Such software matches similar sequences by assigning degrees of identity or homology to the compared sequences.

An "isolated" TR_{1-41} peptide is a TR_{1-41} peptide that has been separated or purified from components which naturally accompany it, e.g., in blood, serum, or urine. Typically, the peptide is considered "isolated" when it is at least 70%, by dry weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, a preparation of the peptide is at least 80%, more preferably at least 90%, and most preferably at least 99%, by dry weight, TR_{1-}

$_{41}$ peptide. Since a TR_{1-41} peptide that is chemically synthesized is separated from components which naturally accompany the first 41 amino acids of the thrombin receptor or the thrombin receptor cleaved fragment, the synthetic peptide is "isolated."

An isolated TR_{1-41} peptide can be obtained, for example, by extraction from a natural source (e.g., from human blood, plasma, serum, or urine); by expression of a recombinant nucleic acid encoding a TR_{1-41} peptide; or by chemical synthesis. A peptide that is produced in a cellular system different from the source from which it naturally originates is "isolated," because it will be separated from components which naturally accompany it. The extent of isolation or purity can be measured by any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

A "TR_{1-41} peptide antibody" is any polyclonal or monoclonal antibody that specifically binds to a TR_{1-41} peptide. An antibody that "specifically binds" to a TR_{1-41} peptide is an antibody which recognizes and binds to a TR_{1-41} peptide, but which does not substantially recognize and bind to other molecules in a sample, e.g., a biological sample, which naturally includes TR_{1-41} peptides.

A "purified antibody" is an antibody which is at least 70%, by dry weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 80%, more preferably at least 90%, and most preferably at least 99%, by dry weight, antibody, e.g., a TR_{1-41} peptide antibody.

The use of TR_{1-41} peptides enables the preparation of polyclonal and monoclonal antibodies that specifically bind to new epitopes on the peptide, which can be used in new diagnostic and therapeutic methods.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Figs. 1A to 1C are a series of line graphs showing TR_{1-41}-, TR_{42-55}-, and thrombin-induced platelet activation. The platelet surface binding of the monoclonal antibodies S12 (directed against P-selectin, Fig. 1A), PACI (directed against the activated glycoprotein (GP) IIb-IIIa complex, Fig. 1B), and F26 (directed against fibrinogen bound to the GPIIb-IIIa complex, Fig. 1C) were determined by whole blood flow cytometry.

Figs. 2A to 2C are a series of graphs showing TR_{1-41}-, TR_{42-55}-, and thrombin-induced platelet aggregation. Washed platelets were analyzed in a platelet aggregometer after the addition (arrowhead) of thrombin 1 U/ml (Fig. 2A), TR_{42-55} 20 μM (Fig. 2B), or TR_{1-41} μM (Fig. 2C).

Fig. 3 is a line graph showing that TR_{1-41} and TR_{42-55} activate platelets synergistically.

Fig. 4 is a bar graph showing intracellular signal transduction pathways involved in TR_{1-41}-induced platelet activation. The platelet surface binding of the P-selectin-specific monoclonal antibody S12 was determined by whole blood flow cytometry.

Fig. 5 is a bar graph showing that TR_{1-41}-mediated platelet activation requires extracellular calcium and does not require either the GPIb-IX complex (absent in Bernard-Soulier syndrome) or the GPIIb-IIIa complex (absent in Glanzmann's thrombasthenia).

Fig. 6 is a graph showing that normal plasma inhibits TR_{1-41}-induced platelet degranulation.

Figs. 7A to 7C are a series of graphs showing the decrease over time in GPIb, GPIX, and GPV on platelets activated by TR_{1-41} and thrombin.

Detailed Description

The new peptides are potent agonists for platelets, and thus can be used to activate platelets *in vitro* and *in vivo*, as well as in methods for determining a patient's level of platelet reactivity, and thrombin inhibitor efficacy. The new peptides also can be used to prepare new antibodies for use in new diagnostic and therapeutic methods. The effects of the new peptides were demonstrated in various experiments, including whole blood flow cytometric studies and experiments using various inhibitors.

Whole blood flow cytometric studies established that the TR_{1-41} peptide induces platelet activation. The TR_{1-41} peptide was shown to be more potent than the TR_{42-55} peptide and almost as potent as thrombin, as determined by the increase in: (i) platelet surface expression of P-selectin (reflecting α granule secretion); (ii)

exposure of the fibrinogen binding site on the GPIIb-IIIa complex; and (iii) fibrinogen binding to the activated GPIIb-IIIa complex.

Experiments with inhibitors (PGI_2, staurosporine, wortmannin, EDRF, and genistein), and with platelets obtained from Bernard-Soulier syndrome and Glanzmann's thrombasthenia patients, demonstrated that TR_{1-41} peptide-induced platelet activation is: (i) inhibited by cyclic AMP; (ii) mediated by protein kinase C, phosphatidyl inositol-3 (PI_3) kinase and myosin light chain kinase; (iii) dependent on extracellular calcium and intracellular protein tyrosine kinase; and (iv) independent of the GPIb-IX complex and the GPIIb-IIa complex. Other tests established that TR_{1-41} peptides and the TR_{42-55} peptide activate platelets synergistically.

Peptides

The new peptides were synthesized using a Rainin Symphony (Rainin, Woburn, MA) 12-port automated peptide synthesizer set to perform FMOC-chemistry with HBTU (N,N,N',N',-tetramethyl-O-[1H-Benzotriazol-1-yl] uronium hexafluorophosphate)-mediated coupling. The peptides were then obtained by automated cleavage from a standard resin using trifluoroacetate and appropriate scavengers. The peptides were purified using high performance liquid chromatography with a 25 x 100 mm DeltaPak™ C18 column (Waters Millipore, Milford, MA) and a linear gradient in CH_3CN with ultraviolet spectrophotometric detection at 280 nm. Other standard synthetic methods can be used to make the TR_{1-41} peptides.

The following peptides were synthesized:

TR_{1-41} - MGPRRLLLVAACFSLCGPLLSART-RARRPESKATNATLDPR (SEQ ID NO:1),

TR_{1-10} - MGPRRLLLVA (SEQ ID NO:2),

TR_{11-21} - ACFSLCGPLL (SEQ ID NO:3),

TR_{21-30} - SARTRARRPE (SEQ ID NO:4),

TR_{31-41} - SKATNATLDPR (SEQ ID NO:5),

TR_{1-21} - MGPRRLLLVAACFSLCGPLL (SEQ ID NO:6),

TR_{21-41} - SARTRARRPESKATNATLDPR (SEQ ID NO:7),

TR_{42-55} - SFLLRNPNDKYEPF (SEQ ID NO:8),

TR_{1-41} (S14A) - MGPRRLLLVAACFALCGPLLSART-RARRPESKATNATLDPR (SEQ ID NO:9)(where S14A indicates that the Ser residue normally found in the wild-type sequence at location 14 was replaced with an Ala residue, shown by underlining in the sequence), and

LRTNASLLVPFLTARAKSSGTREAADPPRLMCLRPLARRCG (SEQ ID NO:10)(a scrambled peptide including all amino acids of TR_{1-41} peptide, but in a random order).

Other peptides have been made, including

TR_{29-45} - PESKATNATLDPRSFLL (SEQ ID NO:11),

TR_{44-55} - LLRNPNDKYEPF (SEQ ID NO:12), and

TR_{42-55} (S42F, F43S) - FSLLRNPNDKYEPF (SEQ ID NO:13).

TR_{44-55} and TR_{42-55} (S42F, F43S) were used as controls and did not activate platelets.

Other useful peptides include TR_{17-22}, TR_{15-24}, TR_{12-26}, TR_{10-30} and TR_{5-35}, which can be synthesized using the standard techniques described above.

Antagonist peptides can be synthesized by selectively altering one or more amino acids compared to the naturally occurring first 41 amino acids of the thrombin receptor (SEQ ID NO:1). Candidate TR_{1-41} peptide antagonists can then be tested for antagonistic activity using a modification of either the whole blood flow cytometry platelet activation assay or the platelet aggregation platelet activation assay described herein. In antagonist assays, platelets are exposed to the full length TR_{1-41} peptide in the presence and absence of the test antagonist. Inhibition of TR_{1-41} peptide-mediated platelet activation by the candidate antagonist would be

an indication that it is indeed a TR_{1-41} peptide antagonist.

The TR_{1-41} peptides also can be prepared using standard techniques by isolating the thrombin receptor cleavage fragment naturally cleaved from the receptor by thrombin. In addition, the TR_{1-41} peptides and antagonist peptides can be produced by standard *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* recombination/genetic recombination, using the nucleotide sequences encoding the appropriate portions of the first 41 amino acids of the thrombin receptor (see, e.g., Fig. 5 in Vu et al., *Cell*, **64**:1057-1068, 1991), or mutant analogs thereof. Methods well known to those skilled in the art can be used to construct expression vectors containing differentially expressed or pathway gene protein coding sequences and appropriate transcriptional/translational control signals. See, for example, the techniques described in Maniatis et al., Molecular Cloning A Laboratory Manual (Cold Spring Harbor Laboratory, N.Y., 1989), and Ausubel et al., Current Protocols in Molecular Biology, (Green Publishing Associates and Wiley Interscience, N.Y., 1989).

A variety of host-expression vector systems can be used to express the TR_{1-41} peptides and antagonist peptides. Such host-expression systems represent vehicles by which the coding sequences of interest can be produced and subsequently purified, but also represent cells that can, when transformed or transfected with the appropriate nucleotide coding sequences, exhibit the differentially expressed or pathway gene protein of the invention *in situ*. These include, but are not limited to, microorganisms such as bacteria, e.g., *E. coli* or, *B. subtilis*, transformed with recombinant bacteriophage DNA, plasmid or cosmid DNA expression vectors containing TR_{1-41} peptide coding sequences; yeast, e.g., *Saccharomyces* or

Pichia, transformed with recombinant yeast expression vectors containing the appropriate coding sequences; insect cell systems infected with recombinant virus expression vectors, e.g., baculovirus; plant cell systems infected with recombinant virus expression vectors, e.g., cauliflower mosaic virus (CaMV) or tobacco mosaic virus (TMV), or transformed with recombinant plasmid expression vectors, e.g., Ti plasmids, containing the appropriate coding sequences; or mammalian cell systems, e.g., COS, CHO, BHK, 293 or 3T3, harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells, e.g., metallothionine promoter, or from mammalian viruses, e.g., the adenovirus late promoter or the vaccinia virus 7.5K promoter.

Peptides of the invention also include those described above, but modified for *in vivo* use by the addition, at either or both the amino- and carboxy-terminal ends, of a blocking agent to facilitate survival of the relevant peptide *in vivo*. This can be useful in those situations in which the peptide termini tend to be degraded by proteases prior to cellular or mitochondrial uptake. Such blocking agents can include, without limitation, additional related or unrelated peptide sequences that can be attached to the amino and/or carboxy terminal residues of the peptide to be administered. This can be done either chemically during the synthesis of the peptide or by recombinant DNA technology by methods familiar to artisans of average skill.

Alternatively, blocking agents such as pyroglutamic acid or other molecules known in the art can be attached to the amino and/or carboxy terminal residues, or the amino group at the amino terminus or carboxyl group at the carboxy terminus can be replaced with a different moiety. Likewise, the peptides can be

covalently or noncovalently coupled to pharmaceutically acceptable "carrier" proteins prior to administration.

