

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
27 April 2006 (27.04.2006)

PCT

(10) International Publication Number
WO 2006/044205 A2

(51) International Patent Classification:
A61K 31/553 (2006.01) *A61K 31/554* (2006.01)

(21) International Application Number:
PCT/US2005/035803

(22) International Filing Date: 4 October 2005 (04.10.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/615,970 4 October 2004 (04.10.2004) US
60/684,484 24 May 2005 (24.05.2005) US
60/718,907 19 September 2005 (19.09.2005) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHODS AND COMPOSITIONS FOR NEEDLELESS DELIVERY OF MACROMOLECULES

(57) Abstract: Methods and compositions for needleless delivery of macromolecules to the bloodstream of a subject are provided herein. In one aspect, the invention provides a delivery construct, comprising a receptor binding domain, a transcytosis domain, a macromolecule to be delivered to a subject, and a cleavable linker. Generally, the cleavable linker is cleavable by an enzyme present in higher concentration at or near the basal-lateral membrane of a polarized epithelial cell or in the plasma than elsewhere in the body, for example, at the apical side of the polarized epithelial cell. In other aspects, the invention provides nucleic acids encoding delivery constructs of the invention, kits comprising delivery constructs of the invention, cells expressing delivery constructs of the invention, and methods of using delivery constructs of the invention.



WO 2006/044205 A2

METHODS AND COMPOSITIONS FOR NEEDLELESS DELIVERY OF MACROMOLECULES

[0001] This application is entitled to and claims benefit of U.S. Provisional Application No. 60/615,970, filed October 4, 2004, of U.S. Provisional Application No. 60/684,484, filed May 24, 2005, and of U.S. Provisional Application No. 60/718,907, filed September 19, 2005, each of which is hereby incorporated by reference in its entirety.

1. FIELD OF THE INVENTION

[0002] The present invention relates, in part, to methods and compositions for needleless delivery of macromolecules to a subject. In one aspect, the methods and compositions involve administering to the subject a delivery construct comprising the macromolecule to be delivered, wherein the macromolecule is linked to the remainder of the construct with a linker that is cleavable at a basal-lateral membrane of a polarized epithelial cell.

2. BACKGROUND

[0003] Advances in biochemistry and molecular biology have resulted identification and characterization of many therapeutic macromolecules, including, for example, growth hormone, erythropoietin, insulin, IGF, and the like. Administration of these molecules can result in drastic improvements in quality of life for subjects afflict with a wide range of ailments.

[0004] However, administration of these therapeutic macromolecules remains problematic. Currently, therapeutic macromolecules are typically administered by injection. Such injections require penetration of the subject's skin and tissues and are associated with pain. Further, penetration of the skin breaches one effective nonspecific mechanism of protection against infection, and thus can lead to potentially serious infection.

[0005] Previous attempts have been made for needleless delivery of macromolecules to subjects. *See, e.g.*, International Patent Publication No. WO 01/30,392. In these efforts, delivery vehicles are used to deliver macromolecules to a subject through polarized epithelial cells. However, these derivatives lack a cleavable linker that is cleavable by an enzyme at the basal-lateral membrane of a polarized epithelial cell. Without cleavage, the probability of induction of an immune response against the delivery vehicle and/or the macromolecule to be delivered is increased. Also, steric hindrance between the delivery vehicle and the

macromolecule can reduce the activity of the macromolecule, reducing efficacy of the treatment.

[0006] Accordingly, there is an unmet need for new methods and compositions that can be used to administer macromolecules to subjects without breaching the skin of the subject. This and other needs are met by the methods and compositions of the present invention.

3. SUMMARY OF THE INVENTION

[0007] The delivery constructs of the invention comprise a macromolecule for delivery to a subject that is linked to the remainder of the construct with a cleavable linker. The linker is cleavable by an enzyme or an environmental cue that is present at the basal-lateral membrane of an epithelial cell.

[0008] Accordingly, in certain aspects, the invention provides a delivery construct comprising a receptor binding domain, a transcytosis domain, a macromolecule to be delivered to a subject, and a cleavable linker. Cleavage at the cleavable linker can separate the macromolecule from the remainder of the delivery construct. In certain embodiments, the cleavable linker can be cleavable by an enzyme that is present at a basal-lateral membrane of a polarized epithelial cell of the subject. In other embodiments, the cleavable linker can be cleavable by an enzyme that is present in the plasma of said subject.

[0009] In another aspect, the invention provides a polynucleotide that encodes a delivery construct comprising a receptor binding domain, a transcytosis domain, a macromolecule to be delivered to a subject, and a cleavable linker. Cleavage at the cleavable linker can separate the macromolecule from the remainder of the delivery construct. In certain embodiments, the cleavable linker can be cleavable by an enzyme that is present at a basal-lateral membrane of a polarized epithelial cell of the subject. In other embodiments, the cleavable linker can be cleavable by an enzyme that is present in the plasma of said subject.

[0010] In other embodiments, the polynucleotide that encodes a delivery construct comprises a nucleic acid sequence encoding a receptor binding domain, a nucleic acid sequence encoding a transcytosis domain, a nucleic acid sequence encoding a cleavable linker, and a nucleic acid sequence comprising a polylinker insertion site. The polylinker insertion site can be oriented relative to the nucleic acid sequence encoding a cleavable linker to allow to cleavage of the cleavable linker to separate a macromolecule that is encoded by a nucleic acid inserted into the polylinker insertion site from the remainder of the encoded delivery

construct. In certain embodiments, the cleavable linker can be cleavable by an enzyme that is present at a basal-lateral membrane of a polarized epithelial cell of the subject. In other embodiments, the cleavable linker can be cleavable by an enzyme that is present in the plasma of said subject.

[0011] In another aspect, the invention provides an expression vector comprising a polynucleotide of the invention.

[0012] In still another aspect, the invention provides a cell comprising an expression vector of the invention.

[0013] In yet another aspect, the invention provides a composition comprising a delivery construct of the invention. In certain embodiments, the composition is a pharmaceutical composition.

[0014] In still another aspect, the invention provides a method for delivering a macromolecule to a subject. The method comprises contacting an apical surface of a polarized epithelial cell of the subject with a delivery construct of the invention. The delivery construct can comprise a receptor binding domain, a transcytosis domain, a cleavable linker, and the macromolecule to be delivered. The transcytosis domain can transcytose the macromolecule to and through the basal-lateral membrane of the epithelial cell. In certain embodiments, the cleavable linker can be cleaved by an enzyme that is present at a basal-lateral membrane of a polarized epithelial cell of the subject. In other embodiments, the cleavable linker can be cleavable by an enzyme that is present in the plasma of the subject. Cleavage at the cleavable linker can separate the macromolecule from the remainder of the delivery construct, and can deliver the macromolecule to the subject free from the remainder of the construct.

4. BRIEF DESCRIPTION OF THE DRAWINGS

[0015] Figures 1A-1D present micrographs showing adhesion to and transport across mouse tracheal epithelium of an exemplary delivery construct comprising green fluorescent protein (GFP) (Panels A-C), while GFP alone does not adhere to the epithelial cells (Panel D).

[0016] Figure 2 presents a time course of serum nt-PE-GFP concentrations following intranasal administration of 100 µg nt-PE-GFP to anesthetized mice.

[0017] Figure 3 presents the amino acid sequence of an exemplary PE.

[0018] Figure 4 presents a western blot showing transport and cleavage of an exemplary delivery construct comprising rat growth hormone (rGH) by human intestinal epithelial cell monolayers. The delivery construct was incubated in contact with the apical side of the epithelial cell monolayer for 4 hours. Media isolated from the basolateral side of the membrane post-incubation (lane 1) contained rGH of native apparent molecular weight, while media from the apical side of the membrane (lane 2) contained intact delivery construct. Lanes 3 and 4 show intact Delivery Construct 2 and recombinant rGH, respectively.

[0019] Figure 5 presents serum rGH concentrations in BALB/c mice which were dosed by subcutaneous (SC) injection of 30 µg of non-glycosylated recombinant rat GH. Individual mice sera were tested at a dilution of 1:10, and the group average of rGH concentration were reported (n=4 mice per time point). Standard error of the mean (SEM) was indicated by the error bars.

[0020] Figure 6 presents serum rGH concentrations in BALB/c mice which were dosed orally with 100 µg of Delivery Construct 2. Individual mice sera were tested at a dilution of 1:10, and the group average of rGH concentration were reported (n=4 mice per time point). Standard error of the mean (SEM) was indicated by the error bars.

[0021] Figure 7 presents a graphical representation comparing pharmacokinetics of rGH delivered subcutaneously and with Delivery Construct 2.

[0022] Figure 8 shows expression levels of IGF-I-BP3 mRNA in the liver of female BALB/c mice treated with 30 µg recombinant rGH by subcutaneous injection or with 100 µg of Delivery Construct 2 by oral gavage. Total RNA extracted from the liver was subjected to quantitative RT-PCR using primers specific for IGF-I-BP3, as described above. Values were normalized to glyceraldehyde-3 phosphate dehydrogenase (GAPDH) and expressed as % of control.

[0023] Figure 9 shows expression levels of growth hormone (GH) receptor mRNA in the liver of female BALB/c mice treated with rGH by subcutaneous injection (30 µg) or Delivery Construct 2 by oral gavage (100 µg). Total RNA extracted from the liver was subjected to quantitative RT-PCR using primers specific for GH receptor, shown above. Values were normalized to glyceraldehyde-3 phosphate dehydrogenase (GAPDH) and expressed as % of control.

[0024] Figure 10 shows expression levels of insulin-like growth factor I (IGF-I) mRNA in the liver of female BALB/c mice treated with rGH by subcutaneous injection (30 µg) or Delivery Construct 2 by oral gavage (100 µg). Total RNA extracted from the liver was subjected to quantitative RT-PCR using primers specific for IGF-I, shown above. Values were normalized to glyceraldehyde-3 phosphate dehydrogenase (GAPDH) and expressed as % of control.

[0025] Figures 11A and B show serum anti-rGH IgG antibodies (diluted 1:25 in Figure 11A and 1:200 in Figure 11B) of mice orally administered 3, 10, or 30 µg Delivery Construct 2 (F2) or subcutaneously administered 3 or 10 µg rGH in graphs with error bars, while Figures 11C and D presents the same data from individual animals.

[0026] Figure 12 shows a nucleotide sequence that encodes Delivery Construct 6 (SEQ ID NO:34), an exemplary Delivery Construct for delivering human growth hormone (hGH).

[0027] Figures 13A and B show the amino acid sequence of Delivery Construct 6 (SEQ ID NO:35), an exemplary Delivery Construct for delivering hGH.

[0028] Figure 14 shows a nucleotide sequence that encodes Delivery Construct 7 (SEQ ID NO:36), an exemplary Delivery Construct for delivering interferon- α (IFN- α).

[0029] Figures 15A and B show the amino acid sequence of Delivery Construct 7 (SEQ ID NO:37), an exemplary Delivery Construct for delivering IFN- α .

[0030] Figure 16 shows 6 presents serum IFN- α concentrations in BALB/c mice which were dosed orally with 100 µg of Delivery Construct 8. Individual mice sera were tested at a dilution of 1:10, and the group average of IFN- α concentration were reported (n=3 mice per time point).

[0031] Figures 17A and B show the amino acid sequence of Delivery Construct 8 (SEQ ID NO:38), an exemplary Delivery Construct for delivering proinsulin.

[0032] Figures 18 A and B shows the amino acid sequence of the two amino acid chains of Delivery Construct 9 (SEQ ID NOs:39 and 40, respectively), an exemplary Delivery Construct for delivering insulin. Disulfide bonds are formed between the cysteine at position 7 of SEQ ID NO:40 (shown in Figure 18B) and the cysteine at position 381 of SEQ ID

NO:39 (shown in Figure 18A) and between the cysteine at position 20 of SEQ ID NO:40 and the cysteine at position 393 of SEQ ID NO:39.

5. DETAILED DESCRIPTION OF THE INVENTION

5.1. DEFINITIONS

[0033] Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

[0034] A "ligand" is a compound that specifically binds to a target molecule. Exemplary ligands include, but are not limited to, an antibody, a cytokine, a substrate, a signaling molecule, and the like.

[0035] A "receptor" is compound that specifically binds to a ligand.

[0036] A ligand or a receptor (*e.g.*, an antibody) "specifically binds to" or "is specifically immunoreactive with" another molecule when the ligand or receptor functions in a binding reaction that indicates the presence of the molecule in a sample of heterogeneous compounds. Thus, under designated assay (*e.g.*, immunoassay) conditions, the ligand or receptor binds preferentially to a particular compound and does not bind in a significant amount to other compounds present in the sample. For example, a polynucleotide specifically binds under hybridization conditions to another polynucleotide comprising a complementary sequence and an antibody specifically binds under immunoassay conditions to an antigen bearing an epitope used to induce the antibody.

[0037] "Immunoassay" refers to a method of detecting an analyte in a sample involving contacting the sample with an antibody that specifically binds to the analyte and detecting binding between the antibody and the analyte. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity. In one example, an antibody that binds a particular antigen with an affinity (K_m) of about 10 μ M specifically binds the antigen.

[0058] "Linker" refers to a molecule that joins two other molecules, either covalently, or through ionic, van der Waals or hydrogen bonds, *e.g.*, a nucleic acid molecule that hybridizes to one complementary sequence at the 5' end and to another complementary sequence at the 3' end, thus joining two non-complementary sequences. A "cleavable linker" refers to a linker that can be degraded or otherwise severed to separate the two components connected by the cleavable linker. Cleavable linkers are generally cleaved by enzymes, typically peptidases, proteases, nucleases, lipases, and the like. Cleavable linkers may also be cleaved by environmental cues, such as, for example, changes in temperature, pH, salt concentration, *etc.* when there is such a change in environment following transcytosis of the delivery construct across a polarized epithelial membrane.

[0039] "Pharmaceutical composition" refers to a composition suitable for pharmaceutical use in an animal. A pharmaceutical composition comprises a pharmacologically effective amount of an active agent and a pharmaceutically acceptable carrier. "Pharmacologically effective amount" refers to that amount of an agent effective to produce the intended pharmacological result. "Pharmaceutically acceptable carrier" refers to any of the standard pharmaceutical carriers, vehicles, buffers, and excipients, such as a phosphate buffered saline solution, 5% aqueous solution of dextrose, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents and/or adjuvants. Suitable pharmaceutical carriers and formulations are described in *Remington's Pharmaceutical Sciences, 21st Ed.* 2005, Mack Publishing Co., Easton. A "pharmaceutically acceptable salt" is a salt that can be formulated into a compound for pharmaceutical use including, *e.g.*, metal salts (sodium, potassium, magnesium, calcium, *etc.*) and salts of ammonia or organic amines.

[0040] Preferred pharmaceutical carriers depend upon the intended mode of administration of the active agent. Typical modes of administration include enteral (*e.g.*, oral, intranasal, rectal, or vaginal) or parenteral (*e.g.*, subcutaneous, intramuscular, intravenous or intraperitoneal injection; or topical (*e.g.*, transdermal, or transmucosal administration).

[0041] "Small organic molecule" refers to organic molecules of a size comparable to those organic molecules generally used in pharmaceuticals. The term excludes organic biopolymers (*e.g.*, proteins, nucleic acids, *etc.*). Preferred small organic molecules range in size up to about 5000 Da, up to about 2000 Da, or up to about 1000 Da.

[0042] A "subject" of diagnosis, treatment, or administration is a human or non-human animal, including a mammal or a primate, and preferably a human.

[0043] "Treatment" refers to prophylactic treatment or therapeutic treatment. A "prophylactic" treatment is a treatment administered to a subject who does not exhibit signs of a disease or exhibits only early signs for the purpose of decreasing the risk of developing pathology. A "therapeutic" treatment is a treatment administered to a subject who exhibits signs of pathology for the purpose of diminishing or eliminating those signs.

[0044] "Pseudomonas exotoxin A" or "PE" is secreted by *Pseudomonas aeruginosa* as a 67 kD protein composed of three prominent globular domains (Ia, II, and III) and one small subdomain (Ib) that connects domains II and III. See A.S. Allured *et al.*, 1986, *Proc. Natl. Acad. Sci.* 83:1320-1324. Without intending to be bound to any particular theory or mechanism of action, domain Ia of PE is believed to mediate cell binding because domain Ia specifically binds to the low density lipoprotein receptor-related protein ("LRP"), also known as the α 2-macroglobulin receptor (" α 2-MR") and CD-91. See M.Z. Kounnas *et al.*, 1992, *J. Biol. Chem.* 267:12420-23. Domain Ia spans amino acids 1-252. Domain II of PE is believed to mediate transcytosis to the interior of a cell following binding of domain Ia to the α 2-MR. Domain II spans amino acids 253-364. Certain portions of this domain may be required for secretion of PE from *Pseudomonas aeruginosa* after its synthesis. See, e.g., Vouloux *et al.*, 2000, *J. Bacteriol.* 182:4051-8. Domain Ib has no known function and spans amino acids 365-399. Domain III mediates cytotoxicity of PE and includes an endoplasmic reticulum retention sequence. PE cytotoxicity is believed to result from ADP ribosylation of elongation factor 2, which inactivates protein synthesis. Domain III spans amino acids 400-613 of PE. Deleting amino acid E553 (" Δ E553") from domain III eliminates EF2 ADP ribosylation activity and detoxifies PE. PE having the mutation Δ E553 is referred to herein as "PE Δ E553." Genetically modified forms of PE are described in, e.g., United States patent nos. 5,602,095; 5,512,658 and 5,458,878 *Pseudomonas exotoxin*, as used herein, also includes genetically modified, allelic, and chemically inactivated forms of PE within this definition. See, e.g., Vasil *et al.*, 1986, *Infect. Immunol.* 52:538-48. Further, reference to the various domains of PE is made herein to the reference PE sequence presented as Figure 3. However, one or more domain from modified PE, e.g., genetically or chemically modified PE, or a portion of such domains, can also be used in the chimeric immunogens of the invention so long as the domains retain functional activity. One of skill in the art can readily

identify such domains of such modified PE based on, for example, homology to the PE sequence exemplified in Figure 3 and test for functional activity using, for example, the assays described below.

[0045] "Polynucleotide" refers to a polymer composed of nucleotide units. Polynucleotides include naturally occurring nucleic acids, such as deoxyribonucleic acid ("DNA") and ribonucleic acid ("RNA") as well as nucleic acid analogs. Nucleic acid analogs include those which include non-naturally occurring bases, nucleotides that engage in linkages with other nucleotides other than the naturally occurring phosphodiester bond or which include bases attached through linkages other than phosphodiester bonds. Thus, nucleotide analogs include, for example and without limitation, phosphorothioates, phosphorodithioates, phosphorotriesters, phosphoramidates, boranophosphates, methylphosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs), and the like. Such polynucleotides can be synthesized, for example, using an automated DNA synthesizer. The term "nucleic acid" typically refers to large polynucleotides. The term "oligonucleotide" typically refers to short polynucleotides, generally no greater than about 50 nucleotides. It will be understood that when a nucleotide sequence is represented by a DNA sequence (*i.e.*, A, T, G, C), this also includes an RNA sequence (*i.e.*, A, U, G, C) in which "U" replaces "T."

[0046] Conventional notation is used herein to describe polynucleotide sequences: the left-hand end of a single-stranded polynucleotide sequence is the 5'-end; the left-hand direction of a double-stranded polynucleotide sequence is referred to as the 5'-direction.

[0047] The direction of 5' to 3' addition of nucleotides to nascent RNA transcripts is referred to as the transcription direction. The DNA strand having the same sequence as an mRNA is referred to as the "coding strand"; sequences on the DNA strand having the same sequence as an mRNA transcribed from that DNA and which are located 5' to the 5'-end of the RNA transcript are referred to as "upstream sequences"; sequences on the DNA strand having the same sequence as the RNA and which are 3' to the 3' end of the coding RNA transcript are referred to as "downstream sequences."

[0048] "Complementary" refers to the topological compatibility or matching together of interacting surfaces of two polynucleotides. Thus, the two molecules can be described as complementary, and furthermore, the contact surface characteristics are complementary to each other. . A first polynucleotide is complementary to a second polynucleotide if the

nucleotide sequence of the first polynucleotide is substantially identical to the nucleotide sequence of the polynucleotide binding partner of the second polynucleotide, or if the first polynucleotide can hybridize to the second polynucleotide under stringent hybridization conditions. Thus, the polynucleotide whose sequence 5'-TATAC-3' is complementary to a polynucleotide whose sequence is 5'-GTATA-3'.

[0049] The term “% sequence identity” is used interchangeably herein with the term “% identity” and refers to the level of amino acid sequence identity between two or more peptide sequences or the level of nucleotide sequence identity between two or more nucleotide sequences, when aligned using a sequence alignment program. For example, as used herein, 80% identity means the same thing as 80% sequence identity determined by a defined algorithm, and means that a given sequence is at least 80% identical to another length of another sequence. Exemplary levels of sequence identity include, but are not limited to, 60, 70, 80, 85, 90, 95, 98% or more sequence identity to a given sequence.

[0050] The term “% sequence homology” is used interchangeably herein with the term “% homology” and refers to the level of amino acid sequence homology between two or more peptide sequences or the level of nucleotide sequence homology between two or more nucleotide sequences, when aligned using a sequence alignment program. For example, as used herein, 80% homology means the same thing as 80% sequence homology determined by a defined algorithm, and accordingly a homologue of a given sequence has greater than 80% sequence homology over a length of the given sequence. Exemplary levels of sequence homology include, but are not limited to, 60, 70, 80, 85, 90, 95, 98% or more sequence homology to a given sequence.

[0051] Exemplary computer programs which can be used to determine identity between two sequences include, but are not limited to, the suite of BLAST programs, *e.g.*, BLASTN, BLASTX, and TBLASTX, BLASTP and TBLASTN, publicly available on the Internet at the NCBI website. *See also* Altschul *et al.*, 1990, *J. Mol. Biol.* 215:403-10 (with special reference to the published default setting, *i.e.*, parameters $w=4$, $t=17$) and Altschul *et al.*, 1997, *Nucleic Acids Res.*, 25:3389-3402. Sequence searches are typically carried out using the BLASTP program when evaluating a given amino acid sequence relative to amino acid sequences in the GenBank Protein Sequences and other public databases. The BLASTX program is preferred for searching nucleic acid sequences that have been translated in all reading frames against amino acid sequences in the GenBank Protein Sequences and other

public databases. Both BLASTP and BLASTX are run using default parameters of an open gap penalty of 11.0, and an extended gap penalty of 1.0, and utilize the BLOSUM-62 matrix. *See id.*

[0052] A preferred alignment of selected sequences in order to determine “% identity” between two or more sequences, is performed using for example, the CLUSTAL-W program in MacVector version 6.5, operated with default parameters, including an open gap penalty of 10.0, an extended gap penalty of 0.1, and a BLOSUM 30 similarity matrix.

[0053] “Polar Amino Acid” refers to a hydrophilic amino acid having a side chain that is uncharged at physiological pH, but which has at least one bond in which the pair of electrons shared in common by two atoms is held more closely by one of the atoms. Genetically encoded polar amino acids include Asn (N), Gln (Q) Ser (S) and Thr (T).

[0054] “Nonpolar Amino Acid” refers to a hydrophobic amino acid having a side chain that is uncharged at physiological pH and which has bonds in which the pair of electrons shared in common by two atoms is generally held equally by each of the two atoms (*i.e.*, the side chain is not polar). Genetically encoded nonpolar amino acids include Ala (A), Gly (G), Ile (I), Leu (L), Met (M) and Val (V) .

[0055] “Hydrophilic Amino Acid” refers to an amino acid exhibiting a hydrophobicity of less than zero according to the normalized consensus hydrophobicity scale of Eisenberg *et al.*, 1984, *J. Mol. Biol.* 179:125-142. Genetically encoded hydrophilic amino acids include Arg (R), Asn (N), Asp (D), Glu (E), Gln (Q), His (H), Lys (K), Ser (S) and Thr (T).

[0056] “Hydrophobic Amino Acid” refers to an amino acid exhibiting a hydrophobicity of greater than zero according to the normalized consensus hydrophobicity scale of Eisenberg *et al.*, 1984, *J. Mol. Biol.* 179:125-142. Genetically encoded hydrophobic amino acids include Ala (A), Gly (G), Ile (I), Leu (L), Met (M), Phe (F), Pro (P), Trp (W), Tyr (Y) and Val (V).

[0057] “Acidic Amino Acid” refers to a hydrophilic amino acid having a side chain pK value of less than 7. Acidic amino acids typically have negatively charged side chains at physiological pH due to loss of a hydrogen ion. Genetically encoded acidic amino acids include Asp (D) and Glu (E).

[0058] "Basic Amino Acid" refers to a hydrophilic amino acid having a side chain pK_a value of greater than 7. Basic amino acids typically have positively charged side chains at physiological pH due to association with a hydrogen ion. Genetically encoded basic amino acids include Arg (R), His (H) and Lys (K).

[0059] "Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (*i.e.*, rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA produced by that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and non-coding strand, used as the template for transcription, of a gene or cDNA can be referred to as encoding the protein or other product of that gene or cDNA. Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may include introns.

[0060] "Amplification" refers to any means by which a polynucleotide sequence is copied and thus expanded into a larger number of polynucleotide molecules, *e.g.*, by reverse transcription, polymerase chain reaction, ligase chain reaction, and the like.

[0061] "Primer" refers to a polynucleotide that is capable of specifically hybridizing to a designated polynucleotide template and providing a point of initiation for synthesis of a complementary polynucleotide. Such synthesis occurs when the polynucleotide primer is placed under conditions in which synthesis is induced, *i.e.*, in the presence of nucleotides, a complementary polynucleotide template, and an agent for polymerization such as DNA polymerase. A primer is typically single-stranded, but may be double-stranded. Primers are typically deoxyribonucleic acids, but a wide variety of synthetic and naturally occurring primers are useful for many applications. A primer is complementary to the template to which it is designed to hybridize to serve as a site for the initiation of synthesis, but need not reflect the exact sequence of the template. In such a case, specific hybridization of the primer to the template depends on the stringency of the hybridization conditions. Primers can be

labeled with, e.g., chromogenic, radioactive, or fluorescent moieties and used as detectable moieties.

[0062] "Probe," when used in reference to a polynucleotide, refers to a polynucleotide that is capable of specifically hybridizing to a designated sequence of another polynucleotide. A probe specifically hybridizes to a target complementary polynucleotide, but need not reflect the exact complementary sequence of the template. In such a case, specific hybridization of the probe to the target depends on the stringency of the hybridization conditions. Probes can be labeled with, e.g., chromogenic, radioactive, or fluorescent moieties and used as detectable moieties. In instances where a probe provides a point of initiation for synthesis of a complementary polynucleotide, a probe can also be a primer.

[0063] "Hybridizing specifically to" or "specific hybridization" or "selectively hybridize to", refers to the binding, duplexing, or hybridizing of a nucleic acid molecule preferentially to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (e.g., total cellular) DNA or RNA.

[0064] The term "stringent conditions" refers to conditions under which a probe will hybridize preferentially to its target subsequence, and to a lesser extent to, or not at all to, other sequences. "Stringent hybridization" and "stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments such as Southern and northern hybridizations are sequence dependent, and are different under different environmental parameters. An extensive guide to the hybridization of nucleic acids can be found in Tijssen, 1993, *Laboratory Techniques in Biochemistry and Molecular Biology - Hybridization with Nucleic Acid Probes*, part I, chapter 2, "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, NY; Sambrook *et al.*, 2001, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, 3rd ed., NY; and Ausubel *et al.*, eds., Current Edition, *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley Interscience, NY.

[0065] Generally, highly stringent hybridization and wash conditions are selected to be about 5° C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the T_m for a particular probe.

[0066] One example of stringent hybridization conditions for hybridization of complementary nucleic acids which have more than about 100 complementary residues on a filter in a Southern or northern blot is 50% formalin with 1 mg of heparin at 42° C, with the hybridization being carried out overnight. An example of highly stringent wash conditions is 0.15 M NaCl at 72° C for about 15 minutes. An example of stringent wash conditions is a 0.2X SSC wash at 65° C for 15 minutes. See Sambrook *et al.* for a description of SSC buffer. A high stringency wash can be preceded by a low stringency wash to remove background probe signal. An exemplary medium stringency wash for a duplex of, *e.g.*, more than about 100 nucleotides, is 1x SSC at 45° C for 15 minutes. An exemplary low stringency wash for a duplex of, *e.g.*, more than about 100 nucleotides, is 4-6x SSC at 40° C for 15 minutes. In general, a signal to noise ratio of 2x (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization.

[0067] "Polypeptide" refers to a polymer composed of amino acid residues, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof linked via peptide bonds, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof. Synthetic polypeptides can be synthesized, for example, using an automated polypeptide synthesizer. Conventional notation is used herein to portray polypeptide sequences; the beginning of a polypeptide sequence is the amino-terminus, while the end of a polypeptide sequence is the carboxyl-terminus.

[0068] The term "protein" typically refers to large polypeptides, for example, polypeptides comprising more than about 50 amino acids. The term "protein" can also refer to dimers, trimers, and multimers that comprise more than one polypeptide.

[0069] "Conservative substitution" refers to the substitution in a polypeptide of an amino acid with a functionally similar amino acid. The following six groups each contain amino acids that are conservative substitutions for one another:

Alanine (A), Serine (S), and Threonine (T)

Aspartic acid (D) and Glutamic acid (E)

Asparagine (N) and Glutamine (Q)

Arginine (R) and Lysine (K)

Isoleucine (I), Leucine (L), Methionine (M), and Valine (V)

Phenylalanine (F), Tyrosine (Y), and Tryptophan (W).

[0070] The term "about," as used herein, unless otherwise indicated, refers to a value that is no more than 10% above or below the value being modified by the term. For example, the term "about 5 µg/kg" means a range of from 4.5 µg/kg to 5.5 µg/kg. As another example, "about 1 hour" means a range of from 48 minutes to 72 minutes.

5.2. Delivery constructs

[0071] Generally, the delivery constructs of the present invention are polypeptides that have structural domains corresponding to domains Ia and II of PE. These structural domains perform certain functions, including, but not limited to, cell recognition and transcytosis, that correspond to the functions of the domains of PE.

[0072] In addition to the portions of the molecule that correspond to PE functional domains, the delivery constructs of this invention can further comprise a macromolecule for delivery to a biological compartment of a subject. The macromolecule can be introduced into any portion of the delivery construct that does not disrupt a cell-binding or transcytosis activity. The macromolecule is connected with the remainder of the delivery construct with a cleavable linker.

[0073] Accordingly, the delivery constructs of the invention generally comprise the following structural elements, each element imparting particular functions to the delivery construct: (1) a "receptor binding domain" that functions as a ligand for a cell surface receptor and that mediates binding of the construct to a cell; (2) a "transcytosis domain" that mediates transcytosis from a lumen bordering the apical surface of a mucous membrane to the basal-lateral side of a mucous membrane; (3) the macromolecule; and (4) a cleavable linker that connects the macromolecule to the remainder of the delivery construct.

[0074] The delivery constructs of the invention offer several advantages over conventional techniques for local or systemic delivery of macromolecules to a subject. Foremost among such advantages is the ability to deliver the macromolecule without using a needle to puncture the skin of the subject. Many subjects require repeated, regular doses of macromolecules. For example, diabetics must inject insulin several times per day to control blood sugar concentrations. Such subjects' quality of life would be greatly improved if the

delivery of a macromolecule could be accomplished without injection, by avoiding pain or potential complications associated therewith.

[0075] Furthermore, many embodiments of the delivery constructs can be constructed and expressed in recombinant systems. Recombinant technology allows one to make a delivery construct having an insertion site designed for introduction of any suitable macromolecule. Such insertion sites allow the skilled artisan to quickly and easily produce delivery constructs for delivery of new macromolecules, should the need to do so arise.

[0076] In addition, connection of the macromolecule to the remainder of the delivery construct with a linker that is cleaved by an enzyme present at a basal-lateral membrane of an epithelial cell allows the macromolecule to be liberated from the delivery construct and released from the remainder of the delivery construct soon after transcytosis across the epithelial membrane. Such liberation reduces the probability of induction of an immune response against the macromolecule. It also allows the macromolecule to interact with its target free from the remainder of the delivery construct.

[0077] Other advantages of the delivery constructs of the invention will be apparent to those of skill in the art.

[0078] In certain embodiments, the invention provides a delivery construct that comprises a receptor binding domain, a transcytosis domain, a macromolecule to be delivered to a subject, and a cleavable linker. Cleavage at the cleavable linker separates the macromolecule from the remainder of the construct. The cleavable linker is cleavable by an enzyme that is present at a basal-lateral membrane of a polarized epithelial cell of the subject or in the plasma of the subject. In certain embodiments, the enzyme that is at a basal-lateral membrane of a polarized epithelial cell exhibits higher activity on the basal-lateral side of a polarized epithelial cell than it does on the apical side of the polarized epithelial cell. In certain embodiments, the enzyme that is in the plasma of the subject exhibits higher activity in the plasma than it does on the apical side of a polarized epithelial cell.