Also of interest are peptidomimetic compounds that are designed based upon the amino acid sequences of the new TR_{1-41} peptides. Peptidomimetic compounds are synthetic compounds having a three-dimensional conformation (i.e., a "peptide motif") that is substantially the same as the three-dimensional conformation of a selected peptide. The peptide motif provides the peptidomimetic compound with the ability to activate platelets or to inhibit activation of platelets that is the same as or greater than the activity of the peptide from which the peptidomimetic was derived. Peptidomimetic compounds can have additional characteristics that enhance their therapeutic application such as increased cell permeability, greater affinity and/or avidity, and prolonged biological half-life.

The peptidomimetics typically have a backbone that is partially or completely non-peptide, but with side groups that are identical to the side groups of the amino acid residues that occur in the peptide on which the peptidomimetic is based. Several types of chemical bonds, e.g. ester, thioester, thioamide, retroamide, reduced carbonyl, dimethylene and ketomethylene bonds, are known in the art to be generally useful substitutes for peptide bonds in the construction of protease-resistant peptidomimetics.

Antibodies

Antibodies that specifically bind to one or more of the new TR_{1-41} peptides can be produced by a variety of methods. Such antibodies can include, but are not limited to, polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain

antibodies, Fab fragments, $F(ab')_2$ fragments, fragments produced by a FAb expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above.

Such antibodies can be used, for example, to detect the naturally occurring thrombin receptor cleaved fragment in a patient's circulating blood, which provides a direct measure of the level of thrombin receptor activation. Such antibodies also can be used in treatment methods for inhibiting excess activation of thrombin receptors and platelets by the thrombin receptor cleaved fragment, and to inhibit binding of the thrombin receptor cleaved fragment to its target cells.

To produce antibodies to the new TR_{1-41} peptides, a host animal is immunized with any one of the peptides. Such host animals can include, but are not limited to, rabbits, mice, and rats. Various adjuvants can be used to increase the immunological response, depending on the host species, including Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as oil emulsions, keyhole limpet hemocyanin (KLH), dinitrophenol (DNP), and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*.

Monoclonal antibodies can be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the standard hybridoma technique of Kohler and Milstein, (*Nature*, **256**:495-497, 1975; and U.S. Patent No. 4,376,110), the human B-cell hybridoma technique (Kosbor et al., *Immunology Today*, **4**:72, 1983; Cole et al., *Proc. Natl. Acad. Sci. USA*, **80**:2026-2030, 1983), and the BV-hybridoma technique (Cole et al., *Monoclonal Antibodies And Cancer Therapy* (Alan R. Liss, Inc. 1985), pp. 77-96. Such antibodies can be of

any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention can be cultivated *in vitro* or *in vivo*. Production of high titers of mAbs *in vivo* makes this the presently preferred method of antibody production.

In addition, "chimeric antibodies" can be made by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity (see, Morrison et al., *Proc. Natl. Acad. Sci.*, **81**:6851-6855, 1984; Neuberger et al., *Nature*, **312**:604-608, 1984; Takeda et al., *Nature*, **314**:452-454, 1985; and U.S. Patent No. 4,816,567). A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a constant region derived from human immunoglobulin.

Alternatively, standard techniques described for the production of single chain antibodies (e.g., U.S. Patent 4,946,778; Bird, *Science*, **242**:423-426, 1988; Huston et al., *Proc. Natl. Acad. Sci. USA*, **85**:5879-5883, 1988; and Ward et al., *Nature*, **334**:544-546, 1989), and for making humanized monoclonal antibodies (U.S. Patent No. 5,225,539), can be used for therapeutic antibodies.

Antibody fragments that recognize specific epitopes can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragments that can be produced by pepsin digestion of the antibody, and the Fab fragments that can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries can be constructed (Huse et al., *Science*, **246**:1275-1281, 1989) to allow rapid and easy

identification of monoclonal Fab fragments with the desired specificity.

Specifically, monoclonal antibodies to TR_{1-41} can be prepared as follows. Mice were immunized with up to 40 μg of TR_{1-41} conjugated to hemocyanin. The antiserum titers were tested by standard ELISA. Briefly, TR_{1-41} is coated onto microtiter plates at 50 ng/well. After blocking the plates, diluted antisera is added and monitored by biotinylated anti-mouse antibody to detect mouse IgG bound to the plates. Streptavidin-alkaline phosphatase is then added and monitored for color development. Mouse spleen cells are fused with the myeloma cells once antiserum titers reach at least 1/10,000. Clones reacting with different domains of the TR_{1-41} peptide are selected by observing the binding of the antibody with synthetic peptides corresponding to the TR_{1-20} and TR_{21-41}. The binding characteristics of the selected clones of antibodies with TR_{1-41} are then determined using standard techniques.

Other monoclonal antibodies used in the following experiments were obtained as follows. Monoclonal antibody S12 is directed against P-selectin, and was obtained from Centocor (Malvern, PA). P-selectin, also referred to as CD62P, GMP-140, and PADGEM protein, is a component of the α granule membrane of resting platelets that is expressed only on the platelet surface after platelet degranulation and secretion. PAC1 (Cell Center, University of Pennsylvania, Philadelphia, PA) is directed against the fibrinogen binding site exposed by a conformational change in the GPIIb-IIIa complex of activated platelets. F26 (Gralnick et al., *J. Lab. Clin. Med.*, **118**:604-613, 1991) is directed against a conformational change in fibrinogen bound to the GPIIb-IIIa complex. Y2/51 (DAKO, Carpenteria, CA) is directed

against GPIIIa and was purchased conjugated to fluorescein isothiocyanate (FITC).

Unlike Y2/51, antibodies PAC1 and F26 do not bind to resting platelets. Y2/51 does not interfere with PAC1 and F26 binding and therefore can be used in the same assays. PAC1 and F26 were biotinylated using standard techniques (see, e.g., Michelson et al., *Blood*, **77**:770-779, 1991). S12, F26 and Y2/51 are IgG, whereas PAC1 is IgM.

TR_{1-41} Peptides Activate Platelets

TR_{1-41} peptides can be tested for platelet activation using a whole blood flow cytometry assay or a platelet aggregation assay. Other standard methods of measuring platelet activation include actin polymerization studies and calcium mobilization studies, e.g., as described in Watson et al. (ed.), Platelets: A Practical Approach (Oxford University Press, Inc., New York, 1996).

Whole Blood Flow Cytometry Assay

The new TR_{1-41} peptides were analyzed in a whole blood flow cytometry assay previously described in detail in Michelson et al., *supra*. There were no centrifugation, gel filtration, vortexing, or stirring steps that could artifactually activate platelets. In brief, peripheral blood was drawn from healthy volunteers who had not ingested aspirin or other anti-platelet drugs during the previous ten days. As indicated, some samples were drawn from a boy with Bernard-Soulier syndrome, and some from a boy with Glanzmann's thrombasthenia. The first 2 ml of drawn blood were discarded. Blood was then drawn into a sodium citrate filled VACUTAINER™ (Becton Dickinson, Rutherford, NJ). Within 15 minutes of drawing, the blood was diluted 1:20 in modified HEPES-Tyrode's buffer (137 mM NaCl, 2.8 mM KCl,

1 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.4 mM Na_2HPO_4, 0.35% bovine serum albumin, 10 mM HEPES, 5.5 mM glucose), pH 7.4.

In some experiments the samples were diluted with autologous plasma along with modified HEPES-Tyrode's buffer, pH 7.4. In these experiments, plasma content varied from 0 to 45% by volume. The peptide glycine-L-prolyl-L-arginyl-L-proline (GPRP, Calbiochem, San Diego, CA) at a concentration of 2.5 mM was added to the samples to prevent fibrin polymerization. The samples were incubated for varying times at 22°C with various concentrations of either (a) the peptides TR_{1-41}, TR_{1-10}, TR_{11-20}, TR_{21-30}, TR_{31-41}, TR_{1-20}, TR_{21-41}, or TR_{42-55} (also known as thrombin receptor activating peptide (TRAP), Calbiochem), (b) purified human α-thrombin, or (c) control buffer.

In some experiments, hirudin 10 U/ml (Calbiochem), a specific thrombin inhibitor, was added concomitantly with the addition of the synthetic peptides or thrombin. In other experiments, the chelating agents EDTA (2 mM) or EGTA (2 mM) were added 10 minutes prior to addition of the synthetic peptides or thrombin.

At various time points up to 300 seconds after the addition of the agonists, all samples were fixed at 22°C for 20 minutes with formaldehyde 1% (final concentration). After fixation, samples were diluted 10-fold in modified Tyrode's buffer, pH 7.4. The samples were then incubated at 22°C for 20 minutes with a near saturating concentration of FITC-conjugated monoclonal antibody Y2/51, and a saturating concentration of biotinylated monoclonal antibody S12, PAC-1, or F26, followed by an incubation at 22° for 20 minutes with 30 μg/ml of phycoerythrin-streptavidin (Jackson ImmunoResarch, West Grove, PA).

Samples were analyzed in an EPICS Profile flow cytometer (Coulter Cytometry, Hialeah, FL). The flow

cytometer was equipped with a 500 mW argon laser (Cyonics, San Jose, CA) operated at 15 mW with an emission wavelength of 488 nm. The fluorescence of FITC and phycoerythrin were detected using 525 nm and 575 nm band pass filters, respectively.

After identification of platelets by gating on both Y2/51-FITC positivity (i.e., GPIIIa-positivity) and their characteristic light scatter, binding of the biotinylated monoclonal antibody (S12, PAC1, or F26) was determined by analyzing 5,000 individual platelets for phycoerythrin fluorescence. Background binding, obtained from parallel samples run with FITC-Y2/51 and purified biotinylated mouse IgG (IgM for PAC1 assays) (Boehringer Mannheim, Indianapolis, IN), was subtracted from each test sample.

Other antibodies that can be used to measure platelet activation include CD63 (Nieuwenhuis, *Blood*, **70**:838, 1987)-, ligand-induced binding site-(LIBS)(Frelinger, *J. Biol. Chem.*, **263**:12397, 1988, and Frelinger, *J. Biol. Chem.*, **265**:6346, 1990) and receptor-induced binding site (RIBS)(Zamarron, *Thromb. Haemost.*, **64**:41, 1990, Abrams, *Blood*, **75**:128, 1990, and Gralnick, *Br. J. Haematol.*, **80**:347, 1992)-, Factor Va (Sims, *J. Biol. Chem.*, **263**:18205, 1988)-, Factor VIII (Gilbert, *J. Biol. Chem.*, **266**:17261, 1991)-, annexin V-, multimerin (Hayward, *J. Biol. Chem.*, **266**:7114, 1991, and Hayward, *J. Clin. Invest.*, **91**:2630, 1993)-, and thrombospondin (Boukerche, *Eur. J. Biochem.*, **171**:383, 1988, and Aiken, *Semin. Thromb. Hemost.*, **13**:307, 1987)-specific antibodies, as well as the 7E3 GPIIb-IIIa complex (Coller et al., *Clin. Invest.*, **72**:325-338, 1983)-, 6D1 GPIb (Coller et al., *Blood*, **61**:99-105, 1983)-, GRP GPIX (Michaelson et al., *Blood*, **87**:1396-1408, 1996)-, and the SW16 GPV (Moderman et al., *J. Biol. Chem.*, **267**:364, 1992)-specific antibodies.

Platelet Aggregation Assay

A platelet aggregation assay also can be used to determine platelet activation. Washed platelets were analyzed in a platelet aggregometer after the addition (arrowhead) of thrombin 1 U/ml (Fig. 2A), TR_{42-55} 20 μM (Fig. 2B), or TR_{1-41} μM (Fig. 2C). Platelet aggregometry was performed in a Payton Series 1000B aggregometer (Payton, Buffalo, NY). Aggregation was recorded as an increase in light transmission.