[0079] In certain embodiments, the delivery construct further comprises a second cleavable linker. In certain embodiments, the first and/or the second cleavable linker comprises an amino acid sequence that is selected from the group consisting of Ala-Ala-Pro-Phe (SEQ ID NO.:4), Gly-Gly-Phe (SEQ ID NO.:5), Ala-Ala-Pro-Val (SEQ ID NO.:6), Gly-Gly-Leu (SEQ ID NO.:7), Ala-Ala-Leu (SEQ ID NO.:8), Phe-Val-Arg (SEQ ID NO.:9), Val-Gly-Arg (SEQ

ID NO.:10). In certain embodiments, the first and/or the second cleavable linker comprises an amino acid sequence that is selected from the group consisting of Ala-Ala-Pro-Phe (SEQ ID NO.:4), Gly-Gly-Phe (SEQ ID NO.:5), Ala-Ala-Pro-Val (SEQ ID NO.:6), Gly-Gly-Leu (SEQ ID NO.:7), Ala-Ala-Leu (SEQ ID NO.:8), Phe-Val-Arg (SEQ ID NO.:9), Val-Gly-Arg (SEQ ID NO.:10) and is cleavable by an enzyme that exhibits higher activity on the basal-lateral side of a polarized epithelial cell than it does on the apical side of the polarized epithelial cell. In certain embodiments, the first and/or the second cleavable linker comprises an amino acid sequence that is selected from the group consisting of Ala-Ala-Pro-Phe (SEQ ID NO.:4), Gly-Gly-Phe (SEQ ID NO.:5), Ala-Ala-Pro-Val (SEQ ID NO.:6), Gly-Gly-Leu (SEQ ID NO.:7), Ala-Ala-Leu (SEQ ID NO.:8), Phe-Val-Arg (SEQ ID NO.:9), Val-Gly-Arg (SEQ ID NO.:10) and is cleavable by an enzyme that exhibits higher activity in the plasma than it does on the apical side of a polarized epithelial cell.

[0080] In certain embodiments, the enzyme that is present at a basal-lateral membrane of a polarized epithelial cell is selected from the group consisting of Cathepsin GI, Chymotrypsin I, Elastase I, Subtilisin AI, Subtilisin AII, Thrombin I, and Urokinase I.

[0081] In certain embodiments, the receptor binding domain is selected from the group consisting of receptor binding domains from *Pseudomonas* exotoxin A, cholera toxin, botulinum toxin, diphtheria toxin, shiga toxin, or shiga-like toxin; monoclonal antibodies; polyclonal antibodies; single-chain antibodies; TGF α ; EGF; IGF-I; IGF-II; IGF-III; IL-1; IL-2; IL-3; IL-6; MIP-1a; MIP-1b; MCAF; and IL-8. In certain embodiments, the receptor binding domain binds to a cell-surface receptor that is selected from the group consisting of α 2-macroglobulin receptor, epidermal growth factor receptor, transferrin receptor, chemokine receptor, CD25, CD11B, CD11C, CD80, CD86, TNF α receptor, TOLL receptor, M-CSF receptor, GM-CSF receptor, scavenger receptor, and VEGF receptor. In further embodiments, the receptor binding domain of *Pseudomonas* exotoxin A is Domain Ia of *Pseudomonas* exotoxin A. In yet further embodiments, the receptor binding domain of *Pseudomonas* exotoxin A has an amino acid sequence that is SEQ ID NO.:1.

[0082] In certain embodiments, the transcytosis domain is selected from the group consisting of transcytosis domains from *Pseudomonas* exotoxin A, botulinum toxin, diphtheria toxin, pertussis toxin, cholera toxin, heat-labile *E. coli* enterotoxin, shiga toxin, and shiga-like toxin. In further embodiments, the transcytosis domain is *Pseudomonas* exotoxin A

transcytosis domain. In still further embodiments, the *Pseudomonas* exotoxin A transcytosis domain has an amino acid sequence that is SEQ ID NO.:2.

[0083] In certain embodiments, the macromolecule is selected from the group of a nucleic acid, a peptide, a polypeptide, a protein, and a lipid. In further embodiments, the polypeptide is selected from the group consisting of polypeptide hormones, cytokines, chemokines, growth factors, and clotting factors. In yet further embodiments, the polypeptide is selected from the group consisting of IGF-I, IGF-II, IGF-III, EGF, IFN- α , IFN- β , IFN- γ , G-CSF, GM-CSF, IL-1, IL-2, IL-3, IL-6, IL-8, IL-12, EPO, growth hormone, factor VII, vasopressin, calcitonin, parathyroid hormone, luteinizing hormone-releasing factor, tissue plasminogen activators, adrenocorticotropin, enkephalin, and glucagon-like peptide 1. In still further embodiments, the polypeptide is human growth hormone. In other embodiments, the protein is human insulin.

[0084] In certain embodiments, the delivery constructs further comprise a second macromolecule that is selected from the group consisting of a nucleic acid, a peptide, a polypeptide, a protein, a lipid, and a small organic molecule and a second cleavable linker, wherein cleavage at said second cleavable linker separates said second macromolecule from the remainder of said construct. In certain embodiments, the first macromolecule is a first polypeptide and said second macromolecule is a second polypeptide. In certain embodiments, the first polypeptide and the second polypeptide associate to form a multimer. In certain embodiments, the multimer is a dimer, tetramer, or octamer. In further embodiments, the dimer is an antibody.

5.2.1. Receptor Binding Domain

[0085] The delivery constructs of the invention generally comprise a receptor binding domain. The receptor binding domain can be any receptor binding domain known to one of skill in the art without limitation to bind to a cell surface receptor that is present on the apical membrane of an epithelial cell. Preferably, the receptor binding domain binds specifically to the cell surface receptor. The receptor binding domain should bind to the cell surface receptor with sufficient affinity to allow endocytosis of the delivery construct.

[0086] In certain embodiments, the receptor binding domain can comprise a peptide, a polypeptide, a protein, a lipid, a carbohydrate, or a small organic molecule, or a combination thereof. Examples of each of these molecules that bind to cell surface receptors present on

the apical membrane of epithelial cells are well known to those of skill in the art. Suitable peptides or polypeptides include, but are not limited to, bacterial toxin receptor binding domains, such as the receptor binding domains from PE, cholera toxin, botulinum toxin, diphtheria toxin, shiga toxin, shiga-like toxin, *etc.*; antibodies, including monoclonal, polyclonal, and single-chain antibodies, or derivatives thereof, growth factors, such as EGF, IGF-I, IGF-II, IGF-III *etc.*; cytokines, such as IL-1, IL-2, IL-3, IL-6, *etc.*; chemokines, such as MIP-1a, MIP-1b, MCAF, IL-8, *etc.*; and other ligands, such as CD4, cell adhesion molecules from the immunoglobulin superfamily, integrins, ligands specific for the IgA receptor, *etc.* See, e.g., Pastan *et al.*, 1992, *Annu. Rev. Biochem.* 61:331-54; and U.S. Patent Nos. 5,668,255, 5,696,237, 5,863,745, 5,965,406, 6,022,950, 6,051,405, 6,251,392, 6,440,419, and 6,488,926. The skilled artisan can select the appropriate receptor binding domain based upon the expression pattern of the receptor to which the receptor binding domain binds.

[0087] Lipids suitable for receptor binding domains include, but are not limited to, lipids that themselves bind cell surface receptors, such as sphingosine-1-phosphate, lysophosphatidic acid, sphingosylphosphorylcholine, retinoic acid, *etc.*; lipoproteins such as apolipoprotein E, apolipoprotein A, *etc.*, and glycolipids such as lipopolysaccharide, *etc.*; glycosphingolipids such as globotriaosylceramide and galabiosylceramide; and the like. Carbohydrates suitable for receptor binding domains include, but are not limited to, monosaccharides, disaccharides, and polysaccharides that comprise simple sugars such as glucose, fructose, galactose, *etc.*; and glycoproteins such as mucins, selectins, and the like. Suitable small organic molecules for receptor binding domains include, but are not limited to, vitamins, such as vitamin A, B₁, B₂, B₃, B₆, B₉, B₁₂, C, D, E, and K, amino acids, and other small molecules that are recognized and/or taken up by receptors present on the apical surface of epithelial cells. U.S. Patent No. 5,807,832 provides an example of such small organic molecule receptor binding domains, vitamin B₁₂.

[0088] In certain embodiments, the receptor binding domain can bind to a receptor found on an epithelial cell. In further embodiments, the receptor binding domain can bind to a receptor found on the apical membrane of an epithelial cell. The receptor binding domain can bind to any receptor known to be present on the apical membrane of an epithelial cell by one of skill in the art without limitation. For example, the receptor binding domain can bind to α 2-MR, EGFR, or IGFR. An example of a receptor binding domain that can bind to α 2-MR is domain Ia of PE. Accordingly, in certain embodiments, the receptor binding domain is

domain Ia of PE. In other embodiments, the receptor binding domain is a portion of domain Ia of PE that can bind to α 2-MR. Exemplary receptor binding domains that can bind to EGFR include, but are not limited to, EGF and TGF α . Examples of receptor binding domains that can bind to IGFR include, but are not limited to, IGF-I, IGF-II, or IGF-III. Thus, in certain embodiments, the receptor binding domain is EGF, IGF-I, IGF-II, or IGF-III. In other embodiments, the receptor binding domain is a portion of EGF, IGF-I, IGF-II, or IGF-III that can bind to the EGF or IGF receptor.

[0089] In certain embodiments, the receptor binding domain binds to a receptor that is highly expressed on the apical membrane of a polarized epithelial cell but is not expressed or expressed at low levels on antigen presenting cells, such as, for example, dendritic cells. Exemplary receptor binding domains that have this kind of expression pattern include, but are not limited to, TGF α , EGF, IGF-I, IGF-II, and IGF-III.

[0090] In certain embodiments, the delivery constructs of the invention comprise more than one domain that can function as a receptor binding domain. For example, the delivery construct can comprise PE domain Ia in addition to another receptor binding domain.

[0091] The receptor binding domain can be attached to the remainder of the delivery construct by any method or means known by one of skill in the art to be useful for attaching such molecules, without limitation. In certain embodiments, the receptor binding domain is expressed together with the remainder of the delivery construct as a fusion protein. Such embodiments are particularly useful when the receptor binding domain and the remainder of the construct are formed from peptides or polypeptides.

[0092] In other embodiments, the receptor binding domain is connected with the remainder of the delivery construct with a linker. In yet other embodiments, the receptor binding domain is connected with the remainder of the delivery construct without a linker. Either of these embodiments are useful when the receptor binding domain comprises a peptide, polypeptide, protein, lipid, carbohydrate, nucleic acid, or small organic molecule.

[0093] In certain embodiments, the linker can form a covalent bond between the receptor binding domain and the remainder of the delivery construct. In certain embodiments, the covalent bond can be a peptide bond. In other embodiments, the linker can link the receptor binding domain to the remainder of the delivery construct with one or more non-covalent interactions of sufficient affinity. One of skill in the art can readily recognize linkers that

interact with each other with sufficient affinity to be useful in the delivery constructs of the invention. For example, biotin can be attached to the receptor binding domain, and streptavidin can be attached to the remainder of the molecule. In certain embodiments, the linker can directly link the receptor binding domain to the remainder of the molecule. In other embodiments, the linker itself comprises two or more molecules that associate in order to link the receptor binding domain to the remainder of the molecule. Exemplary linkers include, but are not limited to, straight or branched-chain carbon linkers, heterocyclic carbon linkers, substituted carbon linkers, unsaturated carbon linkers, aromatic carbon linkers, peptide linkers, *etc.*

[0094] In embodiments where a linker is used to connect the receptor binding domain to the remainder of the delivery construct, the linkers can be attached to the receptor binding domain and/or the remainder of the delivery construct by any means or method known by one of skill in the art without limitation. For example, the linker can be attached to the receptor binding domain and/or the remainder of the delivery construct with an ether, ester, thioether, thioester, amide, imide, disulfide, peptide, or other suitable moiety. The skilled artisan can select the appropriate linker and method for attaching the linker based on the physical and chemical properties of the chosen receptor binding domain and the linker. The linker can be attached to any suitable functional group on the receptor binding domain or the remainder of the molecule. For example, the linker can be attached to sulfhydryl (-S), carboxylic acid (COOH) or free amine (-NH₂) groups, which are available for reaction with a suitable functional group on a linker. These groups can also be used to connect the receptor binding domain directly connected with the remainder of the molecule in the absence of a linker.

[0095] Further, the receptor binding domain and/or the remainder of the delivery construct can be derivatized in order to facilitate attachment of a linker to these moieties. For example, such derivatization can be accomplished by attaching suitable derivative such as those available from Pierce Chemical Company, Rockford, Illinois. Alternatively, derivatization may involve chemical treatment of the receptor binding domain and/or the remainder of the molecule. For example, glycol cleavage of the sugar moiety of a carbohydrate or glycoprotein receptor binding domain with periodate generates free aldehyde groups. These free aldehyde groups may be reacted with free amine or hydrazine groups on the remainder of the molecule in order to connect these portions of the molecule. *See, e.g.,* U.S. Patent No.4,671,958. Further, the skilled artisan can generate free sulfhydryl groups on proteins to

provide a reactive moiety for making a disulfide, thioether, thioester, *etc.* linkage. *See, e.g.*, U.S. Pat. No. 4,659,839.

[0096] Any of these methods for attaching a linker to a receptor binding domain and/or the remainder of a delivery construct can also be used to connect a receptor binding domain with the remainder of the delivery construct in the absence of a linker. In such embodiments, the receptor binding domain is coupled with the remainder of the construct using a method suitable for the particular receptor binding domain. Thus, any method suitable for connecting a protein, peptide, polypeptide, nucleic acid, carbohydrate, lipid, or small organic molecule to the remainder of the delivery construct known to one of skill in the art, without limitation, can be used to connect the receptor binding domain to the remainder of the construct. In addition to the methods for attaching a linker to a receptor binding domain or the remainder of a delivery construct, as described above, the receptor binding domain can be connected with the remainder of the construct as described, for example, in U.S. Patent Nos. 6,673,905; 6,585,973; 6,596,475; 5,856,090; 5,663,312; 5,391,723; 6,171,614; 5,366,958; and 5,614,503.

[0097] In certain embodiments, the receptor binding domain can be a monoclonal antibody. In some of these embodiments, the chimeric immunogen is expressed as a fusion protein that comprises an immunoglobulin heavy chain from an immunoglobulin specific for a receptor on a cell to which the chimeric immunogen is intended to bind. The light chain of the immunoglobulin then can be co-expressed with the chimeric immunogen, thereby forming a light chain-heavy chain dimer. In other embodiments, the antibody can be expressed and assembled separately from the remainder of the chimeric immunogen and chemically linked thereto.

5.2.2. Transcytosis Domain

[0098] The delivery constructs of the invention also comprise a transcytosis domain. The transcytosis domain can be any transcytosis domain known by one of skill in the art to effect transcytosis of chimeric proteins that have bound to a cell surface receptor present on the apical membrane of an epithelial cell. In certain embodiments, the transcytosis domain is a transcytosis domain from PE, diphtheria toxin, pertussis toxin, cholera toxin, heat-labile *E. coli* enterotoxin, shiga toxin, or shiga-like toxin. *See, for example*, U.S. Patent Nos. 5,965,406, and 6,022,950. In preferred embodiments, the transcytosis domain is domain II of PE.

[0099] The transcytosis domain need not, though it may, comprise the entire amino acid sequence of domain II of native PE, which spans residues 253-364 of PE. For example, the transcytosis domain can comprise a portion of PE that spans residues 280-344 of domain II of PE. The amino acids at positions 339 and 343 appear to be necessary for transcytosis. *See* Siegall *et al.*, 1991, *Biochemistry* 30:7154-59. Further, conservative or nonconservative substitutions can be made to the amino acid sequence of the transcytosis domain, as long as transcytosis activity is not substantially eliminated. A representative assay that can routinely be used by one of skill in the art to determine whether a transcytosis domain has transcytosis activity is described below.

[0100] Without intending to be limited to any particular theory or mechanism of action, the transcytosis domain is believed to permit the trafficking of the delivery construct through a polarized epithelial cell after the construct binds to a receptor present on the apical surface of the polarized epithelial cell. Such trafficking through a polarized epithelial cell is referred to herein as "transcytosis." This trafficking permits the release of the delivery construct from the basal-lateral membrane of the polarized epithelial cell.

5.2.3. Macromolecules for Delivery

[0101] The delivery constructs of the invention can also comprise a macromolecule. The macromolecule can be attached to the remainder of the delivery construct by any method known by one of skill in the art, without limitation. In certain embodiments, the macromolecule is expressed together with the remainder of the delivery construct as a fusion protein. In such embodiments, the macromolecule can be inserted into or attached to any portion of the delivery construct, so long as the receptor binding domain, the transcytosis domain, and macromolecule retain their activities. The macromolecule is connected with the remainder of the construct with a cleavable linker, or a combination of cleavable linkers, as described below.

[0102] In native PE, the Ib loop (domain Ib) spans amino acids 365 to 399, and is structurally characterized by a disulfide bond between two cysteines at positions 372 and 379. This portion of PE is not essential for any known activity of PE, including cell binding, transcytosis, ER retention or ADP ribosylation activity. Accordingly, domain Ib can be deleted entirely, or modified to contain a macromolecule.

[0103] Thus, in certain embodiments, the macromolecule can be inserted into domain Ib. If desirable, the macromolecule can be inserted into domain Ib wherein the cysteines at

positions 372 and 379 are not cross-linked. This can be accomplished by reducing the disulfide linkage between the cysteines, by deleting the cysteines entirely from the Ib domain, by mutating the cysteines to other residues, such as, for example, serine, or by other similar techniques. Alternatively, the macromolecule can be inserted into the Ib loop between the cysteines at positions 372 and 379. In such embodiments, the disulfide linkage between the cysteines can be used to constrain the macromolecule if desirable. In any event, in embodiments where the macromolecule is inserted into domain Ib of PE, or into any other portion of the delivery construct, the macromolecule should be flanked by cleavable linkers such that cleavage at the cleavable linkers liberates the macromolecule from the remainder of the construct.

[0104] In other embodiments, the macromolecule can be connected with the N-terminal or C-terminal end of a polypeptide portion of the delivery construct. In such embodiments, the method of connection should be designed to avoid interference with other functions of the delivery construct, such as receptor binding or transcytosis. In yet other embodiments, the macromolecule can be connected with a side chain of an amino acid of the delivery construct. The macromolecule is connected with the remainder of the delivery construct with a cleavable linker, as described below. In such embodiments, the macromolecule to be delivered can be connected with the remainder of the delivery construct with one or more cleavable linkers such that cleavage at the cleavable linker(s) separates the macromolecule from the remainder of the delivery construct. It should be noted that, in certain embodiments, the macromolecule of interest can also comprise a short (1-20 amino acids, preferably 1-10 amino acids, and more preferably 1-5 amino acids) leader peptide in addition to the macromolecule of interest that remains attached to the macromolecule following cleavage of the cleavable linker. Preferably, this leader peptide does not affect the activity or immunogenicity of the macromolecule.

[0105] In embodiments where the macromolecule is expressed together with another portion of the delivery construct as a fusion protein, the macromolecule can be inserted into the delivery construct by any method known to one of skill in the art without limitation. For example, amino acids corresponding to the macromolecule can be inserted directly into the delivery construct, with or without deletion of native amino acid sequences. In certain embodiments, all or part of the Ib domain of PE can be deleted and replaced with the macromolecule. In certain embodiments, the cysteine residues of the Ib loop are deleted

so that the macromolecule remains unconstrained. In other embodiments, the cysteine residues of the Ib loop are linked with a disulfide bond and constrain the macromolecule.

[0106] The macromolecule can be any macromolecule that is desired to be introduced into a subject. Thus, the macromolecule can be a peptide, a polypeptide, a protein, a nucleic acid, a carbohydrate, a lipid, a glycoprotein, synthetic organic and inorganic compounds, or any combination thereof. The macromolecule can also be a detectable compound such as a radiopaque compound, including air and barium and magnetic compounds. In certain embodiments, the macromolecule can be either soluble or insoluble in water. In certain embodiments, the macromolecule can be a macromolecule that can perform a desirable biological activity when introduced to the bloodstream of the subject. For example, the macromolecule can have receptor binding activity, enzymatic activity, messenger activity (*i.e.*, act as a hormone, cytokine, neurotransmitter, or other signaling molecule), luminescent or other detectable activity, or regulatory activity, or any combination thereof. In, for example, diagnostic embodiments, the macromolecule can be conjugated to or can itself be a pharmaceutically acceptable gamma-emitting moiety, including but not limited to, indium and technetium, magnetic particles, radiopaque materials such as air or barium and fluorescent compounds.

[0107] In other embodiments, the macromolecule that is delivered can exert its effects in biological compartments of the subject other than the subject's blood. For example, in certain embodiments, the macromolecule can exert its effects in the lymphatic system. In other embodiments, the macromolecule can exert its effects in an organ or tissue, such as, for example, the subject's liver, heart, lungs, pancreas, kidney, brain, bone marrow, *etc.* In such embodiments, the macromolecule may or may not be present in the blood, lymph, or other biological fluid at detectable concentrations, yet may still accumulate at sufficient concentrations at its site of action to exert a biological effect.

[0108] Further, the macromolecule can be a protein that comprises more than one polypeptide subunit. For example, the protein can be a dimer, trimer, or higher order multimer. In certain embodiments, two or more subunits of the protein can be connected with a covalent bond, such as, for example, a disulfide bond. In other embodiments, the subunits of the protein can be held together with non-covalent interactions. One of skill in the art can routinely identify such proteins and determine whether the subunits are properly associated using, for example, an immunoassay. Exemplary proteins that comprise more than

one polypeptide chain that can be delivered with a delivery construct of the invention include, but are not limited to, antibodies, insulin, IGF I, and the like.

[0109] Accordingly, in certain embodiments, the macromolecule is a peptide, polypeptide, or protein. In certain embodiments, the macromolecule comprises a peptide or polypeptide that comprises about 5, about 8, about 10, about 12, about 15, about 17, about 20, about 25, about 30, about 40, about 50, or about 60, about 70, about 80, about 90, about 100, about 200, about 400, about 600, about 800, or about 1000 amino acids. In certain embodiments, the macromolecule is a protein that comprises 1, 2, 3, 4, 5, 6, 7, 8, or more polypeptides. In certain embodiments, the peptide, polypeptide, or protein is a molecule that is commonly administered to subjects by injection. Exemplary peptides or polypeptides include, but are not limited to, IGF-I, IGF-II, IGF-III, EGF, IFN- α , IFN- β , IFN- γ , G-CSF, GM-CSF, IL-1, IL-2, IL-3, IL-6, IL-8, IL-12, EPO, growth hormone, clotting factors such as factor VII, vasopressin, calcitonin parathyroid hormone, luteinizing hormone-releasing factor, tissue plasminogen activators, adrenocorticotropin, enkephalin, glucagon-like peptide 1, asparaginase, and the like. In a preferred embodiment, the macromolecule is insulin. In certain preferred embodiments, the polypeptide is growth hormone. In even more preferred embodiments, the polypeptide is human growth hormone. In an equally preferred embodiment, the polypeptide is IFN- α , more preferably IFN α -2b. In an equally preferred embodiment, the polypeptide is insulin or proinsulin. In other embodiments, the polypeptide is green fluorescent protein. The sequences of all of these macromolecules are well known to those in the art, and attachment of these macromolecules to the delivery constructs is well within the skill of those in the art using standard techniques, as discussed below.

[0110] In certain embodiments, the macromolecule can be selected to not be cleavable by an enzyme present at the basal-lateral membrane of an epithelial cell. For example, the assays described in the examples can be used to routinely test whether such a cleaving enzyme can cleave the macromolecule to be delivered. If so, the macromolecule can be routinely altered to eliminate the offending amino acid sequence recognized by the cleaving enzyme. The altered macromolecule can then be tested to ensure that it retains activity using methods routine in the art.

[0111] Other examples of macromolecules that can be delivered according to the present invention include, but are not limited to, antineoplastic compounds, such as nitrosoureas, *e.g.*, carmustine, lomustine, semustine, streptozotocin; methylhydrazines, *e.g.*,

procarbazine, dacarbazine; steroid hormones, *e.g.*, glucocorticoids, estrogens, progestins, androgens, tetrahydrodesoxycaricosterone;. immunoactive compounds such as immunosuppressives, *e.g.*, pyrimethamine, trimethoprim, penicillamine, cyclosporine, azathioprine; and immunostimulants, *e.g.*, levamisole, diethyl dithiocarbamate, enkephalins, endorphins; antimicrobial compounds such as antibiotics, *e.g.*, β -lactam, penicillin, cephalosporins, carbapenems and monobactams, β -lactamase inhibitors, aminoglycosides, macrolides, tetracyclins, spectinomycin; antimalarials, amebicides; antiprotazoals; antifungals, *e.g.*, amphotericin β , antivirals, *e.g.*, acyclovir, idoxuridine, ribavirin, trifluridine, vidarbine, gancyclovir; parasiticides; antihelmintics; radiopharmaceuticals; gastrointestinal drugs; hematologic compounds; immunoglobulins; blood clotting proteins, *e.g.*, antihemophilic factor, factor IX complex; anticoagulants, *e.g.*, dicumarol, heparin Na; fibrolysin inhibitors, *e.g.*, tranexamic acid; cardiovascular drugs; peripheral anti-adrenergic drugs; centrally acting antihypertensive drugs, *e.g.*, methyldopa, methyldopa HCl; antihypertensive direct vasodilators, *e.g.*, diazoxide, hydralazine HCl; drugs affecting renin-angiotensin system; peripheral vasodilators, *e.g.*, phentolamine; anti-anginal drugs; cardiac glycosides; inodilators, *e.g.*, amrinone, milrinone, enoximone, fenoximone, imazodan, sulmazole; antidysrhythmics; calcium entry blockers; drugs affecting blood lipids, *e.g.*, ranitidine, bosentan, rezulin; respiratory drugs; sympathomimetic drugs, *e.g.*, albuterol, bitolterol mesylate, dobutamine HCl, dopamine HCl, ephedrine So, epinephrine, fenfluramine HCl, isoproterenol HCl, methoxamine HCl, norepinephrine bitartrate, phenylephrine HCl, ritodrine HCl; cholinomimetic drugs, *e.g.*, acetylcholine Cl; anticholinesterases, *e.g.*, edrophonium Cl; cholinesterase reactivators; adrenergic blocking drugs, *e.g.*, acebutolol HCl, atenolol, esmolol HCl, labetalol HCl, metoprolol, nadolol, phentolamine mesylate, propranolol HCl; antimuscarinic drugs, *e.g.*, anisotropine methylbromide, atropine SO₄, clinidium Br, glycopyrrolate, ipratropium Br, scopolamine HBr; neuromuscular blocking drugs; depolarizing drugs, *e.g.*, atracurium besylate, hexafluorenum Br, metocurine iodide, succinylcholine Cl, tubocurarine Cl, vecuronium Br; centrally acting muscle relaxants, *e.g.*, baclofen; neurotransmitters and neurotransmitter agents, *e.g.*, acetylcholine, adenosine, adenosine triphosphate; amino acid neurotransmitters, *e.g.*, excitatory amino acids, GABA, glycine; biogenic amine neurotransmitters, *e.g.*, dopamine, epinephrine, histamine, norepinephrine, octopamine, serotonin, tyramine; neuropeptides, nitric oxide, K⁺ channel toxins; antiparkinson drugs, *e.g.*, amantadine HCl, benzotropine mesylate, carbidopa; diuretic drugs, *e.g.*, dichlorphenamide, methazolamide, bendroflumethiazide, polythiazide; antimigraine drugs, *e.g.*, carboprost tromethamine mesylate, methysergide maleate.

[0112] Still other examples of macromolecules that can be delivered according to the present invention include, but are not limited to, hormones such as pituitary hormones, *e.g.*, chorionic gonadotropin, cosyntropin, menotropins, somatotropin, iorticotropin, protirelin, thyrotropin, vasopressin, lypressin; adrenal hormones, *e.g.*, beclomethasone dipropionate, betamethasone, dexamethasone, triamcinolone; pancreatic hormones, *e.g.*, glucagon, insulin; parathyroid hormone, *e.g.*, dihydrochysterol; thyroid hormones, *e.g.*, calcitonin etidronate disodium, levothyroxine Na, liothyronine Na, liotrix, thyroglobulin, teriparatide acetate; antithyroid drugs; estrogenic hormones; progestins and antagonists; hormonal contraceptives; testicular hormones; gastrointestinal hormones, *e.g.*, cholecystokinin, enteroglycan, galanin, gastric inhibitory polypeptide, epidermal growth factor-urogastrone, gastric inhibitory polypeptide, gastrin-releasing peptide, gastrins, pentagastrin, tetragastrin, motilin, peptide YY, secretin, vasoactive intestinal peptide, sincalide.

[0113] Still other examples of macromolecules that can be delivered according to the present invention include, but are not limited to, enzymes such as hyaluronidase, streptokinase, tissue plasminogen activator, urokinase, PGE-adenosine deaminase; intravenous anesthetics such as droperidol, etomidate, fetanyl citrate/droperidol, hexobarbital, ketamine HCl, methohexital Na, thiamylal Na, thiopental Na; antiepileptics, *e.g.*, carbamazepine, clonazepam, divalproex Na, ethosuximide, mephentyoin, paramethadione, phenytoin, primidone.

[0114] Still other examples of macromolecules that can be delivered according to the present invention include, but are not limited to, peptides and proteins such as ankyrins, arrestins, bacterial membrane proteins, clathrin, connexins, dystrophin, endothelin receptor, spectrin, selectin, cytokines; chemokines; growth factors, insulin, erythropoietin (EPO), tumor necrosis factor (TNF), neuropeptides, neuropeptide Y, neurotensin, transforming growth factor α , transforming growth factor β , interferon (IFN); hormones, growth inhibitors, *e.g.*, genistein, steroids *etc.*; glycoproteins, *e.g.*, ABC transporters, platelet glycoproteins, GPIb-IX complex, GPIIb-IIIa complex, vitronectin, thrombomodulin, CD4, CD55, CD58, CD59, CD44, lymphocyte function-associated antigen, intercellular adhesion molecule, vascular cell adhesion molecule, Thy-1, antiporters, CA-15-3 antigen, fibronectins, laminin, myelin-associated glycoprotein, GAP, GAP-43.

[0115] Yet other examples of macromolecules that can be delivered according to the present invention include, but are not limited to, cytokines and cytokine receptors such as

Interleukin-1 (IL-1), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-1 receptor, IL-2 receptor, IL-3 receptor, IL-4 receptor, IL-5 receptor, IL-6 receptor, IL-7 receptor, IL-8 receptor, IL-9 receptor, IL-10 receptor, IL-11 receptor, IL-12 receptor, IL-13 receptor, IL-14 receptor, IL-15 receptor, IL-16 receptor, IL-17 receptor, IL-18 receptor, lymphokine inhibitory factor, macrophage colony stimulating factor, platelet derived growth factor, stem cell factor, tumor growth factor β , tumor necrosis factor, lymphotoxin, Fas, granulocyte colony stimulating factor, granulocyte macrophage colony stimulating factor, interferon α , interferon β , and interferon γ .

[0116] Still other examples of macromolecules that can be delivered according to the present invention include, but are not limited to, growth factors and protein hormones such as erythropoietin, angiogenin, hepatocyte growth factor, fibroblast growth factor, keratinocyte growth factor, nerve growth factor, tumor growth factor α , thrombopoietin, thyroid stimulating factor, thyroid releasing hormone, neurotrophin, epidermal growth factor, VEGF, ciliary neurotrophic factor, LDL, somatomedin, insulin growth factor, insulin-like growth factor I and II; chemokines such as ENA-78, ELC, GRO- α , GRO- β , GRO- γ , HRG, LIF, IP-10, MCP-1, MCP-2, MCP-3, MCP-4, MIP-1 α , MIP-1 β , MIG, MDC, NT-3, NT-4, SCF, LIF, leptin, RANTES, lymphotactin, eotaxin-1, eotaxin-2, TARC, TECK, WAP-1, WAP-2, GCP-1, GCP-2; α -chemokine receptors, *e.g.*, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6, CXCR7; and β -chemokine receptors, *e.g.*, CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7.

[0117] Yet other examples of macromolecules that can be delivered according to the present invention include, but are not limited to, chemotherapeutics, such as chemotherapy or anti-tumor agents which are effective against various types of human cancers, including leukemia, lymphomas, carcinomas, sarcomas, myelomas *etc.*, such as, for example, doxorubicin, mitomycin, cisplatin, daunorubicin, bleomycin, actinomycin D, and neocarzinostatin.

[0118] Still other examples of macromolecules that can be delivered according to the present invention include, but are not limited to, antibodies such as anti-cluster of differentiation antigen CD-1 through CD-166 and the ligands or counter receptors for these molecules; anti-cytokine antibodies, *e.g.*, anti-IL-1 through anti-IL-18 and the receptors for these molecules; anti-immune receptor antibodies; antibodies against T cell receptors, major histocompatibility complexes I and II, B cell receptors, selectin killer inhibitory receptors,

killer activating receptors, OX-40, MadCAM-1, Gly-CAM1, integrins, cadherens, sialoadherens, Fas, CTLA-4, Fc γ -receptors, Fc α -receptors, Fc ε -receptors, Fc μ -receptors, and their ligands; anti-metalloproteinase antibodies, *e.g.*, antibodies specific for collagenase, MMP-1 through MMP-8, TIMP-1, TIMP-2; anti-cell lysis/proinflammatory molecules, *e.g.*, perforin, complement components, prostanoids, nitron oxide, thromboxanes; and anti-adhesion molecules, *e.g.*, carcinoembryonic antigens, lamins, fibronectins.