Washed platelets were prepared as previously described in Michelson et al., *Blood*, **76**:2005-2010, 1990. Blood was drawn by venipuncture into a VACUTAINER™, as described above. The citrated blood was centrifuged (150 x g, 15 minutes, 22°C) and the supernatant (platelet-rich plasma, "PRP") was separated. After addition to the PRP of citrate albumin wash buffer (128 mM NaCl, 4.3 mM $Na_2H_2PO_4.H_2O$, 7.5 mM Na_2HPO_4, 4.8 mM sodium citrate, 2.4 mM citric acid, 11 mM glucose, 0.35% bovine serum albumin), pH 6.5 with PGE_1 50 ng/ml, washed platelets were prepared by centrifugation.

The concentration of washed platelets was adjusted to 600,000/μL in modified HEPES-Tyrode's buffer, pH 7.4. The washed platelets were diluted in an equal volume of HEPES-Tyrode's buffer, pH 7.4, or autologous plasma. TR_{1-41} 20 μM, TR_{42-55} 20 μM, or thrombin 1 U/ml were added to a final volume of 500 μL of washed platelets.

TR_{1-41} Peptide Fragments and Mutant Analogs

To assess whether the entire 41 amino acid sequence of the thrombin receptor is required for platelet activation, various TR_{1-41} peptides were synthesized as described above that correspond to the first, second, third, and fourth sets of ten sequential amino acids, and the first and second sets of twenty sequential amino acids of the first 41 amino acids of the

thrombin receptor. None of these peptides (TR_{1-10}, TR_{11-20}, TR_{21-30}, TR_{31-41}, TR_{1-20}, TR_{21-40}) resulted in platelet activation as detected by whole blood flow cytometry (data not shown). In addition, a scrambled peptide (SEQ ID NO:10) was shown in the whole blood flow cytometry and platelet aggregation assays not to activate platelets (data not shown). Other peptides (TR_{44-55} and TR_{42-55} (S42F, F43S)) were also inactive. However, a mutant analog, TR_{1-41} (S14A) (SEQ ID NO:9), did activate platelets (data not shown). This finding shows that analogs of TR_{1-41} containing a mutation, albeit a relatively conservative mutation (S14A), retain agonist activity.

Modulation of the Intracellular Signaling Pathways in TR_{1-41} Peptide-Induced Platelet Activation

To explore the signal transduction mechanisms involved in TR_{1-41} peptide-induced platelet activation, platelet activation by TR_{1-41} peptide (and by thrombin for comparison) was assayed in the presence of agents that modify the intracellular environment, and potentially modulate the TR_{1-41} peptide-induced platelet activation.

In variations of the whole blood flow cytometry assay, either 10 μM prostaglandin (PGI_2) (Sigma, St. Louis, MO) or 10 μM S-nitroso-N-acetylcysteine (SNAC) (an endothelium-derived relaxing factor (EDRF) congener), known inhibitors of platelet activation, were added concomitantly with the addition of the synthetic peptides or thrombin. The EDRF congener (SNAC) was prepared at 22°C by reacting equimolar concentrations of fresh *N*-acetylcysteine with $NaNO_2$ at acidic pH. SNAC was prepared within 10 minutes of use, kept at 4°C, and diluted as necessary into aqueous buffer immediately before addition to assay systems.

In other experiments, the following agents were incubated at 22°C for 30 minutes prior to the addition of the synthetic peptides or thrombin: (a) staurosporine 10 μM (Sigma), an inhibitor of protein kinase C; (b) wortmannin (Sigma) at 100 nM is an inhibitor of phosphatidyl inositol-3(PI_3)-kinase and at 1 μM is an inhibitor of myosin light chain kinase (MLCK) and PI_3-kinase; or (c) genistein 100 μM (Sigma), an inhibitor of protein tyrosine kinases.

In addition to using normal blood samples, some samples were drawn from a boy with Bernard-Soulier syndrome, or a boy with Glanzmann's thrombasthenia.

Synergy of TR_{1-41} Peptides and TR_{42-55} Peptide

In other experiments, various concentrations of TR_{1-41} peptide and TR_{42-55} peptide were added together for 10 minutes at 22°C to determine whether these two different peptides might interact in a greater than additive, i.e., synergistic, manner. Diluted whole blood was incubated (22°C, 10 minutes) with the TR_{1-41} peptide at concentrations of 0, 5, 10, and 20 μM, and then incubated (22°C, 10 minutes) with TR_{42-55} at concentrations of 0, 5, 10, and 20 μM. The platelet surface binding of the P-selectin-specific monoclonal antibody S12 was determined by flow cytometry. Binding was expressed as a percent of the binding with maximal thrombin (2 U/ml). The results indicate that at least at the lower concentrations there is a synergistic effect (Fig. 3).

Effect of Plasma on TR_{1-41}-Induced Platelet Activation

The addition of increasing volumes of platelet-poor plasma to diluted whole blood resulted in increasing inhibition of the platelet activating effects of TR_{1-41}, as determined by the platelet surface binding of S12

(Fig. 6) and PAC1 (data not shown). After dilution of whole blood with the indicated concentration of autologous platelet-poor plasma, samples were incubated (22°C, 10 minutes) with TR_{1-41} 25 μM. The platelet surface binding of the P-selectin-specific monoclonal antibody S12 was determined by flow cytometry.

This plasma inhibitor of TR_{1-41} may be a physiological mechanism for the prevention of undesirable TR_{1-41}-induced platelet activation at points distal to local thrombus formation.

Uses of the TR_{1-41} Peptides, Antagonist Peptides, and Antibodies

Coronary artery disease is increasingly being understood as a thrombotic disorder. Markers of platelet activation are present in patients with acute coronary syndromes and myocardial infarction, and platelets from patients with these conditions are more aggregable than platelets from normal subjects. However, it is unlikely that activated platelets circulate in the blood for a significant amount of time because a) activated platelets adhere to endothelial surfaces, b) aggregate to one another, c) adhere to monocytes and neutrophils, and/or d) are cleared via the reticulo-endothelial system. Thus, it is difficult to detect circulating activated platelets in patients at risk for the development of cardiovascular thrombotic disorders.

Not only is there evidence of platelet activation in patients with acute coronary artery syndromes, there is direct evidence of increased thrombin production in these syndromes. Thrombin is the most physiologically active and potent platelet activator. By cleaving its receptor between amino acids 41 and 42, thrombin activates platelets via two synergistic pathways: 1) via the tethered ligand which is mimicked using the thrombin

receptor agonist peptides (TRAPs) such as the TR_{42-55} peptide; and 2) via the newly discovered TR_{1-41} peptide pathway. The enhanced thrombin generation in coronary artery disease results in increased platelet reactivity by generating subthreshold amounts of circulating TR_{1-41} peptide that interact with the circulating platelets and lower their threshold for activation.

These "primed" platelets should be poised to become fully activated under conditions in which normal platelets would not be activated. Thus patients with "primed" platelets may be at increased risk of thrombotic events such as unstable angina, myocardial infarction, and stroke. Identification of these patients allows individual tailoring of anti-platelet therapy (use of more potent agents for example), or more careful clinical follow-up. Furthermore, identification of patients without "primed" platelets would identify a group at low risk for acute complications from interventional procedures such as percutaneous transluminal coronary angioplasty (PTCA) and intracoronary stent insertion.

Primed platelets can be detected using either flow cytometry or platelet aggregation as described above, and can be detected in whole blood, platelet rich plasma, or with washed platelets. Platelet activation is measured either as the binding of specific activation dependent monoclonal antibodies to the platelet surface (flow cytometry), or by platelet aggregation (impedance method or optical method). Platelet samples from the subject are activated at increasing concentrations of agonists, including thrombin, TRAP, TR_{1-41}, epinephrine, ADP, the thromboxane A2 analog U46619, and others, and are compared to established normal controls.

For example, samples can be incubated for varying times at 22°C with various concentrations of a) TR_{1-41} peptide (2.5, 5.0, 10, 15, 20, and 25 μM); b) TRAP (2.5,

5.0, 10, 15, 20, and 25 μM); c) epinephrine (1, 2, 5, and 10 μM); d) ADP (1, 2, 5, and 10 μM); e) U46619 (thromboxane A2 analog)(0.5, 1.0, 1.5, 2.0, and 5.0 μM); or f) thrombin (0.1 0.5, 1.0, and 2.0 U/ml). At 300 seconds after the addition of the agonists, all samples are fixed at 22°C for 20 minutes with formaldehyde 1% (final concentration). After fixation, samples are diluted 10-fold in modified Tyrode's buffer, pH 7.4. The samples are then incubated at 22°C for 20 minutes with a near saturating concentration of FITC-conjugated monoclonal antibody Y2/51 and a saturating concentration of biotinylated monoclonal antibody S12, PAC-1 or F26, followed by an incubation at 22°C for 20 minutes with 30 μg/ml of phycoerythrin-streptavidin (Jackson ImmunoResearch, West Grove, PA). As described above, the samples are analyzed in an EPICS Profile flow cytometer (Coulter Cytometry, Hialeah, FL.).

Activation is expressed as either the percentage of fluorescent intensity compared to a maximally stimulated platelet, or as a percentage of platelets labelled with an activation-dependent antibody. A normal platelet control population is studied to obtain baseline normal values. Activated platelets are those whose percent maximal fluorescent antibody binding or percent positive cells is greater than two standard deviations from the values of normal, non-stimulated, non-activated platelets.

Thrombin inhibitors such as heparin, low molecular weight heparin, and hirudin are frequently used in the treatment of thrombotic disorders including unstable angina and myocardial infarction. They are also routinely used during angioplasty and cardiac catheterization. While these agents are effective anticoagulants, they have significant bleeding risks limiting their use. Development of specific anti-

thrombin agents, such as those that inhibit either TRAP or TR_{1-41} platelet activation pathways directly or competitively, may produce enough of an anti-platelet effect without significantly increasing the bleeding risk. For example, the TR_{1-41} peptide mutant analog, TR_{1-41} (S14A), an inactive form of the TR_{1-41} peptide, will competitively inhibit TR_{1-41} peptide-induced platelet activation and thus be an effective anti-thrombin, or more specifically, an anti-TR_{1-41} peptide agent.

Furthermore, development of any anti-thrombin compounds, either directed against the enzyme or its receptor, must be tested for their efficacy in blocking individually both pathways of thrombin-induced platelet activation, that is the pathway mediated via the tethered ligand (TRAP) and the pathway mediated by the TR_{1-41} peptide. Full inhibition of thrombin interacting with its receptor will prevent both pathways from being activated. However, a significant anticoagulant effect will result, potentially limiting its usage. Pharmacologic compounds which inhibit either the tethered ligand pathway (TRAP) or TR_{1-41} pathway should provide an anti-thrombin induced platelet effect without a significant anticoagulant defect. Any such compound must be tested for its ability to inhibit either TRAP, TR_{1-41}, or thrombin-induced platelet activation.

For example, any specific TRAP inhibitor must a) inhibit TRAP-induced platelet activation, and b) allow TR_{1-41} peptide-induced platelet activation to continue. Thus, TR_{1-41} peptide can be used in the platelet activation assays described herein to test the TRAP specificity of the new compound. Similarly, a TR_{1-41} inhibitor must inhibit TR_{1-41}-induced platelet activation. This too can be tested in the presence of TR_{1-41} peptide using the assays described herein.

Thus, TR_{1-41} peptides can be used to induce thrombosis *in vivo* as a hemostatic device. Artificially induced thrombus formation is beneficial in situations such as in surgery where wounds are continuously bleeding, and arterial punctures during interventional cardiovascular or radiological procedures. TR_{1-41} peptides may prove more beneficial than either thrombin or TRAP compounds because the platelet activating effect is not as intense. TR_{1-41} peptides can be given topically in either a gel, lotion, or solution to instigate platelet activation. Other modes of administration are described below.

In another aspect, the local concentration of endogenous TR_{1-41} peptide at sites of thrombus formation, such as vessel injury, is likely to be sufficient to cause TR_{1-41}-induced platelet activation. Thus, TR_{1-41} peptides can be administered therapeutically to augment platelet activation in a growing thrombus. Such a role for the TR_{1-41} peptide is consistent with the slower kinetics of TR_{1-41}-induced platelet activation compared to thrombin- or TR_{42-55}-induced platelet activation. The inhibition of TR_{1-41} peptide-induced platelet activation by a plasma component suggests a physiological mechanism for the prevention of undesirable TR_{1-41}-induced platelet activation at points distal to a local thrombus.

Furthermore, certain medical situations such as cardiopulmonary bypass are associated with a defect in platelet function. TR_{1-41} peptides can be administered either intravenously or mixed into the bypass machine to stimulate platelets to a moderate degree, thus overriding the platelet defect.

On the other hand, the TR_{1-41} peptide antagonists are useful therapeutically in clinical conditions in which thrombin-induced cellular activation is part of the pathophysiological process, for example, angina, coronary

artery disease, unstable angina, myocardial infarction, congestive heart failure, cardiomyopathy, ventricular and atrial arrhythmias, and stroke. In addition, they can be administered to patients receiving thrombolytic therapy, and patients receiving angioplasty. Moreover, the antagonists can be beneficial for patients needing prophylaxis for venous thrombosis such as those having deep venous thrombosis, recent surgery (any type), continuous bed rest, venous stasis, or pulmonary embolism. In addition, the antagonists can be used to reduce the restenosis rate in patients undergoing angioplasty.

The TR_{1-41} peptides and peptide antagonists, as well as TR_{1-41} peptide antibodies, are administered in conventional formulations for systemic administration as is known in the art. Typical formulations may be found, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton PA, latest edition.

Preferred forms of systemic administration of peptides include injection, typically by intravenous injection. Other injection routes, such as subcutaneous, intramuscular, or intraperitoneal, can also be used. More recently, alternative means for systemic administration of peptides have been devised which include transmucosal and transdermal administration using penetrants such as bile salts or fusidic acids or other detergents. In addition, if properly formulated in enteric or encapsulated formulations, oral administration is also possible.

The dosage range required depends on the choice of peptide, peptide antagonist or antibody, the route of administration, the nature of the formulation, the nature of the patient's illness, and the judgment of the attending physician. Suitable dosage ranges, however, are in the range of 0.1-100.0 μg/kg of the patient. Wide

variations in the needed dosage, however, are to be expected in view of the variety of peptides and peptide antagonists available and the differing efficiencies of various routes of administration. For example, oral administration would be expected to require higher dosages than administration by intravenous injection. Variations in these dosage levels can be adjusted using standard empirical routines for optimization as is well understood in the art.

In addition to the above described methods of peptide administration, TR_{1-41} peptides and peptide antagonists can be delivered to a patient by administration of a nucleic acid encoding the peptides, an expression vector encoding the peptides, or cells, e.g., a patient's own cells, transduced with such a vector.

Such expression vectors contain a nucleic acid in which a polynucleotide sequence encoding a TR_{1-41} peptide or peptide antagonist is operatively linked to a promoter or enhancer-promoter combination. A promoter is a trancriptional regulatory element composed of a region of a DNA molecule, typically within 100 nucleotide pairs upstream of the point at which transcription starts.

Another transcriptional regulatory element is an enhancer, which provides expression specificity in terms of time, location, and level. Unlike a promoter, an enhancer can function when located at variable distances from the transcription site, provided a promoter is present. An enhancer can also be located downstream of the transcription initiation site. The coding sequence of the expression vector is operatively linked to a transcription terminating region. To bring a coding sequence under the control of a promoter, it is necessary to position the translation initiation site of the translational reading frame of the peptide or polypeptide

between one and about fifty nucleotides downstream (3′) of the promoter. Suitable vectors include plasmids, and viral vectors such as herpes viruses, retroviruses, vaccinia viruses, attenuated vaccinia viruses, canary pox viruses, adenoviruses and adeno-associated viruses, among others.

The application of agonist or antagonist encoding genes to the modulation of platelet activity in humans can utilize either *in vivo* or *ex vivo* based approaches. The *ex vivo* method includes the steps of harvesting cells (e.g., fibroblasts or hematopoietic stem cells) from a subject, culturing the cells, transducing them with an expression vector, and maintaining the cells under conditions suitable for expression of the therapeutic peptide. These methods are known in the art of molecular biology. The transduction step is accomplished by any standard means used for *ex vivo* gene therapy, including calcium phosphate, lipofection, electroporation, viral infection, and biolistic gene transfer. Cells that have been successfully transduced are then selected, for example, for expression of a drug resistance gene. The cells may then be lethally irradiated (if desired) and injected or implanted into the patient.

The *in vivo* approach requires delivery of a genetic construct encoding a therapeutic TR_{1-41} peptide or antagonist into a patient, preferably targeted to the cells or tissue of interest (e.g., coronary arteries in the case of coronary artery disease). This can be achieved by administering it directly to the relevant tissue (e.g., by injection into cardiac tissue during cardiac surgery).

Tissue specific targeting can also be achieved by the use of a molecular conjugate composed of a plasmid or other vector attached to poly-L-lysine by electrostatic or covalent forces. Poly-L-lysine binds to a ligand that

can bind to a receptor on target cells (Cristiano et al. *J. Mol. Med.* 73:479, 1995). Similarly, cell-specific antibodies can be bound to vectors and thereby target them to the relevant cells. A promoter inducing tissue or cell-specific expression can be used to achieve a further level of targeting. Appropriate tissue-specific promoters include, for example, the cardiac myosin light chain promoter (Lee et al., *J. Biol. Chem.* 267:15875, 1992) or the α-cardiac myosin heavy chain promoter (Gulick et al., *J. Biol. Chem.* 266:9180, 1991). These promoters would allow production of the antagonists of the invention, for example, in cardiac tissue and could thus be useful in therapy of coronary heart disease or myocardial infarction, for example.

Vectors can also be incorporated into liposomes or an appropriate biodegradable polymeric microparticle (also referred to as a "microsphere," "nanosphere," "nanoparticle," or "microcapsule") and delivered in this form. The vectors can be incorporated alone into these delivery vehicles or co-incorporated with tissue-specific antibodies.

DNA or transfected cells can be administered in a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are biologically compatible vehicles which are suitable for administration to a human, e.g., physiological saline. A therapeutically effective amount is an amount of the DNA of the invention which is capable of producing a medically desirable result in a treated animal. As is well known in the medical arts, the dosage for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Dosages will vary, but a preferred dosage for administration of DNA is from

approximately 10^6 to 10^{12} copies of the DNA molecule. This dose can be repeatedly administered, as needed. Routes of administration will be regional, as exemplified above, systemic (e.g., intravenous, subcutaneous, intramuscular or intraperitoneal) or transmucosal (e.g., intrarectal, intravaginal or intranasal).

Current methods (including platelet aggregometry and flow cytometry) of measuring platelet function use panels of platelet agonists, most of which are weaker agonists than TR_{1-41} peptides. TR_{1-41} peptide is one of the strongest platelet agonists and therefore, presumably one of the most physiologically important. Thus, TR_{1-41} should be added to the panels of platelet agonists used in all platelet function tests.

More generally, TR_{1-41} peptides can be used to test *ex vivo* or *in vitro* platelet function and reactivity in clinical settings where altered platelet function may indicate a high risk of future thrombotic or clinical events. These situations include assaying platelet function in patients with angina, coronary artery disease, unstable angina, myocardial infarction, congestive heart failure, cardiomyopathy, ventricular and atrial arrhythmias, sudden death, stroke, patients receiving thrombolytic therapy, and patients receiving angioplasty. These tests are based on the whole blood flow cytometry and aggregation assays described herein.

In addition, TR_{1-41} peptides can be used to test *ex vivo* or *in vitro* platelet function and reactivity in research settings to better elucidate the signaling pathways of the thrombin receptor. Using standard cell biology, protein biochemistry, and molecular biology techniques, and activating platelets with either TRAP (representing the tethered ligand) or TR_{1-41} peptides, the individual signal transduction mechanisms can be studied.

TR_{1-41} peptide antibodies, either polyclonal or monoclonal, can be used to inhibit the function of the naturally occurring thrombin receptor cleaved fragment on its target cells. TR_{1-41} peptide is thought to be involved in physiological and pathological processes. Like thrombin itself, TR_{1-41} can regulate cell proliferation, growth factors, cytokines, wound healing, inflammation, etc. The monoclonal anti-TR_{1-41} antibodies should be capable of blocking the interactions of TR_{1-41} with its target cells. The inhibition of TR_{1-41}-mediated processes *in vivo* thus may exhibit therapeutic effects.

EXAMPLES

1. TR_{1-41} Peptides Activate Platelets

Exposure of diluted whole blood to TR_{1-41} peptide resulted in a concentration-dependent increase in the surface expression of various activation-dependent antigens, as determined by the whole blood flow cytometry assay described above (Figs. 1A to 1C, binding is expressed as a percent of the binding with maximal thrombin (2 U/ml), and data are mean $\pm$ S.E.M., n = 6). These results indicate that the platelets in the whole blood were activated.

TR_{1-41} peptide resulted in increased platelet surface expression of P-selectin (reflecting α-granule secretion; S12 monoclonal antibody binding) (Fig. 1A). TR_{1-41} peptide also resulted in the increased expression of the activated conformation of the GPIIb-IIIa complex, as reported by PAC1 binding (Fig. 1B). The TR_{1-41} peptide-induced increase in the platelet surface binding of monoclonal antibody F26 demonstrated that TR_{1-41} resulted in fibrinogen binding to this activated GPIIb-IIIa complex (Fig. 1C).

The maximal TR_{1-41} peptide-induced binding of S12, PAC1, and F26 (O at 25 μM) was significantly greater than

with TR_{42-55} (□ at 25 μM) and almost as great was with thrombin (■ at 0.5 to 1.0 U/ml)(Figs. 1A to 1C). To assess whether thrombin is required for TR_{1-41} peptide-induced platelet activation, platelets were activated with TR_{1-41} peptide in the presence of saturating amounts of hirudin (10 U/ml), a specific thrombin inhibitor. Hirudin did not inhibit the ability of TR_{1-41} or TR_{42-55} to activate platelets but, as expected, did inhibit thrombin-induced platelet activation (data not shown).

The increase in platelet surface expression of P-selectin detected by flow cytometry was more rapid in response to thrombin and TR_{42-55} than to TR_{1-41} (data not shown). After one minute of agonist-induced platelet activation, thrombin 2 U/ml resulted in greater than 80% of maximal degranulation, TR_{42-55} 25 μM resulted in greater than 60% of maximal platelet degranulation, whereas TR_{1-41} peptide (25 μM) resulted in only minimal platelet degranulation. Thrombin and TR_{42-55} resulted in maximal platelet degranulation within 1.5 minutes, whereas TR_{1-41} peptide resulted in maximal platelet degranulation after 5 minutes.