[0119] Yet other examples of macromolecules that can be delivered according to the present invention include, but are not limited to, antiviral agents such as reverse transcriptase inhibitors and nucleoside analogs, *e.g.*, ddI, ddC, 3TC, ddA, AZT; protease inhibitors, *e.g.*, Invirase, ABT-538; and inhibitors of in RNA processing, *e.g.*, ribavirin.

[0120] Further, specific examples of macromolecules that can be delivered with the delivery constructs of the present invention Capoten, Monopril, Pravachol, Avapro, Plavix, Cefzil, Duricef/Ultracel, Azactam, Videx, Zerit, Maxipime, VePesid, Paraplatin, Platinol, Taxol, UFT, Buspar, Serzone, Stadol NS, Estrace, Glucophage (Bristol-Myers Squibb); Ceclor, Lorabid, Dynabac, Prozac, Darvon, Permax, Zyprexa, Humalog, Axid, Gemzar, Evista (Eli Lilly); Vasotec/Vaseretic, Mevacor, Zocor, Prinivil/Prinizide, Plendil, Cozaar/Hyzaar, Pepcid, Prilosec, Primaxin, Noroxin, Recombivax HB, Varivax, Timoptic/XE, Trusopt, Proscar, Fosamax, Sinemet, Crixivan, Propecia, Vioxx, Singulair, Maxalt, Ivermectin (Merck & Co.); Diflucan, Unasyn, Sulperazon, Zithromax, Trovan, Procardia XL, Cardura, Norvasc, Dofetilide, Feldene, Zolof, Zeldox, Glucotrol XL, Zyrtec, Eletriptan, Viagra, Droloxifene, Aricept, Lipitor (Pfizer); Vantin, Rescriptor, Vistide, Genotropin, Micronase/Glyn./Glyb., Fragmin, Total Medrol, Xanax/alprazolam, Sermion, Halcion/triazolam, Freedox, Dostinex, Edronax, Mirapex, Pharmorubicin, Adriamycin, Camptosar, Remisar, Depo-Provera, Caverject, Detrusitol, Estring, Healon, Xalatan, Rogaine (Pharmacia & Upjohn); Lopid, Accrupil, Dilantin, Cognex, Neurontin, Loestrin, Dilzem, Fempatch, Estrostep, Rezulin, Lipitor, Omnicef, FemHRT, Suramin, and Clinafloxacin (Warner Lambert).

[0121] Yet further examples of macromolecules which may be delivered by the delivery constructs of the present invention may be found in: Goodman and Gilman's The Pharmacological Basis of Therapeutics, 9th ed. McGraw-Hill 1996, incorporated herein by reference in its entirety.

[0122] In certain embodiments, the macromolecule can be inactive or in a less active form when administered, then be activated in the subject. For example, the macromolecule can be a peptide or polypeptide with a masked active site. The peptide or polypeptide can be activated by removing the masking moiety. Such removal can be accomplished by peptidases or proteases in the cases of peptide or polypeptide masking agents. Alternatively, the masking agent can be a chemical moiety that is removed by an enzyme present in the subject. This strategy can be used when it is desirable for the macromolecule to be active in limited circumstances. For example, it may be useful for a macromolecule to be active only in the liver of the subject. In such cases, the macromolecule can be selected to have a masking moiety that can be removed by an enzyme that is present in the liver, but not in other organs or tissues. Exemplary methods and compositions for making and using such masked macromolecules can be found in U.S. Patent Nos. 6,080,575, 6,265,540, and 6,670,147.

[0123] In another example of such embodiments, the macromolecule can be a pro-macromolecule that is activated by a biological activity, for example by processing, present in the subject. For example, the exemplary macromolecule proinsulin can be delivered with a delivery construct of the present invention. Following delivery of the pro-macromolecule, it can be activated in the subject by appropriate processing enzymes. While it is believed that proinsulin is processed by enzymes (the endoproteases PC2 and PC3) present in highest concentration in secretory granules of pancreatic beta-cells, it is also believed that such enzyme are present in sufficient concentration in other compartments to permit activation of the pro-macromolecule into its fully active form. Further, it should be noted that many pro-macromolecules, including, for example, proinsulin, also exhibit activity similar to that of the fully active molecule. *See, for example, Desbuquois et al., 2003, Endocrinology 12:5308-5321.* Thus, even if not all of the pro-macromolecule is converted to the fully active form, the pro-molecule can in many cases still exert a desirable biological activity in the subject.

5.2.4. Cleavable Linkers

[0124] In the delivery constructs of the invention, the macromolecule to be delivered to the subject is connected with the remainder of the delivery construct with one or more cleavable linkers. The number of cleavable linkers present in the construct depends, at least in part, on the location of the macromolecule in relation to the remainder of the delivery construct and the nature of the macromolecule. When the macromolecule is inserted into the delivery construct, the macromolecule can be flanked by cleavable linkers, such that cleavage

at both linkers separates the macromolecule. The flanking cleavable linkers can be the same or different from each other. When the macromolecule can be separated from the remainder of the delivery construct with cleavage at a single linker, the delivery constructs can comprise a single cleavable linker. Further, where the macromolecule is, *e.g.*, a dimer or other multimer, each subunit of the macromolecule can be separated from the remainder of the delivery construct and/or the other subunits of the macromolecule by cleavage at the cleavable linker.

[0125] The cleavable linkers are generally cleavable by a cleaving enzyme that is present at or near the basal-lateral membrane of an epithelial cell. By selecting the cleavable linker to be cleaved by such enzymes, the macromolecule can be liberated from the remainder of the construct following transcytosis across the mucous membrane and release from the epithelial cell into the cellular matrix on the basal-lateral side of the membrane. Further, cleaving enzymes could be used that are present inside the epithelial cell, such that the cleavable linker is cleaved prior to release of the delivery construct from the basal-lateral membrane, so long as the cleaving enzyme does not cleave the delivery construct before the delivery construct enters the trafficking pathway in the polarized epithelial cell that results in release of the delivery construct and macromolecule from the basal-lateral membrane of the cell.

[0126] In certain embodiments, the cleaving enzyme is a peptidase. In other embodiments, the cleaving enzyme is an RNase. In yet other embodiments, the cleaving enzyme can cleave carbohydrates. Preferred peptidases include, but are not limited to, Cathepsin GI, Chymotrypsin I, Elastase I, Subtilisin AI, Subtilisin AII, Thrombin I, and Urokinase I. Table 1 presents these enzymes together with an amino acid sequence that is recognized and cleaved by the particular peptidase.

Table 1	
Peptidases Present Near Basal-Lateral Mucous Membranes	
Peptidase	Amino Acid Sequence Recognized and Cleaved
Cathepsin GI	Ala-Ala-Pro-Phe (SEQ ID NO.:4)
Chymotrypsin I	Gly-Gly-Phe (SEQ ID NO.:5)
Elastase I	Ala-Ala-Pro-Val (SEQ ID NO.:6)
Subtilisin AI	Gly-Gly-Leu (SEQ ID NO.:7)

Subtilisin AII	Ala-Ala-Leu (SEQ ID NO.:8)
Thrombin I	Phe-Val-Arg (SEQ ID NO.:9)
Urokinase I	Val-Gly-Arg (SEQ ID NO.:10)

[0127] In certain embodiments, the delivery construct can comprise more than one cleavable linker, wherein cleavage at either cleavable linker can separate the macromolecule to be delivered from the delivery construct. In certain embodiments, the cleavable linker can be selected based on the sequence, in the case of peptide, polypeptide, or protein macromolecules for delivery, to avoid the use of cleavable linkers that comprise sequences present in the macromolecule to be delivered. For example, if the macromolecule comprises AAL, the cleavable linker can be selected to be cleaved by an enzyme that does not recognize this sequence.

[0128] Further, the cleavable linker preferably exhibits a greater propensity for cleavage than the remainder of the delivery construct. As one skilled in the art is aware, many peptide and polypeptide sequences can be cleaved by peptidases and proteases. In certain embodiments, the cleavable linker is selected to be preferentially cleaved relative to other amino acid sequences present in the delivery construct during administration of the delivery construct. In certain embodiments, the receptor binding domain is substantially (*e.g.*, about 99%, about 95%, about 90%, about 85%, about 80, or about 75%) intact following delivery of the delivery construct to the bloodstream of the subject. In certain embodiments, the translocation domain is substantially (*e.g.*, about 99%, about 95%, about 90%, about 85%, about 80, or about 75%) intact following delivery of the delivery construct to the bloodstream of the subject. In certain embodiments, the macromolecule is substantially (*e.g.*, about 99%, about 95%, about 90%, about 85%, about 80, or about 75%) intact following delivery of the delivery construct to the bloodstream of the subject. In certain embodiments, the cleavable linker is substantially (*e.g.*, about 99%, about 95%, about 90%, about 85%, about 80, or about 75%) cleaved following delivery of the delivery construct to the bloodstream of the subject.

[0129] In other embodiments, the cleavable linker is cleaved by a cleaving enzyme found in the plasma of the subject. Any cleaving enzyme known by one of skill in the art to be present in the plasma of the subject can be used to cleave the cleavable linker. Use of such enzymes to cleave the cleavable linkers is less preferred than use of cleaving enzymes found

near the basal-lateral membrane of a polarized epithelial cell because it is believed that more efficient cleavage will occur in near the basal-lateral membrane. However, if the skilled artisan determines that cleavage mediated by a plasma enzyme is sufficiently efficient to allow cleavage of a sufficient fraction of the delivery constructs to avoid adverse effects, such plasma cleaving enzymes can be used to cleave the delivery constructs. Accordingly, in certain embodiments, the cleavable linker can be cleaved with an enzyme that is selected from the group consisting of caspase-1, caspase-3, proprotein convertase 1, proprotein convertase 2, proprotein convertase 4, proprotein convertase 4 PACE 4, prolyl oligopeptidase, endothelin cleaving enzyme, dipeptidyl-peptidase IV, signal peptidase, neprilysin, renin, and esterase. *See, e.g.*, U.S. Patent No. 6,673,574. Table 2 presents these enzymes together with an amino acid sequence(s) recognized by the particular peptidase. The peptidase cleaves a peptide comprising these sequences at the N-terminal side of the amino acid identified with an asterisk.

Table 2	
Plasma Peptidases	
Peptidase	Amino Acid Sequence Recognized and Cleaved
Caspase-1	Tyr-Val-Ala-Asp-Xaa* (SEQ ID NO.:11)
Caspase-3	Asp-Xaa-Xaa-Asp-Xaa* (SEQ ID NO.:12)
Proprotein convertase 1	Arg-(Xaa) _n -Arg-Xaa*; n = 0, 2, 4 or 6 (SEQ ID NO.:13)
Proprotein convertase 2	Lys-(Xaa) _n -Arg-Xaa*; n = 0, 2, 4, or 6 (SEQ ID NO.:14)
Proprotein convertase 4	Glp-Arg-Thr-Lys-Arg-Xaa* (SEQ ID NO.:15)
Proprotein convertase 4 PACE 4	Arg-Val-Arg-Arg-Xaa* (SEQ ID NO.:16) Decanoyl-Arg-Val-Arg-Arg-Xaa* (SEQ ID NO.:17)
Prolyl oligopeptidase Endothelin cleaving enzyme in combination with dipeptidyl-peptidase IV	Pro-Xaa*-Trp-Val-Pro-Xaa (SEQ ID NO.:18)
Signal peptidase	Trp-Val*-Ala-Xaa (SEQ ID NO.:19)
Neprilysin in combination with dipeptidyl-peptidase IV	Xaa-Phe*-Xaa-Xaa (SEQ ID NO.:20) Xaa-Tyr*-Xaa-Xaa (SEQ ID NO.:21)

	Xaa-Trp*-Xaa-Xaa (SEQ ID NO.:22)
Renin in combination with dipeptidyl-peptidase IV	Asp-Arg-Tyr-Ile-Pro-Phe-His-Leu*-Leu-(Val, Ala or Pro)-Tyr-(Ser, Pro, or Ala) (SEQ ID NO.:23)

[0130] Thus, in certain more preferred embodiments, the cleavable linker can be any cleavable linker known by one of skill in the art to be cleavable by an enzyme that is present at the basal-lateral membrane of an epithelial cell. In certain embodiments, the cleavable linker comprises a peptide. In other embodiments, the cleavable linker comprises a nucleic acid, such as RNA or DNA. In still other embodiments, the cleavable linker comprises a carbohydrate, such as a disaccharide or a trisaccharide. In certain embodiments, the cleavable linker is a peptide that comprises an amino acid sequence that is selected from the group consisting of Ala-Ala-Pro-Phe (SEQ ID NO.:4), Gly-Gly-Phe (SEQ ID NO.:5), Ala-Ala-Pro-Val (SEQ ID NO.:6), Gly-Gly-Leu (SEQ ID NO.:7), Ala-Ala-Leu (SEQ ID NO.:8), Phe-Val-Arg (SEQ ID NO.:9), Val-Gly-Arg (SEQ ID NO.:10).

[0131] Alternatively, in less preferred embodiments, the cleavable linker can be any cleavable linker known by one of skill in the art to be cleavable by an enzyme that is present in the plasma of the subject to whom the delivery construct is administered. In certain embodiments, the cleavable linker comprises a peptide. In other embodiments, the cleavable linker comprises a nucleic acid, such as RNA or DNA. In still other embodiments, the cleavable linker comprises a carbohydrate, such as a disaccharide or a trisaccharide. In certain embodiments, the cleavable linker is a peptide that comprises an amino acid sequence that is selected from the group consisting of amino acid sequences presented in Table 2.

[0132] In certain embodiments, the delivery construct comprises more than one cleavable linker. In certain embodiments, cleavage at any of the cleavable linkers will separate the macromolecule to be delivered from the remainder of the delivery construct. In certain embodiments, the delivery construct comprises a cleavable linker cleavable by an enzyme present at the basal-lateral side of a polarized epithelial membrane and a cleavable linkers cleavable by an enzyme that is present in the plasma of the subject to whom the delivery construct is administered.

[0133] Further, Tables 4 and 5, below, present results of experiments testing the ability of peptidases to cleave substrates when applied to the basal-lateral or apical surface of a polarized epithelial membrane. The sequences recognized by these enzymes are well-known in the art. Thus, in certain embodiments, the delivery construct comprises a cleavable linker that is cleavable by an enzyme listed in Tables 4 and 5. Preferred peptidases exhibit higher activity on the basolateral side of the membrane. Particularly preferred peptidases exhibit much higher (*e.g.*, 100%, 200%, or more increase in activity relative to the apical side) on the basolateral side. Thus, in certain embodiments, the cleavable linker is cleavable by an enzyme that exhibits 50% higher activity on the basal-lateral side of the membrane than on the apical side of the membrane. In certain embodiments, the cleavable linker is cleavable by an enzyme that exhibits 100% higher activity on the basal-lateral side of the membrane than on the apical side of the membrane. In certain embodiments, the cleavable linker is cleavable by an enzyme that exhibits 200% higher activity on the basal-lateral side of the membrane than on the apical side of the membrane. In certain embodiments, the cleavable linker is cleavable by an enzyme that exhibits 500% higher activity on the basal-lateral side of the membrane than on the apical side of the membrane. In certain embodiments, the cleavable linker is cleavable by an enzyme that exhibits 1,000% higher activity on the basal-lateral side of the membrane than on the apical side of the membrane. In certain embodiments, the cleavable linker is cleavable by an enzyme that exhibits 2,000% higher activity on the basal-lateral side of the membrane than on the apical side of the membrane. In certain embodiments, the cleavable linker is cleavable by an enzyme that exhibits 3,000% higher activity on the basal-lateral side of the membrane than on the apical side of the membrane. In certain embodiments, the cleavable linker is cleavable by an enzyme that exhibits 5,000% higher activity on the basal-lateral side of the membrane than on the apical side of the membrane. In certain embodiments, the cleavable linker is cleavable by an enzyme that exhibits 10,000% higher activity on the basal-lateral side of the membrane than on the apical side of the membrane.