2. TR_{1-41} Peptides Cause Platelet Aggregation

Figs. 2A to 2C are a series of graphs showing the results of this TR_{1-41}-, TR_{42-55}-, and thrombin-induced platelet aggregation. Washed platelets were analyzed in a platelet aggregometer after the addition (arrowhead) of thrombin 1 U/ml (Fig. 2A), TR_{42-55} 20 μM (Fig. 2B), or TR_{1-41} 20 μM (Fig. 2C). As shown in Figs. 2A to 2C, the maximum amplitude of the aggregation curve is similar among the different agonists. However, the lag phase and slope of the TR_{1-41} aggregation curve were less than that of thrombin and TR_{42-55}, demonstrating the slower kinetics of TR_{1-41}-induced platelet aggregation.

Similar results are also depicted in Tables 1 and 2 below, in which thrombin at a concentration of 2 U/ml was used as a 100% level of aggregation. Table 1 shows the aggregation results for 6 samples of washed platelets, whereas Table 2 shows the aggregation results for 6 samples of platelet rich plasma. The data in Table 1 shows that TR_{1-41} peptide induces slower platelet aggregation and onset of activation than thrombin or TR_{42-55} peptide. The scrambled $TR_{1-41}(s)$, TR_{29-45}, TR_{1-10}, TR_{44-55}, and TR_{42-55} are inactive.

In Table 2, the data show the plasma-inhibiting effect on the activity of the TR_{1-41} peptide, which is described further in Example 6. Here, plasma inhibits the ability of TR_{1-41} peptide to aggregate platelets.

Table 1
Aggregation with Washed Platelets
(n = 6)

Agonist	Thrombin	TR_{1-41}	TR_{42-55}	TR_{29-15}	TR_{1-10}	TR_{1-41^S}	TR_{44-55}	TR_{42-55}
Amplitude of Light Transmission (% of maximum)	93.83 ± 1.2	68 ± 11	85 ± 4.7	1.5 ± 0.7	1.0 ± 0.6	1.0 ± 0.6	1.0 ± 0.4	1.0 ± 0.6
Maximum Slope (% light transmission/ secs)	1.47 ± 0.1	0.82 ± 0.19	1.53 ± 0.33	N/A	N/A	N/A	N/A	N/A
Concentration to Achieve Maximum Aggregation	0.28 ± 0.13 U/ml	15.63 ± 2.85 μM	12 ± 1.25 μM	N/A	N/A	N/A	N/A	N/A
Time Delay to Maximum Aggregation (secs)	47.50 ± 10.3	62.5 ± 16.8	42.7 ± 19.3	N/A	N/A	N/A	N/A	N/A
EC_{50}	0.09 ± 0.01 U/ml	7.25 ± 1.08 μM	13 ± 2.34 μM	N/A	N/A	N/A	N/A	N/A

Table 2
Aggregation with Platelet Rich Plasma
(n = 6)

Agonist	Thrombin	TR_{1-41}	TR_{42-55}	TR_{29-15}	TR_{1-10}	TR_{1-41^s}	TR_{44-55}	TR_{42-55}
Amplitude of Light Transmission (% of maximum)	90 ± 3.95	0	83 ± 2.2	3.0 ± 0.97	2.0 ± 0.8	2.7 ± 0.8	3.8 ± 0.9	4.0 ± 0.56
Maximum Slope (% light transmission/ secs)	1.93 ± 0.26	0	1.88 ± 0.05	N/A	N/A	N/A	N/A	N/A
Concentration to Achieve Maximum Aggregation	0.94 ± 0.053 U/ml	0 μM	22 ± 2.68 μM	N/A	N/A	N/A	N/A	N/A
Time Delay to Maximum Aggregation (secs)	24.34 ± 6.3	0	15.6 ± 1.5	N/A	N/A	N/A	N/A	N/A
EC_{50}	0.63 ± 0.07 U/ml	0 μM	6.9 ± 0.9 μM	N/A	N/A	N/A	N/A	N/A

3. TR_{1-41} and TR_{42-55} Peptides Interact Synergistically

As shown in the graph of Fig. 3, TR_{1-41} peptide augmented TR_{42-55}-induced platelet activation, and TR_{42-55} augmented TR_{1-41}-induced platelet activation in a concentration-dependent manner, thereby demonstrating that these two peptides interact synergistically, i.e., greater than additively. The graphs show the interaction of TR_{1-41} peptide concentrations of 0 μM (○), 5 μM (●), 10 μM (▽), and 20 μM (▼), with TR_{1-41} concentrations of 0, 5, 10, 15, and 20 μM. Data in Fig. 3 are mean ± S.E.M. (n = 3).

4. Intracellular Signalling Pathways Involved in TR_{1-41} Peptide-Induced Platelet Activation

Platelet activation by TR_{1-41} peptide (and by TR_{42-55}, a combination of TR_{1-41} and TR_{42-55} or thrombin for comparison) was assayed in the presence of agents that modify the intracellular environment. As shown in Fig. 4, these data demonstrate that TR_{1-41} peptide-induced platelet activation is modulated, at least in part, via cAMP, protein kinase C, phosphatidyl inositol-3-kinase, intracellular protein tyrosine kinases and myosin light chain kinase.

Figs. 4 and 5 are bar graphs showing intracellular signal transduction pathways involved in TR_{1-41} peptide-induced platelet activation. After either a) preincubation (22°C, 30 minutes) with staurosporine 10 μM, wortmannin 1 μM or 100 nM, or genistein 100 μM, or b) co-incubation with buffer only (control), prostaglandin I_2 (PGI_2) 10 μM, or the EDRF congener SNAC 10 μM, diluted whole blood samples were incubated (22°C, 10 minutes) with either TR_{1-41} peptide 15 μM (dotted bars), TR_{42-55} peptide 15 μM (diagonally hatched bars), a mixture of TR_{1-41} 15 μM peptide and TR_{42-55} peptide 15 μM (horizontally

hatched bars) or thrombin 0.1 U/ml (solid bars), and then fixed (Fig. 4).

In studies of platelets of patients with Bernard-Soulier syndrome and Glanzmann's thrombasthenia, respectively, the blood samples were not preincubated with any inhibitors, but were incubated with TR_{1-41} 20 μM or thrombin 5 U/ml (Fig. 5). The platelet surface binding of the P-selectin-specific monoclonal antibody S12 was determined by whole blood flow cytometry. Binding is expressed as a percent of the binding with maximal thrombin (2 U/ml) in normal volunteers. Data are mean ± S.E.M., n=6, except for data for Bernard-Soulier syndrome and Glanzmann's thrombasthenia which are means of two experiments.

Elevation of intraplatelet cyclic AMP (cAMP) by PGI_2 almost completely inhibited TR_{1-41}-, TR_{42-55}-, concomitant TR_{1-41} and TR_{42-55}-, and thrombin (0.1 U/ml) induced platelet degranulation (as detected by S12 binding) (Fig. 4), and expression of activated GPIIb-IIIa (as detected by PAC1 and F26 binding, data not shown). The EDRF congener SNAC partially inhibited thrombin- and TR_{42-55}-induced platelet activation, but had only a minimal effect on TR_{1-41}- or concomitant TR_{1-41} and TR_{42-55}-induced platelet activation (Fig. 4). The protein kinase C inhibitor staurosporine almost completely inhibited TR_{1-41}-, TR_{42-55}-, concomitant TR_{1-41} and TR_{42-55}-, and thrombin-induced S12 binding (Fig. 4) and PAC-1 binding (data not shown).

At nanomolar concentrations, wortmannin selectively inhibits PI_3 kinase but, at higher concentrations, it also inhibits myosin light chain kinase (Kovacsovics et al., *J. Biol. Chem.* 270:11358, 1995; Thomason et al., *J. Biol. Chem.* 269:16525, 1994). At a concentration of 100 nM or 1 μM, wortmannin reduced

platelet surface P-selectin expression in response to TR_{1-41} or TR_{42-55} more than platelet surface P-selectin expression in response to concomitant TR_{1-41} and TR_{42-55} or thrombin (Fig. 4).

Inhibition of intracellular tyrosine kinase activity by the addition of genistein resulted in a decrease in
TR_{1-41}-, TR_{42-55}-, concomitant TR_{1-41} and TR_{42-55}-, and thrombin-induced platelet activation (Fig. 4).

The absence of the GPIb-IX complex (Bernard-Soulier syndrome) or the GPIIb-IIIa complex (Glanzmann's thrombasthenia) had no effect on TR_{1-41}-induced platelet activation (Fig. 5). In normal platelets, inhibition of the binding of fibrinogen to GPIIb-IIIa by the addition of RGD-containing peptides also did not interfere with TR_{1-41}-induced platelet activation (data not shown).

5. TR_{1-41} Requires Extra-Cellular Calcium to Activate Platelets

In the presence of either the divalent cation chelator EDTA (2.8 mM) or the calcium chelator EGTA (2.8 mM), the TR_{1-41} peptide (25 μM) did not cause any platelet degranulation, as indicated by the absence of platelet surface P-selectin (Fig. 5). Neither EDTA nor EGTA inhibited thrombin (2 U/ml) or TR_{42-55} (25 μM) induced platelet degranulation (Fig. 5). Because the platelet surface expression of P-selectin is not calcium dependent, these experiments demonstrate that the TR_{1-41} peptide requires extra-cellular calcium to activate platelets.

6. Plasma Inhibits TR_{1-41}-Induced Platelet Activation

Addition of increasing volumes of platelet-poor plasma to diluted whole blood resulted in increasing inhibition of the platelet activating effects of the TR_{1-}

$_{41}$ peptide, as determined by the platelet surface binding of S12 (Fig. 6) and PAC1 (data not shown). The added volumes of plasma also inhibited TR_{42-55}- and thrombin-induced platelet degranulation and expression of the activated GPIIb-IIIa receptor, but not to the same extent as for TR_{1-41} (data not shown). Plasma inhibitors of thrombin include anti-thrombin III. Binding in Fig. 6 is expressed as a percent of the binding with maximal thrombin (2 U/ml). Data are mean ± S.E.M., n = 6.

7. TR_{1-41} and Thrombin Induced Decreases in Platelet Surface Expression of GPIb, GPIX and GPV

Exposure of platelets to either TR_{1-41} 20 μM (closed circles) or thrombin 1 U/ml (open circles) resulted in a decrease in platelet GPIb (Fig. 7A), GPIX (Fig. 7B), and GPV (Fig. 7C) expression, as indicated by surface binding of antibody 6D1, antibody GRP and antibody SW16, respectively. The samples were incubated with either TR_{1-41} or thrombin for the indicated periods of time (up to 4 minutes), fixed, stained with the FITC conjugated antibodies and analyzed by flow cytometry. Binding is expressed as a percent of maximal binding without thrombin or TR_{1-41}. Thus a decrease in surface expression of the GPIb-IX-V complex is an additional marker of platelet activation.

Other Embodiments

TR_{1-41} peptides are also agonists for cells other than platelets. For example, TR_{1-41} peptides are chemotactic for monocytes and neutrophils. Other data indicate that GPIb and GPIIb-GPIIIa are not the binding site for the TR_{1-41} peptides.

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, that the foregoing description is

intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

What is claimed is:

1. An isolated TR_{1-41} peptide consisting of a sequence of at least 6 and up to 41 amino acids, wherein said sequence is substantially identical to at least 6 consecutive amino acids within the sequence:

MGPRRLLLVAACFSLCGPLLSARTRARRPESKATNATLDPR (SEQ ID NO:1),

and including at least a core region of amino acids substantially identical to amino acids 17 to 22 of SEQ ID NO:1.

2. A peptide of claim 1, wherein said peptide consists of a sequence of at least 10 amino acids, said sequence being substantially identical to at least 10 consecutive amino acids within the sequence with SEQ ID NO:1 and including at least a core region of amino acids substantially identical to amino acids 15 to 24 of SEQ ID NO:1.

3. A peptide of claim 1, wherein said peptide consists of a sequence of at least 15 amino acids, said sequence being substantially identical to at least 15 consecutive amino acids within the sequence with SEQ ID NO:1 and including at least a core region of amino acids substantially identical to amino acids 12 to 26 of SEQ ID NO:1.

4. A peptide of claim 1, wherein said peptide consists of a sequence of at least 21 amino acids, said sequence being substantially identical to at least 21 consecutive amino acids within the sequence with SEQ ID NO:1 and including at least a core region of amino acids

substantially identical to amino acids 10 to 30 of SEQ ID NO:1.

5. A peptide of claim 1, wherein said peptide has a length of 41 amino acids, and has a sequence substantially identical to the sequence of SEQ ID NO:1.

6. A peptide of claim 1, wherein said peptide has the sequence of SEQ ID NO:1.

7. A peptide of claim 1, wherein said peptide has a length of 31 amino acids, and has a sequence substantially identical to the sequence of amino acids 5 to 35 of SEQ ID NO:1.

8. A peptide of claim 1, wherein said peptide has a length of 31 amino acids, and the sequence of amino acids 5 to 35 of SEQ ID NO:1.

9. A peptide of claim 1, wherein said peptide has a length of 21 amino acids, and has a sequence substantially identical to the sequence of amino acids 10 to 30 of SEQ ID NO:1.

10. A peptide of claim 1, wherein said peptide has a length of 21 amino acids, and the sequence of amino acids 10 to 30 of SEQ ID NO:1.

11. A method of activating platelets, the method comprising providing a TR_{1-41} peptide of claim 1, and exposing the platelets to an amount of the peptide effective to achieve platelet activation.

12. A method of claim 11, wherein the method is carried out *in vitro*.

13. A method of claim 11, wherein the method is carried out *ex vivo*.

14. A method of claim 11, wherein the method is carried out *in vivo* and induces thrombosis.

15. A method of claim 11, further comprising the step of exposing the platelets to peptide TR_{42-55} in an amount sufficient to achieve a synergistic platelet activation effect together with the TR_{1-41} peptide.

16. A method of assaying platelet reactivity, the method comprising

obtaining a biological sample containing platelets;

incubating the sample with a TR_{1-41} peptide of claim 1; and

detecting platelet activation as a measure of platelet reactivity.

17. A method of claim 16, wherein platelet activation is detected by labeling the sample with an activation-dependent label, and detecting any labeled platelets, wherein the presence of labeled platelets indicates the reactivity of the platelets in the sample.

18. A method of claim 17, wherein the activation-dependent label is fluorescein isothiocyanate, fibrinogen, or an antibody selected from the group consisting of PAC1, F26, S12, 7E3, 6D1, SW16, ligand induced binding site-specific antibody, and receptor induced binding site-specific antibody.

19. A method of claim 16, wherein platelet activation is detected by labeling the sample with an

activation-dependent label, detecting any labeled platelets, and comparing the percentage of labeling with control values of known activated platelets as an indication of the degree of reactivity of the platelets in the sample.

20. A method of claim 16, wherein platelet activation is detected by measuring platelet aggregation.

21. A method of claim 16, wherein platelet activation is detected by measuring a change in calcium mobilization in the platelets.

22. A method of claim 16, wherein the TR_{1-41} peptide is added in an amount effective to activate normal platelets.

23. A method of claim 16, wherein said TR_{1-41} peptide is added to separate samples in a range of different concentrations.

24. A method of claim 16, wherein the platelets are considered activated when the percentage of labeling compared to a non-activated platelet control value is greater than two standard deviations from the control value.

25. A method of claim 16, wherein the sample is from a patient having angina, coronary artery disease, unstable angina, myocardial infarction, congestive heart failure, cardiomyopathy, ventricular and atrial arrhythmias, or a stroke.

26. A method of claim 16, further comprising incubating the sample with a TR_{42-55} peptide.

27. An isolated antagonist of a TR_{1-41} peptide, the antagonist comprising a compound that inhibits binding of a TR_{1-41} peptide to platelets.

28. An antagonist of claim 27, wherein the antagonist is an antibody that binds specifically to a TR_{1-41} peptide.

29. An antagonist of claim 27 for use in inhibiting TR_{1-41} peptide-induced platelet activation in a patient, wherein the platelets are exposed to an amount of an antagonist effective to prevent TR_{1-41} peptide-induced platelet activation.

30. A method of inhibiting TR_{1-41} peptide-induced platelet activation in a patient, the method comprising exposing the platelets of the patient to an amount of an antagonist of claim 27 effective to prevent TR_{1-41} peptide-induced platelet activation in the patient.

31. A method of claim 30, wherein the patient has undergone surgery or angioplasty, or has had deep venous thrombosis, venous stasis, a pulmonary embolism, angina, coronary artery disease, unstable angina, myocardial infarction, congestive heart failure, cardiomyopathy, ventricular or atrial arrhythmias, or a stroke.

32. A method of claim 30, wherein the platelets are exposed to the antagonist by administration of the antagonist to the patient.

33. A method of claim 30, wherein the platelets are exposed to the antagonist by administration to the patient of a nucleic acid encoding the antagonist.

34. A nucleic acid comprising a nucleotide sequence that encodes the TR_{1-41} peptide for use in activating platelets in a patient, wherein the platelets are exposed to an amount of the peptide effective to achieve platelet activation.