[0134] Still further, the results in Tables 4 and 5 indicate that certain enzymes are present in higher concentration or exhibit greater activity in certain epithelial lineages as compared to other epithelial lineages. Thus, the experiments described below can be used to test whether the particular epithelial cell lineage through which a macromolecule will be delivered exhibits the desired cleavage activity. In certain embodiments, the cleavage activity is present in tracheal epithelial cells, but not intestinal epithelial cells. In other

embodiments, the cleavage activity is present in intestinal epithelial cells but not tracheal epithelial cells. In certain embodiments, the cleavage activity is present in intestinal epithelial cells and tracheal epithelial cells.

[0135] In certain embodiments, the cleavable linker may be cleavable by any enzyme that preferentially cleaves at the basolateral side of an epithelial membrane as compared to the apical side of the membrane. Example 6.4, below, describes an assay that can be used to assess the activity of such enzymes, while Table 7, appended to the end of this document, provides short names and accession numbers for every known human protease or peptidase. Any cleavage sequence recognized by such proteases or peptidases that preferentially cleaves a test substrate on the basolateral side of an epithelial membrane, or in the plasma, as compared to the apical side of such a membrane can also be used in the methods and compositions of the present invention. In such embodiments, one of skill in the art can readily determine the amino acid sequence recognized by such peptidases or proteases according to standard procedures known in the art or according to the known sequences recognized by the proteases and peptidases.

[0136] The examples below provide methods for identifying cleaving enzymes that are present at or near the basal-lateral membrane of a polarized epithelial cell. The skilled artisan can routinely use such methods to identify additional cleaving enzymes and the chemical structure(s) identified and cleaved by such cleaving enzymes. Delivery constructs comprising such cleavable linkers and methods of delivering macromolecules using delivery constructs comprising such cleavable linkers are also within the scope of the present invention, whether or not such cleaving enzymes are presented in Table 7.

[0137] In other embodiments, the cleavable linker can be a cleavable linker that is cleaved following a change in the environment of the delivery construct. For example, the cleavable linker can be a cleavable linker that is pH sensitive and is cleaved by a change in pH that is experienced when the delivery construct is released from the basal-lateral membrane of a polarized epithelial cell. For instance, the intestinal lumen is strongly alkaline, while plasma is essentially neutral. Thus, a cleavable linker can be a moiety that is cleaved upon a shift from alkaline to neutral pH. The change in the environment of the delivery construct that cleaves the cleavable linker can be any environmental change that is experienced when the delivery construct is released from the basal-lateral membrane of a polarized epithelial cell known by one of skill in the art, without limitation.

5.3. Methods for Delivering a Macromolecule

[0138] In another aspect, the invention provides methods for local or systemic delivery of a macromolecule to a subject. These methods generally comprise administering a delivery construct of the invention to a mucous membrane of the subject to whom the macromolecule is delivered. The delivery construct is typically administered in the form of a pharmaceutical composition, as described below.

[0139] Thus, in certain aspects, the invention provides a method for delivering a macromolecule to a subject. The method comprises contacting an apical surface of a polarized epithelial cell of the subject with a delivery construct. In certain embodiments, the delivery construct comprises a receptor binding domain, a transcytosis domain, a cleavable linker, and the macromolecule to be delivered. The transcytosis domain can transcytose the macromolecule to and through the basal-lateral membrane of said epithelial cell. The cleavable linker can be cleaved by an enzyme that is present at a basal-lateral membrane of a polarized epithelial cell of the subject or in the plasma of the subject. Cleavage at the cleavable linker separates the macromolecule from the remainder of the delivery construct, thereby delivering the macromolecule to the subject.

[0140] In certain embodiments, the enzyme that is present at or near a basal-lateral membrane of a polarized epithelial cell is selected from the group consisting of Cathepsin GI, Chymotrypsin I, Elastase I, Subtilisin AI, Subtilisin AII, Thrombin I, and Urokinase I. In certain embodiments, the cleavable linker comprises an amino acid sequence that is selected from the group consisting of Ala-Ala-Pro-Phe (SEQ ID NO.:4), Gly-Gly-Phe (SEQ ID NO.:5), Ala-Ala-Pro-Val (SEQ ID NO.:6), Gly-Gly-Leu (SEQ ID NO.:7), Ala-Ala-Leu (SEQ ID NO.:8), Phe-Val-Arg (SEQ ID NO.:9), Val-Gly-Arg (SEQ ID NO.:10).

[0141] In certain embodiments, the receptor binding domain is selected from the group consisting of receptor binding domains from *Pseudomonas* exotoxin A, cholera toxin, diphtheria toxin, shiga toxin, or shiga-like toxin; monoclonal antibodies; polyclonal antibodies; single-chain antibodies; TGF α ; EGF; IGF-I; IGF-II; IGF-III; IL-1; IL-2; IL-3; IL-6; MIP-1a; MIP-1b; MCAF; and IL-8. In certain embodiments, the receptor binding domain binds to a cell surface receptor selected from the group consisting of α 2-macroglobulin receptor, EGFR, IGFR, transferrin receptor, chemokine receptor, CD25, CD11B, CD11C, CD80, CD86, TNF α receptor, TOLL receptor, M-CSF receptor, GM-CSF receptor, scavenger receptor, and VEGF receptor.

[0142] In certain embodiments, the transcytosis domain is selected from the group consisting of transcytosis domains from *Pseudomonas* exotoxin A, diphtheria toxin, pertussis toxin, cholera toxin, heat-labile *E. coli* enterotoxin, shiga toxin, and shiga-like toxin.

[0143] In certain embodiments, the macromolecule is selected from the group consisting of a peptide, a polypeptide, a protein, a nucleic acid, and a lipid. In a preferred embodiment, the macromolecule is growth hormone. Even more preferably, the macromolecule is human growth hormone.

[0144] In certain embodiments, the invention provides a method for delivering a macromolecule to the bloodstream of a subject that results in at least about 30% bioavailability of the macromolecule, comprising administering a delivery construct comprising the macromolecule to the subject, thereby delivering at least about 30% of the total macromolecule administered to the blood of the subject in a bioavailable form of the macromolecule. In certain embodiments, at least about 10% of the total macromolecule administered is bioavailable to the subject. In certain embodiments, at least about 15% of the total macromolecule administered is bioavailable to the subject. In certain embodiments, at least about 20% of the total macromolecule administered is bioavailable to the subject. In certain embodiments, at least about 25% of the total macromolecule administered is bioavailable to the subject. In certain embodiments, at least about 35% of the total macromolecule administered is bioavailable to the subject. In certain embodiments, at least about 40% of the total macromolecule administered is bioavailable to the subject. In certain embodiments, at least about 45% of the total macromolecule administered is bioavailable to the subject. In certain embodiments, at least about 50% of the total macromolecule administered is bioavailable to the subject. In certain embodiments, at least about 55% of the total macromolecule administered is bioavailable to the subject. In certain embodiments, at least about 60% of the total macromolecule administered is bioavailable to the subject. In certain embodiments, at least about 65% of the total macromolecule administered is bioavailable to the subject. In certain embodiments, at least about 70% of the total macromolecule administered is bioavailable to the subject. In certain embodiments, at least about 75% of the total macromolecule administered is bioavailable to the subject. In certain embodiments, at least about 80% of the total macromolecule administered is bioavailable to the subject. In certain embodiments, at least about 85% of the total macromolecule administered is bioavailable to the subject. In certain embodiments, at least about 90% of the

total macromolecule administered is bioavailable to the subject. In certain embodiments, at least about 95% of the total macromolecule administered is bioavailable to the subject. In certain embodiments, the percentage of bioavailability of the macromolecule is determined by comparing the amount of macromolecule present in a subject's blood following administration of a delivery construct comprising the macromolecule to the amount of macromolecule present in a subject's blood following administration of the macromolecule through another route of administration. In certain embodiments, the other route of administration is injection, *e.g.*, subcutaneous injection, intravenous injection, intra-arterial injection, *etc.* In other embodiments, the percentage of bioavailability of the macromolecule is determined by comparing the amount of macromolecule present in a subject's blood following administration of a delivery construct comprising the macromolecule to the total amount of macromolecule administered as part of the delivery construct.

[0145] In certain embodiments, peak plasma concentrations of the delivered macromolecule in the subject are achieved about 10 minutes after administration. In certain embodiments, peak plasma concentrations of the delivered macromolecule in the subject are achieved about 15 minutes after administration. In certain embodiments, peak plasma concentrations of the delivered macromolecule in the subject are achieved about 5 minutes after administration. In certain embodiments, peak plasma concentrations of the delivered macromolecule in the subject are achieved about 20 minutes after administration. In certain embodiments, peak plasma concentrations of the delivered macromolecule in the subject are achieved about 25 minutes after administration. In certain embodiments, peak plasma concentrations of the delivered macromolecule in the subject are achieved about 30 minutes after administration. In certain embodiments, peak plasma concentrations of the delivered macromolecule in the subject are achieved about 35 minutes after administration. In certain embodiments, peak plasma concentrations of the delivered macromolecule in the subject are achieved about 40 minutes after administration. In certain embodiments, peak plasma concentrations of the delivered macromolecule in the subject are achieved about 45 minutes after administration. In certain embodiments, peak plasma concentrations of the delivered macromolecule in the subject are achieved about 50 minutes after administration. In certain embodiments, peak plasma concentrations of the delivered macromolecule in the subject are achieved about 55 minutes after administration. In certain embodiments, peak plasma concentrations of the delivered macromolecule in the subject are achieved about 60 minutes after administration. In certain embodiments, peak plasma concentrations of the delivered

macromolecule in the subject are achieved about 90 minutes after administration. In certain embodiments, peak plasma concentrations of the delivered macromolecule in the subject are achieved about 120 minutes after administration.

[0146] In certain embodiments, the peak plasma concentration of the delivered macromolecule is between about 0.01 ng/ml plasma and about 10 µg/ml plasma. In certain embodiments, the peak plasma concentration of the delivered macromolecule is between about 0.01 ng/ml plasma and about 1 µg/ml plasma. In certain embodiments, the peak plasma concentration of the delivered macromolecule is between about 0.01 ng/ml plasma and about 0.1 µg/ml plasma. In certain embodiments, the peak plasma concentration of the delivered macromolecule is between about 0.01 ng/ml plasma and about 10 ng/ml plasma. In certain embodiments, the peak plasma concentration of the delivered macromolecule is between about 1 ng/ml plasma and about 10 µg/ml plasma. In certain embodiments, the peak plasma concentration of the delivered macromolecule is between about 1 ng/ml plasma and about 1 µg/ml plasma. In certain embodiments, the peak plasma concentration of the delivered macromolecule is between about 1 ng/ml plasma and about 0.5 µg/ml plasma. In certain embodiments, the peak plasma concentration of the delivered macromolecule is between about 1 ng/ml plasma and about .1 µg/ml plasma. In certain embodiments, the peak plasma concentration of the delivered macromolecule is between about 10 ng/ml plasma and about 1 µg/ml plasma. In certain embodiments, the peak plasma concentration of the delivered macromolecule is between about 10 ng/ml plasma and about 0.5 µg/ml plasma.

[0147] In certain embodiments, the peak plasma concentration of the delivered macromolecule is at least about 10 µg/ml plasma. In certain embodiments, the peak plasma concentration of the delivered macromolecule is at least about 5 µg/ml plasma. In certain embodiments, the peak plasma concentration of the delivered macromolecule is at least about 1 µg/ml plasma. In certain embodiments, the peak plasma concentration of the delivered macromolecule is at least about 500 ng/ml plasma. In certain embodiments, the peak plasma concentration of the delivered macromolecule is at least about 250 ng/ml plasma. In certain embodiments, the peak plasma concentration of the delivered macromolecule is at least about 100 ng/ml plasma. In certain embodiments, the peak plasma concentration of the delivered macromolecule is at least about 50 ng/ml plasma. In certain embodiments, the peak plasma concentration of the delivered macromolecule is at least about 10 ng/ml plasma. In certain embodiments, the peak plasma concentration of the delivered macromolecule is at least about

5 ng/ml plasma. In certain embodiments, the peak plasma concentration of the delivered macromolecule is at least about 1 ng/ml plasma. In certain embodiments, the peak plasma concentration of the delivered macromolecule is at least about 0.1 ng/ml plasma.

[0148] Moreover, without intending to be bound to any particular theory or mechanism of action, it is believed that oral administration of a delivery construct can deliver a higher effective concentration of the delivered macromolecule to the liver of the subject than is observed in the subject's plasma. "Effective concentration," in this context, refers to the concentration experienced by targets of the macromolecule and can be determined by monitoring and/or quantifying downstream effects of macromolecule-target interactions. While still not bound to any particular theory, it is believed that oral administration of the delivery construct results in absorption of the delivery construct through polarized epithelial cells of the digestive mucosa, *e.g.*, the intestinal mucosa, followed by cleavage of the construct and release of the macromolecule at the basolateral side of the mucous membrane. As one of skill in the art will recognize, the blood at the basolateral membrane of such digestive mucosa is carried from this location to the liver *via* the portal venous system. Thus, when the macromolecule exerts a biological activity in the liver, such as, for example, activities mediated by growth hormone, insulin, IGF-I, *etc.* binding to their cognate receptors, the macromolecule is believed to exert an effect in excess of what would be expected based on the plasma concentrations observed in the subject. Accordingly, in certain embodiments, the invention provides a method of administering a macromolecule to a subject that comprises orally administering a delivery construct comprising the macromolecule to the subject, wherein the macromolecule is delivered to the subject's liver at a higher effective concentration than observed in the subject's plasma.

[0149] In certain embodiments, the epithelial cell is selected from the group consisting of nasal epithelial cells, oral epithelial cells, intestinal epithelial cells, rectal epithelial cells, vaginal epithelial cells, and pulmonary epithelial cells.

[0150] In certain embodiments, the subject is a mammal. In further embodiments, the subject is a rodent, a lagomorph, or a primate. In yet further embodiments, the rodent is a mouse or rat. In other embodiments, the lagomorph is a rabbit. In still other embodiments, the primate is a human, monkey, or ape. In a preferred embodiment, the subject is a human.

[0151] In another aspect, the invention provides a method for delivering a macromolecule to the bloodstream of a subject that induces a lower titer of antibodies against the macromolecule than other routes of administration. Without intending to be bound by any particular theory or mechanism of action, it is believed that entry of the macromolecule through a mucous membrane, *e.g.*, through the intestinal mucosa, causes the immune system to tolerate the macromolecule better than if the macromolecule were, for example, injected. Thus, a lower titer of antibodies against the macromolecule can be produced in the subject by delivering the macromolecule with a delivery construct of the invention through the mucosa rather than injecting the macromolecule, for example, subcutaneously, intravenously, intra-arterially, intraperitoneally, or otherwise. Generally, the time at which the lower titer of antibodies detected for the alternate routes of administration is detected should be roughly comparable; for example, the titer of antibodies can be determined at about 1 week, at about 2 weeks, at about 3 weeks, at about 4 weeks, at about 2 months, or at about 6 months following administration of the macromolecule with the delivery construct or by injection.

[0152] Accordingly, in certain embodiments, the invention provides a method for delivering a macromolecule to the bloodstream a subject that comprises contacting a delivery construct of the invention that comprises the macromolecule to be delivered to an apical surface of a polarized epithelial cell of the subject, such that the macromolecule is administered to the bloodstream of the subject, wherein a lower titer of antibodies specific for the macromolecule is induced in the serum of the subject than is induced by subcutaneously administering the macromolecule separately from the remainder of the delivery construct to a subject.

[0153] In certain embodiments, the macromolecule is selected from the group consisting of a peptide, a polypeptide, a protein, a nucleic acid, and a lipid. In certain embodiments, the macromolecule is selected from the group consisting of polypeptide hormones, cytokines, chemokines, growth factors, and clotting factors. In certain embodiments, the macromolecule is selected from the group consisting of IGF-I, IGF-II, IGF-III, EGF, IFN- α , IFN- β , IFN- γ , G-CSF, GM-CSF, IL-1, IL-2, IL-3, IL-6, IL-8, IL-12, EPO, growth hormone, factor VII, vasopressin, calcitonin, parathyroid hormone, luteinizing hormone-releasing factor, tissue plasminogen activators, adrenocorticotropin, enkephalin, and glucagon-like peptide 1. In certain embodiments, the macromolecule is human growth

normone. In certain embodiments, the macromolecule is human insulin. In certain embodiments, the subject is a mouse, rat, dog, goat, or human.