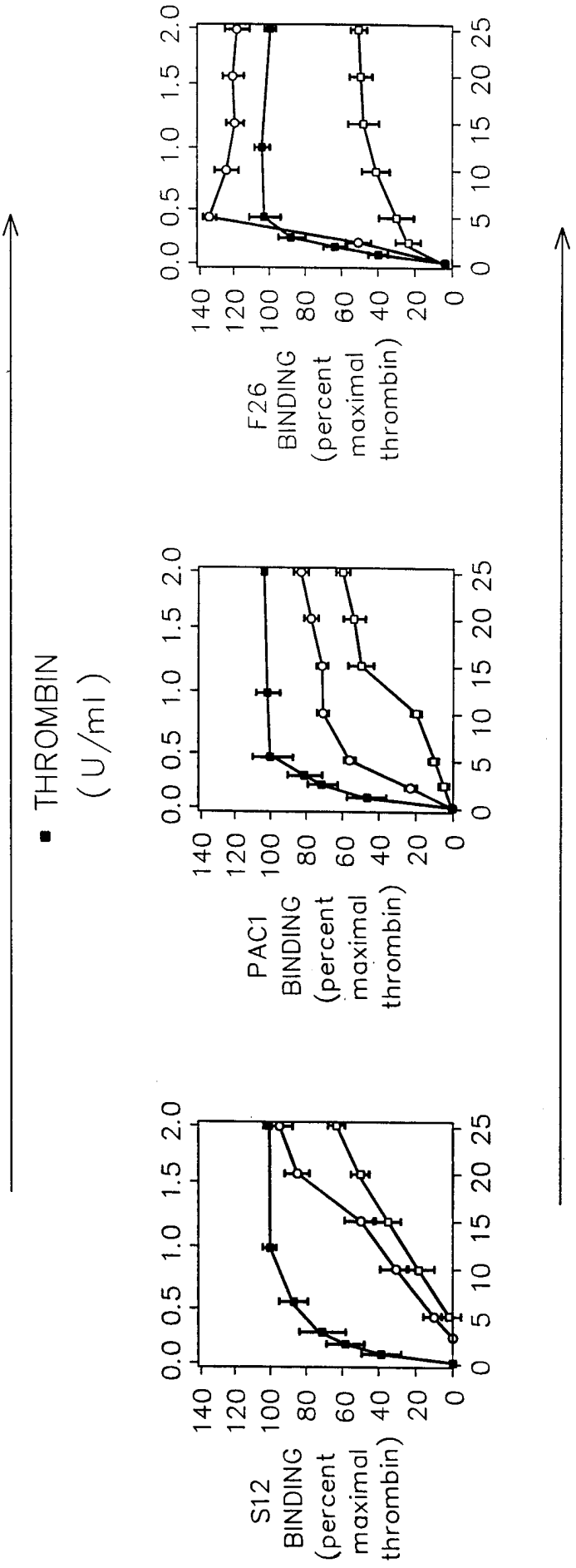

FIG. 1A FIG. 1B FIG. 1C

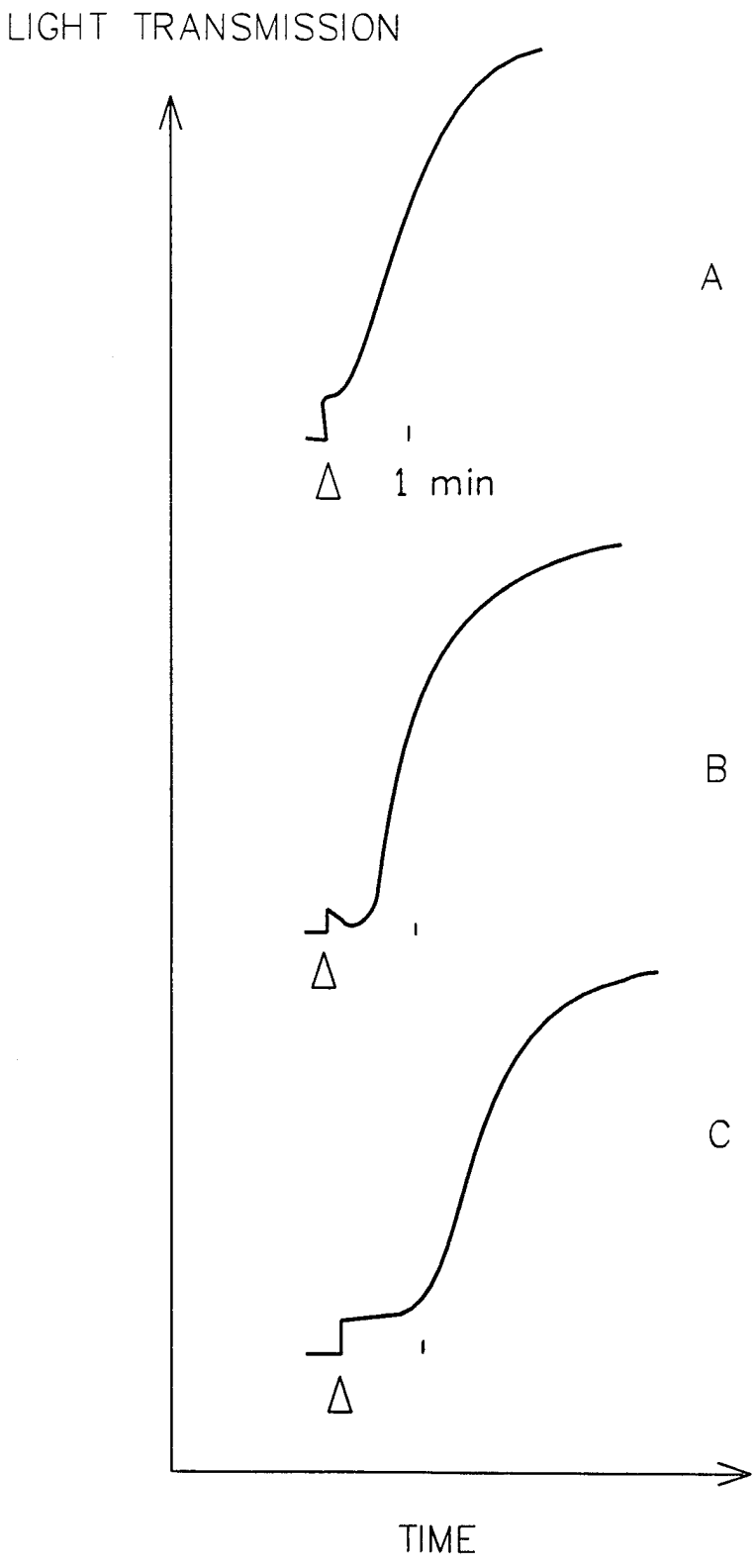

FIG. 2A

FIG. 2B

FIG. 2C

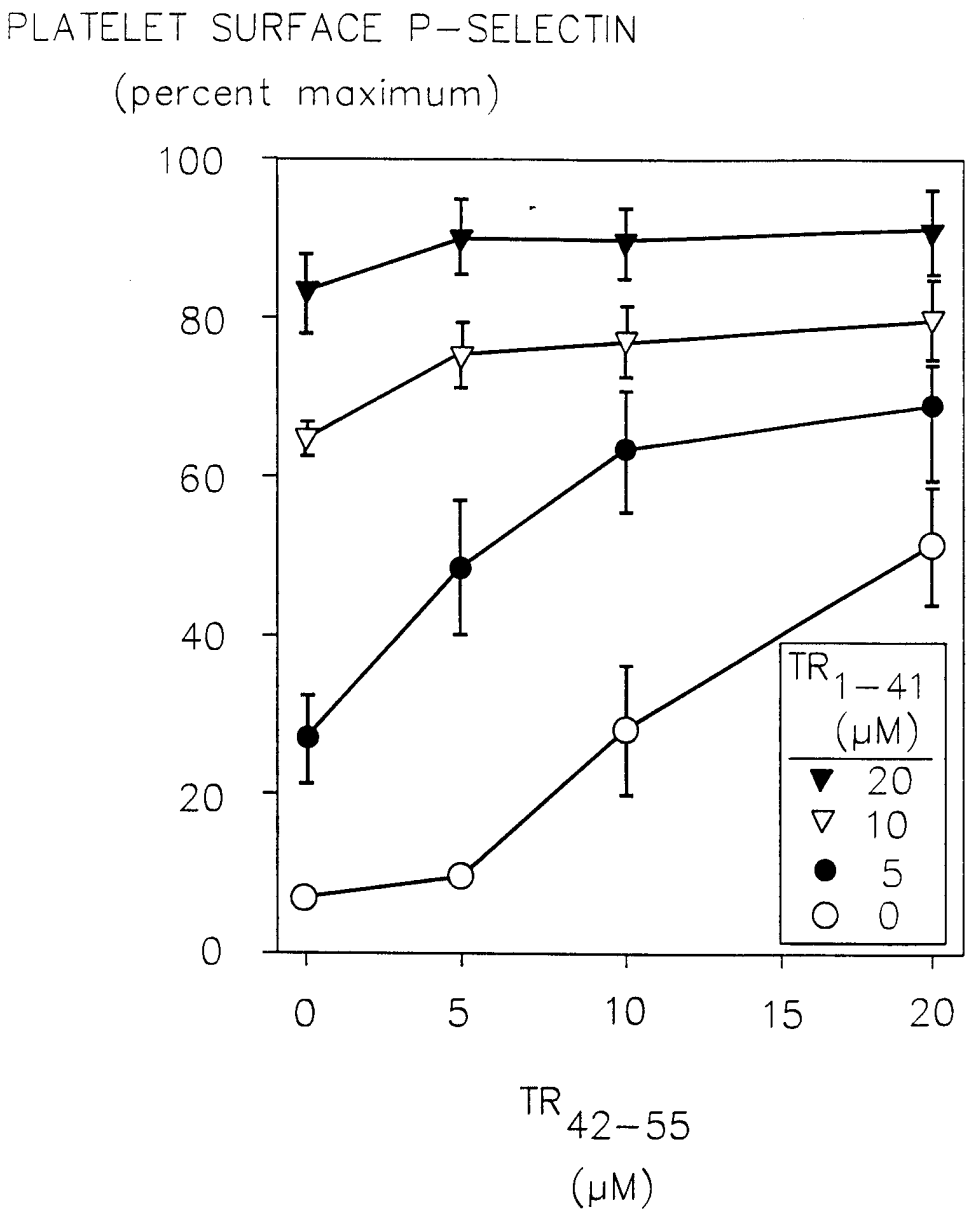
PLATELET SURFACE P-SELECTIN
(percent maximum)
100
80
60
40
20
0
0
5
10
15
20
TR1-41 (μM)
20
10
5
0
TR42-55
(μM)

FIG. 3

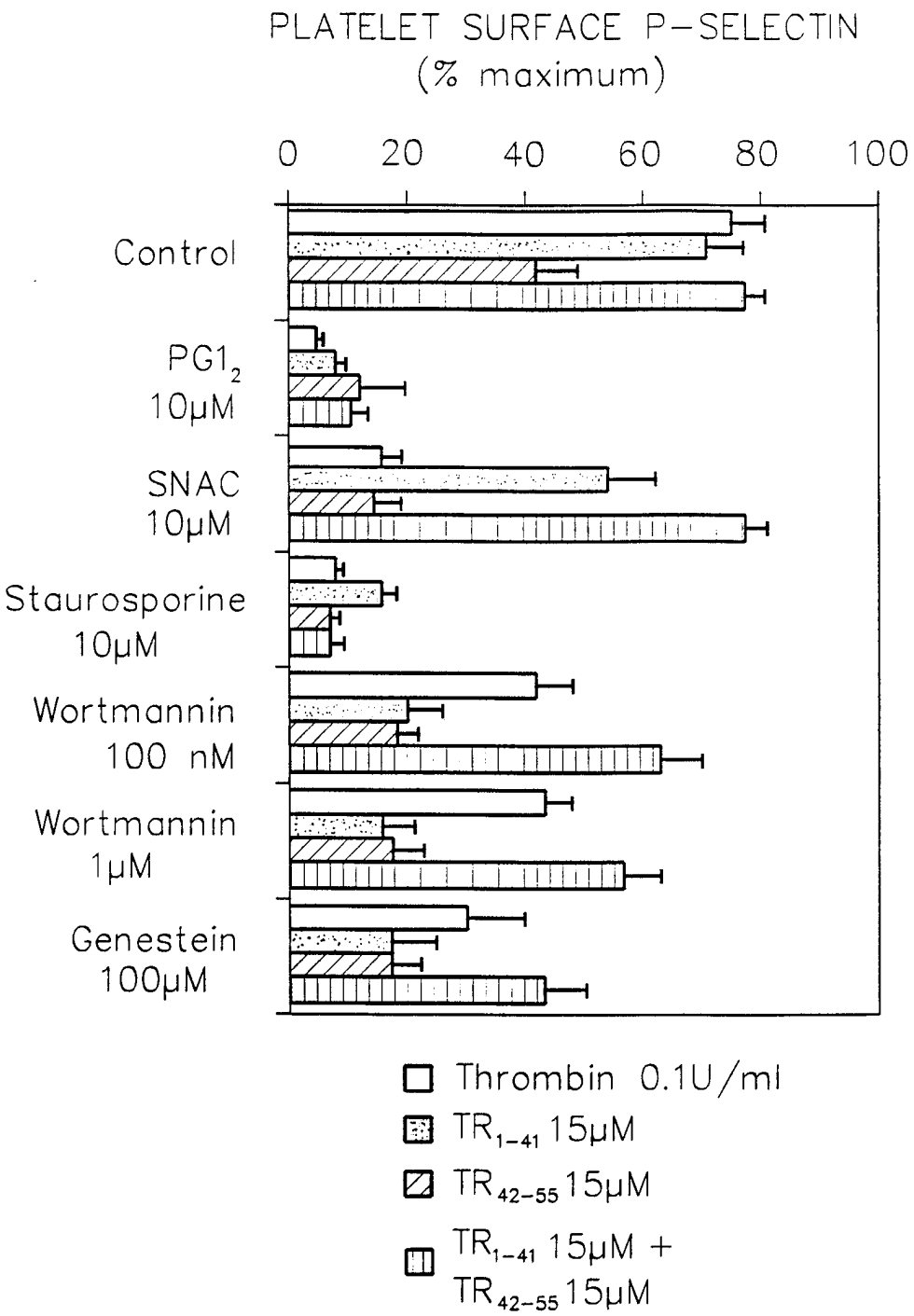
PLATELET SURFACE P-SELECTIN
(% maximum)
0
20
40
60
80
100
Control
PG1$_2$ 10μM
SNAC 10μM
Staurosporine 10μM
Wortmannin 100 nM
Wortmannin 1μM
Genestein 100μM
Thrombin 0.1U/ml
TR$_{1-41}$ 15μM
TR$_{42-55}$ 15μM
TR$_{1-41}$ 15μM + TR$_{42-55}$ 15μM

FIG. 4

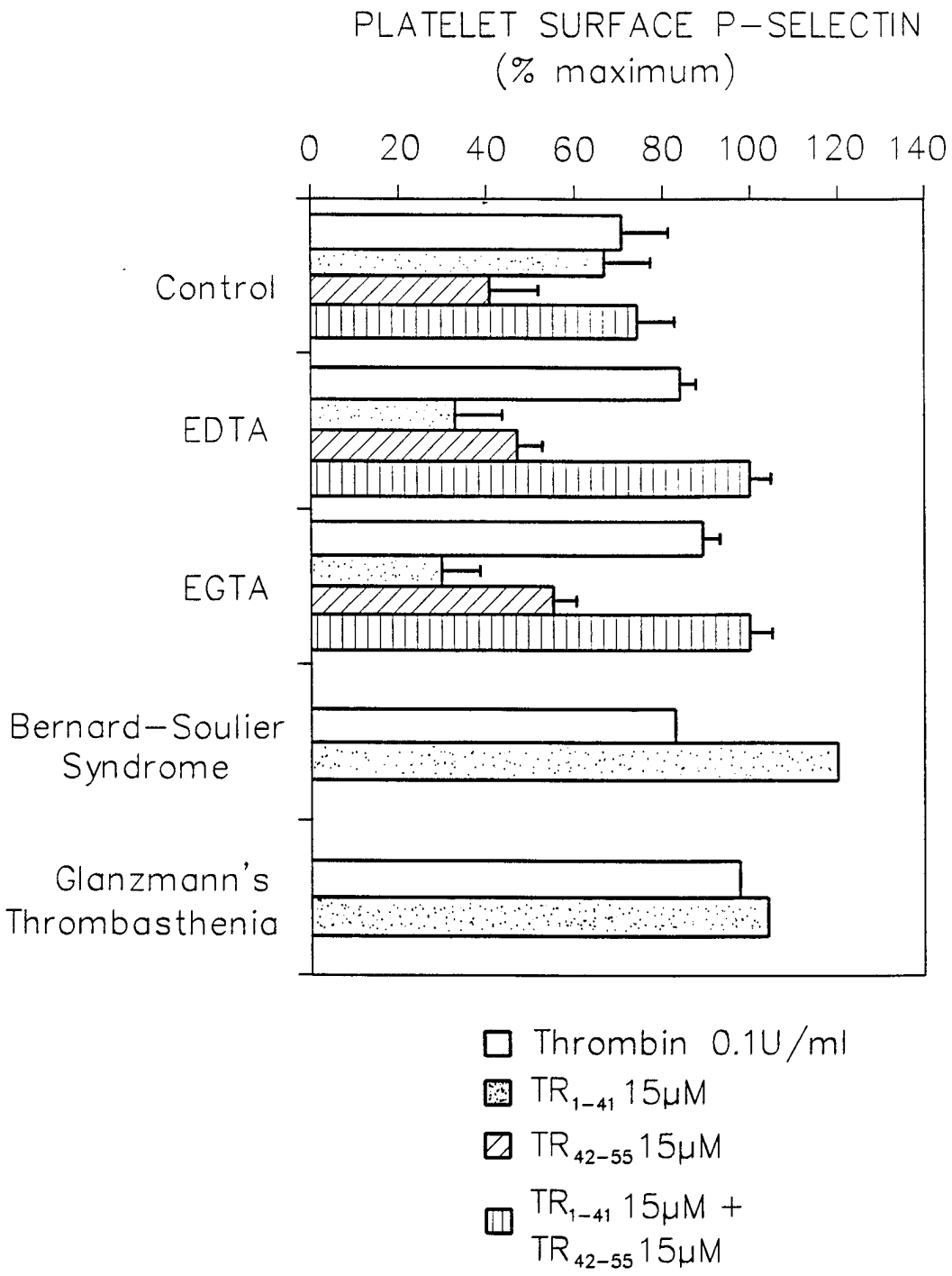
PLATELET SURFACE P-SELECTIN
(% maximum)
0
20
40
60
80
100
120
140
Control
EDTA
EGTA
Bernard-Soulier Syndrome
Glanzmann's Thrombasthenia
Thrombin 0.1U/ml
TR1-41 15μM
TR42-55 15μM
TR1-41 15μM + TR42-55 15μM

FIG. 5

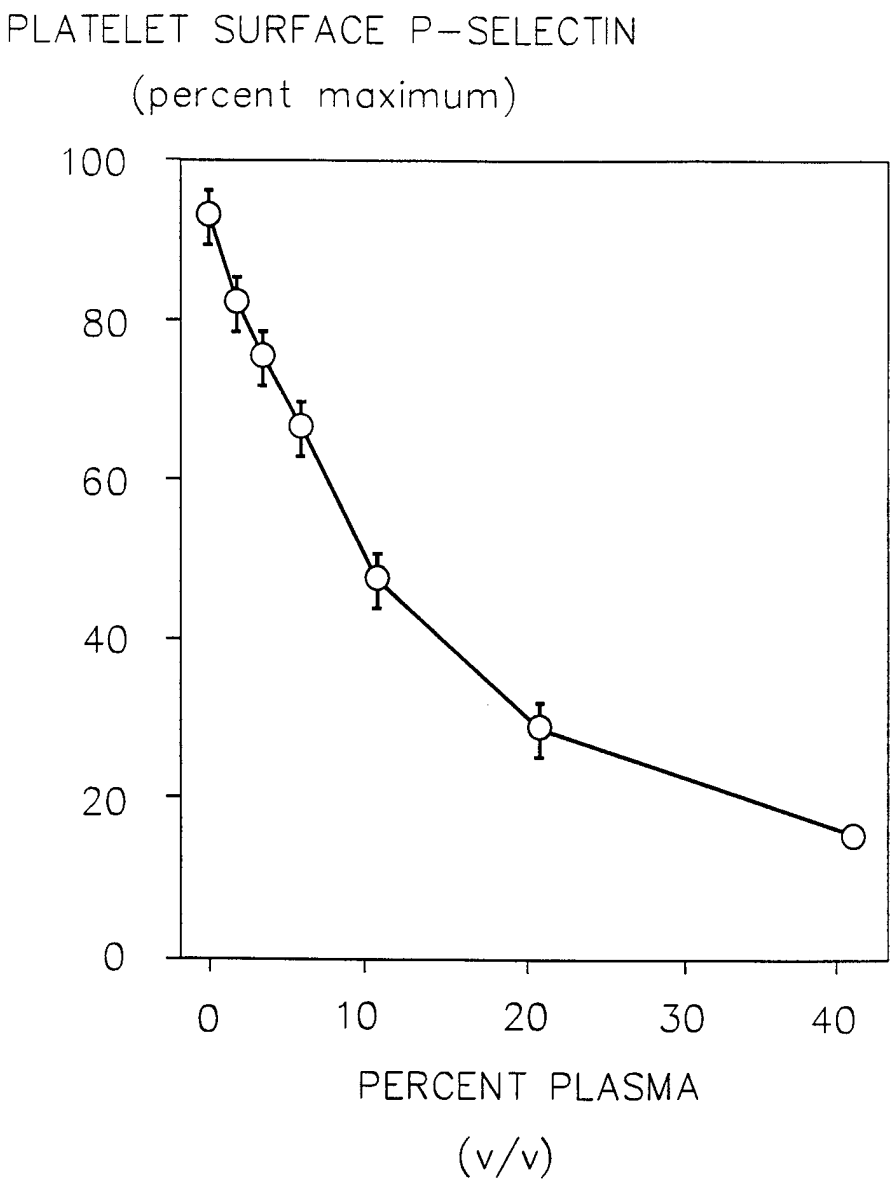
PLATELET SURFACE P-SELECTIN
(percent maximum)
100
80
60
40
20
0
0
10
20
30
40
PERCENT PLASMA
(v/v)

FIG. 6

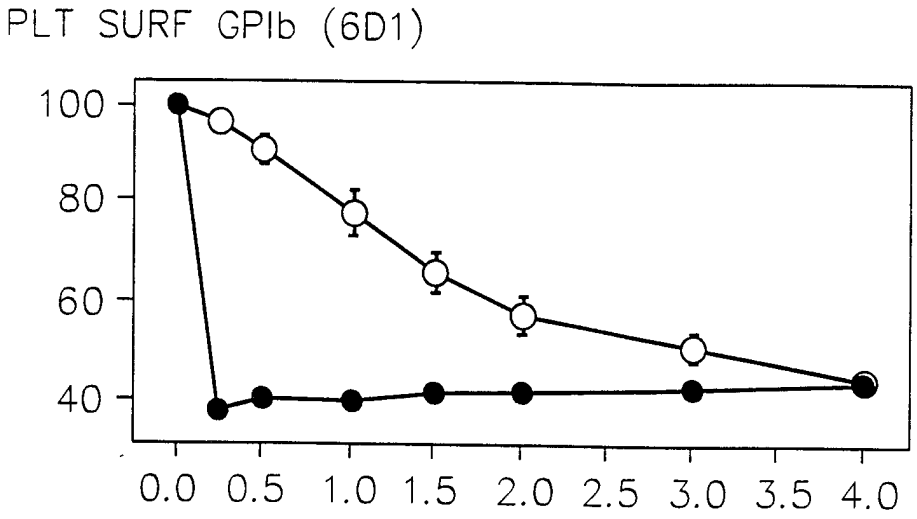

FIG. 7A

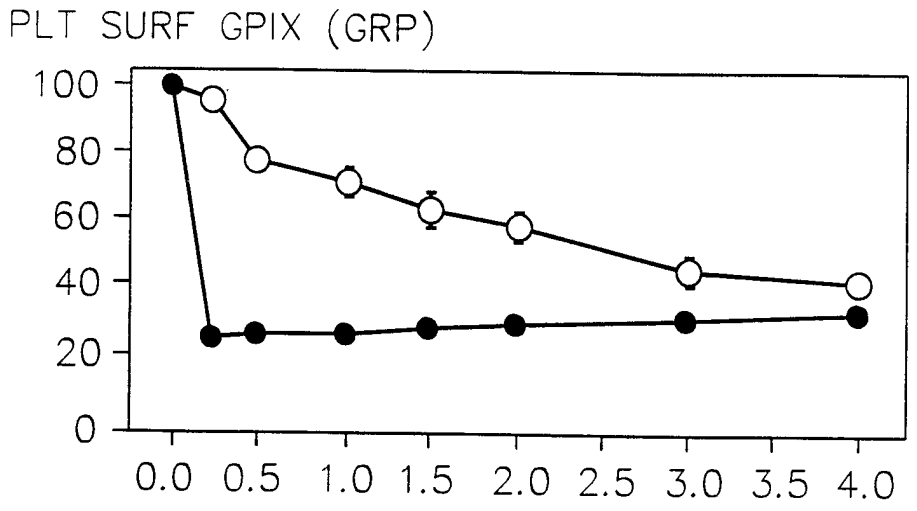

FIG. 7B

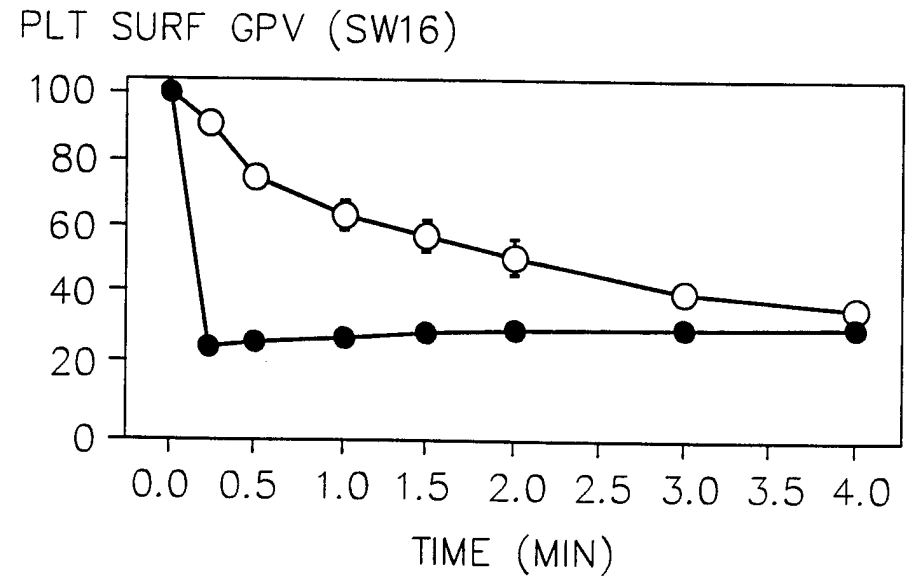

FIG. 7C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/18742

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :530/329, 328, 327, 326, 324; 536/23.5; 514/2, 12, 13, 14, 15, 17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/329, 328, 327, 326, 324; 536/23.5; 514/2, 12, 13, 14, 15, 17

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, CAPLUS, WPIDS
search terms: thrombin, receptor, peptide, platelet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,256,766 A (COUGHLIN) 26 October 1993, Figure 1A and column 4, lines 33-37.	1-6, 34 --- 7-10
X --- Y	VU, T.-K. H. et al. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell. 22 March 1991. Vol. 64, pages 1057-1068, especially Figure 5 and page 1064, column 2, last paragraph.	1- 6 , 34 --- 7-10

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 17 DECEMBER 1997	Date of mailing of the international search report 30 JAN 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ELIANE LAZAR-WESLEY Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/18742

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-10 and 34

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/18742

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07K 7/06, 7/08, 14/00, 14/435, 14/705; C12N 15/12; A61K 38/08, 38/10, 38/17

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claims 1-10 and 34, drawn to an isolated thrombin-receptor peptide (TR 1-41)and a nucleic acid comprising a nucleotide sequence that encodes the peptide.
Group II, claims 11-15, drawn to a method of activating platelets.
Group III, claims 16-26, drawn to a method of assaying platelet reactivity.
Group IV, claims 27-29, drawn to an antagonist of a TR 1-41 peptide.
Group V, claims 30-33, drawn to a method of inhibiting TR 1-41 peptide-induced platelet activation.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Group I is not novel, as evidenced by US 5,256,766 A (COUGHLIN) 26 October 1993, and therefore does not constitute a special technical feature which links the invention of Group I with any other group.