[0154] In certain embodiments, the titer of antibodies specific for the macromolecule induced in the serum of the subject by the macromolecule delivered by the delivery construct is less than about 95% of the titer of antibodies induced by subcutaneously administering the macromolecule separately from the remainder of the delivery construct. In certain embodiments, the titer of antibodies specific for the macromolecule induced in the serum of the subject by the macromolecule delivered by the delivery construct is less than about 90% of the titer of antibodies induced by subcutaneously administering the macromolecule separately from the remainder of the delivery construct. In certain embodiments, the titer of antibodies specific for the macromolecule induced in the serum of the subject by the macromolecule delivered by the delivery construct is less than about 85% of the titer of antibodies induced by subcutaneously administering the macromolecule separately from the remainder of the delivery construct. In certain embodiments, the titer of antibodies specific for the macromolecule induced in the serum of the subject by the macromolecule delivered by the delivery construct is less than about 80% of the titer of antibodies induced by subcutaneously administering the macromolecule separately from the remainder of the delivery construct. In certain embodiments, the titer of antibodies specific for the macromolecule induced in the serum of the subject by the macromolecule delivered by the delivery construct is less than about 75% of the titer of antibodies induced by subcutaneously administering the macromolecule separately from the remainder of the delivery construct.

[0155] In certain embodiments, the titer of antibodies specific for the macromolecule induced in the serum of the subject by the macromolecule delivered by the delivery construct is less than about 70% of the titer of antibodies induced by subcutaneously administering the macromolecule separately from the remainder of the delivery construct. In certain embodiments, the titer of antibodies specific for the macromolecule induced in the serum of the subject by the macromolecule delivered by the delivery construct is less than about 65% of the titer of antibodies induced by subcutaneously administering the macromolecule separately from the remainder of the delivery construct. In certain embodiments, the titer of antibodies specific for the macromolecule induced in the serum of the subject by the macromolecule delivered by the delivery construct is less than about 60% of the titer of antibodies induced by subcutaneously administering the macromolecule separately from the

remainder of the delivery construct. In certain embodiments, the titer of antibodies specific for the macromolecule induced in the serum of the subject by the macromolecule delivered by the delivery construct is less than about 55% of the titer of antibodies induced by subcutaneously administering the macromolecule separately from the remainder of the delivery construct. In certain embodiments, the titer of antibodies specific for the macromolecule induced in the serum of the subject by the macromolecule delivered by the delivery construct is less than about 55% of the titer of antibodies induced by subcutaneously administering the macromolecule separately from the remainder of the delivery construct.

[0156] In certain embodiments, the titer of antibodies specific for the macromolecule induced in the serum of the subject by the macromolecule delivered by the delivery construct is less than about 50% of the titer of antibodies induced by subcutaneously administering the macromolecule separately from the remainder of the delivery construct. In certain embodiments, the titer of antibodies specific for the macromolecule induced in the serum of the subject by the macromolecule delivered by the delivery construct is less than about 45% of the titer of antibodies induced by subcutaneously administering the macromolecule separately from the remainder of the delivery construct. In certain embodiments, the titer of antibodies specific for the macromolecule induced in the serum of the subject by the macromolecule delivered by the delivery construct is less than about 40% of the titer of antibodies induced by subcutaneously administering the macromolecule separately from the remainder of the delivery construct. In certain embodiments, the titer of antibodies specific for the macromolecule induced in the serum of the subject by the macromolecule delivered by the delivery construct is less than about 35% of the titer of antibodies induced by subcutaneously administering the macromolecule separately from the remainder of the delivery construct. In certain embodiments, the titer of antibodies specific for the macromolecule induced in the serum of the subject by the macromolecule delivered by the delivery construct is less than about 30% of the titer of antibodies induced by subcutaneously administering the macromolecule separately from the remainder of the delivery construct.

[0157] In certain embodiments, the titer of antibodies specific for the macromolecule induced in the serum of the subject by the macromolecule delivered by the delivery construct is less than about 25% of the titer of antibodies induced by subcutaneously administering the macromolecule separately from the remainder of the delivery construct. In certain embodiments, the titer of antibodies specific for the macromolecule induced in the serum of

the subject by the macromolecule delivered by the delivery construct is less than 20% of the titer of antibodies induced by subcutaneously administering the macromolecule separately from the remainder of the delivery construct. In certain embodiments, the titer of antibodies specific for the macromolecule induced in the serum of the subject by the macromolecule delivered by the delivery construct is less than about 15% of the titer of antibodies induced by subcutaneously administering the macromolecule separately from the remainder of the delivery construct. In certain embodiments, the titer of antibodies specific for the macromolecule induced in the serum of the subject by the macromolecule delivered by the delivery construct is less than about 10% of the titer of antibodies induced by subcutaneously administering the macromolecule separately from the remainder of the delivery construct. In certain embodiments, the titer of antibodies specific for the macromolecule induced in the serum of the subject by the macromolecule delivered by the delivery construct is less than about 5% of the titer of antibodies induced by subcutaneously administering the macromolecule separately from the remainder of the delivery construct. In certain embodiments, the titer of antibodies specific for the macromolecule induced in the serum of the subject by the macromolecule delivered by the delivery construct is less than about 1% of the titer of antibodies induced by subcutaneously administering the macromolecule separately from the remainder of the delivery construct.

5.3.1. Methods of Administration

[0158] The delivery constructs of the invention can be administered to a subject by any method known to one of skill in the art. In certain embodiments, the delivery constructs are contacted to a mucosal membrane of the subject. For example, the mucosal membrane can be present in the eye, nose, mouth, trachea, lungs, esophagus, stomach, small intestine, large intestine, rectum, anus, sweat glands, vulva, vagina, or penis of the subject. Preferably, the mucosal membrane is a mucosal membrane present in the digestive tract of the subject, such as a mucosal membrane in the mouth, esophagus, stomach, small intestine, large intestine, or rectum of the subject.

[0159] In such embodiments, the delivery constructs are preferably administered to the subject orally. Thus, the delivery construct can be formulated to protect the delivery construct from degradation in the acid environment of the stomach, if necessary. For example, many embodiments of the delivery constructs of the invention comprise polypeptide domains with defined activities. Unless such delivery constructs are protected from acid

and/or enzymatic hydrolysis in the stomach, the constructs will generally be digested before delivery of substantial amounts of the macromolecule to be delivered. Accordingly, composition formulations that protect the delivery construct from degradation can be used in administration of these delivery constructs.

5.3.2. Dosage

[0160] Generally, a pharmaceutically effective amount of the delivery construct of the invention is administered to a subject. The skilled artisan can readily determine if the dosage of the delivery construct is sufficient to deliver an effective amount of the macromolecule, as described below. In certain embodiments, between about 1 μ g and about 1 g of delivery construct is administered. In other embodiments, between about 10 μ g and about 500 mg of delivery construct is administered. In still other embodiments, between about 10 μ g and about 100 mg of delivery construct is administered. In yet other embodiments, between about 10 μ g and about 1000 μ g of delivery construct is administered. In still other embodiments, between about 10 μ g and about 250 μ g of delivery construct is administered. In yet other embodiments, between about 10 μ g and about 100 μ g of delivery construct is administered. Preferably, between about 10 μ g and about 50 μ g of delivery construct is administered.

[0161] The volume of a composition comprising the delivery construct that is administered will generally depend on the concentration of delivery construct and the formulation of the composition. In certain embodiments, a unit dose of the delivery construct composition is between about 0.05 ml and about 1 ml, preferably about 0.5 ml. The delivery construct compositions can be prepared in dosage forms containing between 1 and 50 doses (e.g., 0.5 ml to 25 ml), more usually between 1 and 10 doses (e.g., 0.5 ml to 5 ml).

[0162] The delivery construct compositions of the invention can be administered in one dose or in multiple doses. A dose can be followed by one or more doses spaced by about 1 to about 6 hours, by about 6 to about 12 hours, by about 12 to about 24 hours, by about 1 day to about 3 days, by about 1 day to about 1 week, by about 1 week to about 2 weeks, by about 2 weeks to about 1 month, by about 4 to about 8 weeks, by about 1 to about 3 months, or by about 1 to about 6 months.

[0163] The macromolecules to be delivered are generally macromolecules for which a large amount of knowledge regarding dosage, frequency of administration, and methods for

assessing effective concentrations in subjects has accumulated. Such knowledge can be used to assess efficiency of delivery, effective concentration of the macromolecule in the subject, and frequency of administration. Thus, the knowledge of those skilled in the art can be used to determine whether, for example, the amount of macromolecule delivered to the subject is an effective amount, the dosage should be increased or decreased, the subject should be administered the delivery construct more or less frequently, and the like.

5.3.3. Determining Amounts of Macromolecule Delivered

[0164] The methods of the invention can be used to deliver, either locally or systemically, a pharmaceutically effective amount of a macromolecule to a subject. The skilled artisan can determine whether the methods result in delivery of such a pharmaceutically effective amount of the macromolecule. The exact methods will depend on the macromolecule that is delivered, but generally will rely on either determining the concentration of the macromolecule in the blood of the subject or in the biological compartment of the subject where the macromolecule exerts its effects. Alternatively or additionally, the effects of the macromolecule on the subject can be monitored.

[0165] For example, in certain embodiments of the present invention, the macromolecule that is delivered is insulin, *e.g.*, human insulin. In such embodiments, the skilled artisan can determine whether a pharmaceutically effective amount of human insulin had been delivered to the subject by, for example, taking a plasma sample from the subject and determining the concentration of human insulin therein. One exemplary method for determining the concentration of human insulin is by performing an ELISA assay, but any other suitable assay known to the skilled artisan can be used.

[0166] Alternatively, one of skill in the art can determine if an effective amount of human insulin had been delivered to the subject by monitoring the blood sugar concentrations of the subject. As is well-known in the art, human insulin, among other activities, acts on hepatocytes to promote glycogen formation, thereby reducing plasma glucose concentrations. Accordingly, the subject's plasma glucose concentration can be monitored to determine whether an effective amount of insulin had been delivered.

[0167] Any effect of a macromolecule that is administered that is known by one of skill in the art, without limitation, can be assessed in determining whether an effective amount of the macromolecule has been administered. Exemplary effects include, but are not limited to,

receptor binding, receptor activation, downstream effects of receptor binding, downstream effects of receptor activation, coordination of compounds, effective blood clotting, bone growth, wound healing, cellular proliferation, *etc.* The exact effect that is assessed will depend on the macromolecule that is delivered.

5.4. Polynucleotides Encoding Delivery Constructs

[0168] In another aspect, the invention provides polynucleotides comprising a nucleotide sequence encoding the delivery constructs. These polynucleotides are useful, for example, for making the delivery constructs. In yet another aspect, the invention provides an expression system that comprises a recombinant polynucleotide sequence encoding a receptor binding domain, a transcytosis domain, and a polylinker insertion site for a polynucleotide sequence encoding a macromolecule. The polylinker insertion site can be anywhere in the polynucleotide sequence so long as the polylinker insertion does not disrupt the receptor binding domain or the transcytosis domain. The polylinker insertion site should be oriented near a polynucleotide sequence that encodes a cleavable linker so that cleavage at the cleavable linker separates a macromolecule encoded by a nucleic acid inserted into the polylinker insertion site from the remainder of the encoded delivery construct. Thus, in embodiments where the polylinker insertion site is at an end of the encoded construct, the polynucleotide comprises one nucleotide sequence encoding a cleavable linker between the polylinker insertion site and the remainder of the polynucleotide. In embodiments where the polylinker insertion site is not at the end of the encoded construct, the polylinker insertion site can be flanked by nucleotide sequences that each encode a cleavable linker.

[0169] In certain embodiments, the recombinant polynucleotides are based on polynucleotides encoding PE, or portions or derivatives thereof. In other embodiments, the recombinant polynucleotides are based on polynucleotides that hybridize to a polynucleotide that encodes PE under stringent hybridization conditions. A nucleotide sequence encoding PE is presented as SEQ ID NO.:3. This sequence can be used to prepare PCR primers for isolating a nucleic acid that encodes any portion of this sequence that is desired. For example, PCR can be used to isolate a nucleic acid that encodes one or more of the functional domains of PE. A nucleic acid so isolated can then be joined to nucleic acids encoding other functional domains of the delivery constructs using standard recombinant techniques.

[0170] Other *in vitro* methods that can be used to prepare a polynucleotide encoding PE, PE domains, or any other functional domain useful in the delivery constructs of the

invention include, but are not limited to, reverse transcription, the polymerase chain reaction (PCR), the ligase chain reaction (LCR), the transcription-based amplification system (TAS), the self-sustained sequence replication system (3SR) and the QP replicase amplification system (QB). Any such technique known by one of skill in the art to be useful in construction of recombinant nucleic acids can be used. For example, a polynucleotide encoding the protein or a portion thereof can be isolated by polymerase chain reaction of cDNA using primers based on the DNA sequence of PE or a nucleotide encoding a receptor binding domain.

[0171] Guidance for using these cloning and *in vitro* amplification methodologies are described in, for example, U.S. Patent No. 4,683,195; Mullis *et al.*, 1987, *Cold Spring Harbor Symp. Quant. Biol.* 51:263; and Erlich, *ed.*, 1989, *PCR Technology*, Stockton Press, NY. Polynucleotides encoding a delivery construct or a portion thereof also can be isolated by screening genomic or cDNA libraries with probes selected from the sequences of the desired polynucleotide under stringent, moderately stringent, or highly stringent hybridization conditions.

[0172] Construction of nucleic acids encoding the delivery constructs of the invention can be facilitated by introducing an insertion site for a nucleic acid encoding the macromolecule into the construct. In certain embodiments, an insertion site for the macromolecule can be introduced between the nucleotides encoding the cysteine residues of domain Ib. In other embodiments, the insertion site can be introduced anywhere in the nucleic acid encoding the construct so long as the insertion does not disrupt the functional domains encoded thereby. In certain embodiments, the insertion site can be in the ER retention domain.

[0173] In more specific embodiments, a nucleotide sequence encoding a portion of the Ib domain between the cysteine-encoding residues can be removed and replaced with a nucleotide sequence that includes a cloning site cleaved by a restriction enzyme. For example, the cloning site can be recognized and cleaved by PstI. In such examples, a polynucleotide encoding macromolecule that is flanked by PstI sequences can be inserted into the vector.

[0174] Further, the polynucleotides can also encode a secretory sequence at the amino terminus of the encoded delivery construct. Such constructs are useful for producing the delivery constructs in mammalian cells as they simplify isolation of the immunogen.

[0175] Furthermore, the polynucleotides of the invention also encompass derivative versions of polynucleotides encoding a delivery construct. Such derivatives can be made by any method known by one of skill in the art without limitation. For example, derivatives can be made by site-specific mutagenesis, including substitution, insertion, or deletion of one, two, three, five, ten or more nucleotides, of polynucleotides encoding the delivery construct. Alternatively, derivatives can be made by random mutagenesis. One method for randomly mutagenizing a nucleic acid comprises amplifying the nucleic acid in a PCR reaction in the presence of 0.1 mM $MnCl_2$ and unbalanced nucleotide concentrations. These conditions increase the misincorporation rate of the polymerase used in the PCR reaction and result in random mutagenesis of the amplified nucleic acid.

[0176] Several site-specific mutations and deletions in chimeric molecules derived from PE have been made and characterized. For example, deletion of nucleotides encoding amino acids 1-252 of PE yields a construct referred to as "PE40." Deleting nucleotides encoding amino acids 1-279 of PE yields a construct referred to as "PE37." See U.S. Patent No. 5,602,095. In both of these constructs, the receptor binding domain of PE, *i.e.*, domain Ia, has been deleted. Nucleic acids encoding a receptor binding domain can be ligated to these constructs to produce delivery constructs that are targeted to the cell surface receptor recognized by the receptor binding domain. Of course, these recombinant polynucleotides are particularly useful for expressing delivery constructs that have a receptor binding domain that is not domain Ia of PE. The recombinant polynucleotides can optionally encode an amino-terminal methionine to assist in expression of the construct. In certain embodiments, the receptor binding domain can be ligated to the 5' end of the polynucleotide encoding the transcytosis domain.

[0177] Other nucleic acids encoding mutant forms of PE that can be used as a source of nucleic acids for constructing the delivery constructs of the invention include, but are not limited to, PE Δ 553 and those described in U.S. Patent Nos. 5,602,095; 5,512,658 and 5,458,878, and in Vasil *et al.*, 1986, *Infect. Immunol.* 52:538-48.

[0178] Accordingly, in certain embodiments, the invention provides a polynucleotide that encodes a delivery construct. The delivery construct comprises a receptor binding domain, a transcytosis domain, a macromolecule to be delivered to a subject, and a cleavable linker. Cleavage at the cleavable linker can separate the macromolecule from the remainder of the construct. The cleavable linker can be cleaved by an enzyme that is present at a basal-lateral membrane of a polarized epithelial cell of the subject or in the plasma of the subject.

[0179] In certain embodiments, the polynucleotide hybridizes under stringent hybridization conditions to any polynucleotide of this invention. In further embodiments, the polynucleotide hybridizes under stringent conditions to a nucleic acid that encodes any delivery construct of the invention.

[0180] In certain embodiments, the polynucleotide encodes a delivery construct that further comprises a second cleavable linker. In certain embodiments, the first and/or second cleavable linker comprises an amino acid sequence that is selected from the group consisting of Ala-Ala-Pro-Phe (SEQ ID NO.:4), Gly-Gly-Phe (SEQ ID NO.:5), Ala-Ala-Pro-Val (SEQ ID NO.:6), Gly-Gly-Leu (SEQ ID NO.:7), Ala-Ala-Leu (SEQ ID NO.:8), Phe-Val-Arg (SEQ ID NO.:9), Val-Gly-Arg (SEQ ID NO.:10). In certain embodiments, the first and/or second cleavable linker encoded by the polynucleotide is cleavable by an enzyme that is selected from the group consisting of Cathepsin GI, Chymotrypsin I, Elastase I, Subtilisin AI, Subtilisin AII, Thrombin I, and Urokinase I.

[0181] In certain embodiments, the receptor binding domain encoded by the polynucleotide is selected from the group consisting of receptor binding domains from *Pseudomonas* exotoxin A, cholera toxin, diphtheria toxin, shiga toxin, or shiga-like toxin; monoclonal antibodies; polyclonal antibodies; single-chain antibodies; TGF α ; EGF; IGF-I; IGF-II; IGF-III; IL-1; IL-2; IL-3; IL-6; MIP-1a; MIP-1b; MCAF; and IL-8. In certain embodiments, the receptor binding domain encoded by the polynucleotide binds to a cell-surface receptor that is selected from the group consisting of α 2-macroglobulin receptor, EGFR, IGFR, transferrin receptor, chemokine receptor, CD25, CD11B, CD11C, CD80, CD86, TNF α receptor, TOLL receptor, M-CSF receptor, GM-CSF receptor, scavenger receptor, and VEGF receptor. In further embodiments, the receptor binding domain encoded by the polynucleotide is Domain Ia of *Pseudomonas* exotoxin A. In yet further embodiments, the receptor binding domain encoded by the polynucleotide has an amino acid sequence that is SEQ ID NO.:1.

[0182] In certain embodiments, the transcytosis domain encoded by the polynucleotide is selected from the group consisting of transcytosis domains from *Pseudomonas* exotoxin A, diphtheria toxin, pertussis toxin, cholera toxin, heat-labile *E. coli* enterotoxin, shiga toxin, and shiga-like toxin. In further embodiments, the transcytosis domain is *Pseudomonas* exotoxin A transcytosis domain. In still further embodiments, the *Pseudomonas* exotoxin A transcytosis domain has an amino acid sequence that is SEQ ID NO.:2.

[0183] In certain embodiments, the macromolecule encoded by the polynucleotide is selected from the group of a peptide, a polypeptide, and a protein. In certain embodiments, the polypeptide is selected from the group consisting of polypeptide hormones, cytokines, chemokines, growth factors, and clotting factors. In further embodiments, the polypeptide is selected from the group consisting of IGF-I, IGF-II, IGF-III, EGF, IFN- α , IFN- β , IFN- γ , G-CSF, GM-CSF, IL-1, IL-2, IL-3, IL-6, IL-8, IL-12, EPO, growth hormone, factor VII, vasopressin, calcitonin, parathyroid hormone, luteinizing hormone-releasing factor, tissue plasminogen activators, adrenocorticotropin, enkephalin, and glucagon-like peptide 1. In still further embodiments, the polypeptide is human growth hormone. In other embodiments, the protein is human insulin.

[0184] In other embodiments, the invention provides a polynucleotide that encodes a delivery construct that comprises a nucleic acid sequence encoding a receptor binding domain, a nucleic acid sequence encoding a transcytosis domain, a nucleic acid sequence encoding a cleavable linker, and a nucleic acid sequence comprising a polylinker insertion site. The polylinker insertion site can be oriented relative to the nucleic acid sequence encoding a cleavable linker to allow to cleavage of the cleavable linker to separate a macromolecule that is encoded by a nucleic acid inserted into the polylinker insertion site from the remainder of said delivery construct. The cleavable linker can be cleavable by an enzyme that is present at a basal-lateral membrane of a polarized epithelial cell of said subject or in the plasma of said subject.

5.5. Expression Vectors

[0185] In still another aspect, the invention provides expression vectors for expressing the delivery constructs. Generally, expression vectors are recombinant polynucleotide molecules comprising expression control sequences operatively linked to a nucleotide sequence encoding a polypeptide. Expression vectors can readily be adapted for function in prokaryotes or eukaryotes by inclusion of appropriate promoters, replication sequences,

selectable markers, *etc.* to result in stable transcription and translation of mRNA. Techniques for construction of expression vectors and expression of genes in cells comprising the expression vectors are well known in the art. *See, e.g.,* Sambrook *et al.*, 2001, *Molecular Cloning -- A Laboratory Manual*, 3rd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, and Ausubel *et al.*, eds., Current Edition, *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley Interscience, NY.

[0186] Useful promoters for use in expression vectors include, but are not limited to, a metallothionein promoter, a constitutive adenovirus major late promoter, a dexamethasone-inducible MMTV promoter, a SV40 promoter, a MRP pol III promoter, a constitutive MPSV promoter, a tetracycline-inducible CMV promoter (such as the human immediate-early CMV promoter), and a constitutive CMV promoter.

[0187] The expression vectors should contain expression and replication signals compatible with the cell in which the delivery constructs are expressed. Expression vectors useful for expressing delivery constructs include viral vectors such as retroviruses, adenoviruses and adenoassociated viruses, plasmid vectors, cosmids, and the like. Viral and plasmid vectors are preferred for transfecting the expression vectors into mammalian cells. For example, the expression vector pcDNA1 (Invitrogen, San Diego, CA), in which the expression control sequence comprises the CMV promoter, provides good rates of transfection and expression into such cells.

[0188] The expression vectors can be introduced into the cell for expression of the delivery constructs by any method known to one of skill in the art without limitation. Such methods include, but are not limited to, *e.g.*, direct uptake of the molecule by a cell from solution; facilitated uptake through lipofection using, *e.g.*, liposomes or immunoliposomes; particle-mediated transfection; *etc.* *See, e.g.*, U.S. Patent No. 5,272,065; Goeddel *et al.*, eds, 1990, *Methods in Enzymology*, vol. 185, Academic Press, Inc., CA; Krieger, 1990, *Gene Transfer and Expression -- A Laboratory Manual*, Stockton Press, NY; Sambrook *et al.*, 1989, *Molecular Cloning -- A Laboratory Manual*, Cold Spring Harbor Laboratory, NY; and Ausubel *et al.*, eds., Current Edition, *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley Interscience, NY.

[0189] The expression vectors can also contain a purification moiety that simplifies isolation of the delivery construct. For example, a polyhistidine moiety of, *e.g.*, six histidine

residues, can be incorporated at the amino terminal end of the protein. The polyhistidine moiety allows convenient isolation of the protein in a single step by nickel-chelate chromatography. In certain embodiments, the purification moiety can be cleaved from the remainder of the delivery construct following purification. In other embodiments, the moiety does not interfere with the function of the functional domains of the delivery construct and thus need not be cleaved.

5.6. Cell for Expressing a Delivery Construct

[0190] In yet another aspect, the invention provides a cell comprising an expression vector for expression of the delivery constructs, or portions thereof. The cell is preferably selected for its ability to express high concentrations of the delivery construct to facilitate purification of the protein. In certain embodiments, the cell is a prokaryotic cell, for example, *E. coli*. As described in the examples, the delivery constructs are properly folded and comprise the appropriate disulfide linkages when expressed in *E. coli*.

[0191] In other embodiments, the cell is a eukaryotic cell. Useful eukaryotic cells include yeast and mammalian cells. Any mammalian cell known by one of skill in the art to be useful for expressing a recombinant polypeptide, without limitation, can be used to express the delivery constructs. For example, Chinese hamster ovary (CHO) cells can be used to express the delivery constructs.

5.7. Compositions Comprising Delivery Constructs

[0192] The delivery constructs of the invention can be formulated as compositions. The compositions are generally formulated appropriately for the immediate use intended for the delivery construct. For example, if the delivery construct is not to be administered immediately, the delivery construct can be formulated in a composition suitable for storage. One such composition is a lyophilized preparation of the delivery construct together with a suitable stabilizer. Alternatively, the delivery construct composition can be formulated for storage in a solution with one or more suitable stabilizers. Any such stabilizer known to one of skill in the art without limitation can be used. For example, stabilizers suitable for lyophilized preparations include, but are not limited to, sugars, salts, surfactants, proteins, chaotropic agents, lipids, and amino acids. Stabilizers suitable for liquid preparations include, but are not limited to, sugars, salts, surfactants, proteins, chaotropic agents, lipids, and amino acids. Specific stabilizers that can be used in the compositions include, but are not limited to, trehalose, serum albumin, phosphatidylcholine, lecithin, and arginine. Other

compounds, compositions, and methods for stabilizing a lyophilized or liquid preparation of the delivery constructs may be found, for example, in U.S. Patent Nos. 6,573,237, 6,525,102, 6,391,296, 6,255,284, 6,133,229, 6,007,791, 5,997,856, and 5,917,021.

[0193] Further, the delivery construct compositions of the invention can be formulated for administration to a subject. Such vaccine compositions generally comprise one or more delivery constructs of the invention and a pharmaceutically acceptable excipient, diluent, carrier, or vehicle. Any such pharmaceutically acceptable excipient, diluent, carrier, or vehicle known to one of skill in the art without limitation can be used. Examples of a suitable excipient, diluent, carrier, or vehicle can be found in *Remington's Pharmaceutical Sciences*, 21st Ed. 2005, Mack Publishing Co., Easton.

[0194] In certain embodiments, the delivery construct compositions are formulated for oral administration. In such embodiments, the compositions are formulated to protect the delivery construct from acid and/or enzymatic degradation in the stomach. Upon passage to the neutral to alkaline environment of the duodenum, the delivery construct then contacts a mucous membrane and is transported across the polarized epithelial membrane. The delivery constructs may be formulated in such compositions by any method known by one of skill in the art, without limitation.

[0195] In certain embodiments, the oral formulation comprises a delivery construct and one or more compounds that can protect the delivery construct while it is in the stomach. For example, the protective compound should be able to prevent acid and/or enzymatic hydrolysis of the delivery construct. In certain embodiments, the oral formulation comprises a delivery construct and one or more compounds that can facilitate transit of the construct from the stomach to the small intestine. In certain embodiments, the one or more compounds that can protect the delivery construct from degradation in the stomach can also facilitate transit of the construct from the stomach to the small intestine. Preferably, the oral formulation comprises one or more compounds that can protect the delivery construct from degradation in the stomach and facilitate transit of the construct from the stomach to the small intestine. For example, inclusion of sodium bicarbonate can be useful in facilitating the rapid movement of intra-gastric delivered materials from the stomach to the duodenum as described in Mrsny *et al.*, 1999, *Vaccine* 17:1425-1433.

[0196] Other methods for formulating compositions so that the delivery constructs can pass through the stomach and contact polarized epithelial membranes in the small intestine include, but are not limited to, enteric-coating technologies as described in DeYoung, 1989, *Int J Pancreatol.* 5 Suppl:31-6, and the methods provided in U.S. Patent Nos. 6,613,332, 6,174,529, 6,086,918, 5,922,680, and 5,807,832.

5.7.1. Kits Comprising Compositions

[0197] In yet another aspect, the invention provides a kit that comprises a composition of the invention. In certain embodiments, the kit further comprises instructions that direct administration of the composition to a mucous membrane of the subject to whom the composition is administered. In certain embodiments, the kit further comprises instructions that direct oral administration of the composition to the subject to whom the composition is administered.

[0198] In certain embodiments, the kit comprises a composition of the invention in more or more containers. In certain embodiments, the composition can be in a unit dosage form, *e.g.*, a tablet, lozenge, capsule, *etc.* In certain embodiments, the composition can be provided in or with a device for administering the composition, such as, for example, a device configured to administer a single-unit dose of the composition, *e.g.*, an inhaler.

5.8. Making and Testing Delivery Constructs

[0199] The delivery constructs of the invention are preferably produced recombinantly, as described below. However, the delivery constructs may also be produced by chemical synthesis using methods known to those of skill in the art.

5.8.1. Manufacture of Delivery Constructs

[0200] Methods for expressing and purifying the delivery constructs of the invention are described extensively in the examples below. Generally, the methods rely on introduction of an expression vector encoding the delivery construct to a cell that can express the delivery construct from the vector. The delivery construct can then be purified for administration to a subject.

5.8.2. Testing Delivery Constructs

[0201] Having selected the domains of the delivery construct, the function of these domains, and of the delivery constructs as a whole, can be routinely tested to ensure that the constructs can deliver a macromolecule across mucous membranes of a subject free from the

remainder of the construct. For example, the delivery constructs can be tested for cell recognition, transcytosis and cleavage using routine assays. The entire chimeric protein can be tested, or, the function of various domains can be tested by substituting them for native domains of the wild-type toxin.

5.8.2.1. Receptor binding/Cell recognition

[0202] Receptor binding domain function can be tested by monitoring the delivery construct's ability to bind to the target receptor. Such testing can be accomplished using cell-based assays, with the target receptor present on a cell surface, or in cell-free assays. For example, delivery construct binding to a target can be assessed with affinity chromatography. The construct can be attached to a matrix in an affinity column, and binding of the receptor to the matrix detected, or vice versa. Alternatively, if antibodies have been identified that bind to either the receptor binding domain or its cognate receptor, the antibodies can be used, for example, to detect the receptor binding domain in the delivery construct by immunoassay, or in a competition assay for the cognate receptor. An exemplary cell-based assay that detects delivery construct binding to receptors on cells comprises labeling the construct and detecting its binding to cells by, *e.g.*, fluorescent cell sorting, autoradiography, *etc.*

5.8.2.2. Transcytosis

[0203] The function of the transcytosis domain can be tested as a function of the delivery construct's ability to pass through an epithelial membrane. Because transcytosis first requires binding to the cell, these assays can also be used to assess the function of the cell recognition domain.

[0204] The delivery construct's transcytosis activity can be tested by any method known by one of skill in the art, without limitation. In certain embodiments, transcytosis activity can be tested by assessing the ability of a delivery construct to enter a non-polarized cell to which it binds. Without intending to be bound to any particular theory or mechanism of action, it is believed that the same property that allows a transcytosis domain to pass through a polarized epithelial cell also allows molecules bearing the transcytosis domain to enter non-polarized cells. Thus, the delivery construct's ability to enter the cell can be assessed, for example, by detecting the physical presence of the construct in the interior of the cell. For example, the delivery construct can be labeled with, for example, a fluorescent marker, and the delivery construct exposed to the cell. Then, the cells can be washed, removing any delivery construct that has not entered the cell, and the amount of label

remaining determined. Detecting the label in this fraction indicates that the delivery construct has entered the cell.

[0205] In other embodiments, the delivery construct's transcytosis ability can be tested by assessing the delivery construct's ability to pass through a polarized epithelial cell. For example, the delivery construct can be labeled with, for example, a fluorescent marker and contacted to the apical membranes of a layer of epithelial cells. Fluorescence detected on the basal-lateral side of the membrane formed by the epithelial cells indicates that the transcytosis domain is functioning properly.

5.8.2.3. Cleavable Linker Cleavage

[0206] The function of the cleavable linker can generally be tested in a cleavage assay. Any suitable cleavage assay known by one of skill in the art, without limitation, can be used to test the cleavable linkers. Both cell-based and cell-free assays can be used to test the ability of an enzyme to cleave the cleavable linkers.

[0207] An exemplary cell-free assay for testing cleavage of cleavable linkers comprises preparing extracts of polarized epithelial cells and exposing a labeled delivery construct bearing a cleavable linker to the fraction of the extract that corresponds to membrane-associated enzymes. In such assays, the label can be attached to either the macromolecule to be delivered or to the remainder of the delivery construct. Among these enzymes are cleavage enzymes found near the basal-lateral membrane of a polarized epithelial cell, as described above. Cleavage can be detected, for example, by binding the delivery construct with, for example, an antibody and washing off unbound molecules. If label is attached to the macromolecule to be delivered, then little or no label should be observed on the molecule bound to the antibodies. Alternatively, the binding agent used in the assay can be specific for the macromolecule, and the remainder of the construct can be labeled. In either case, cleavage can be assessed.

[0208] Cleavage can also be tested using cell-based assays that test cleavage by polarized epithelial cells assembled into membranes. For example, a labeled delivery construct, or portion of a delivery construct comprising the cleavable linker, can be contacted to either the apical or basolateral side of a monolayer of suitable epithelial cells, such as, for example, Coco-2 cells, under conditions that permit cleavage of the linker. Cleavage can be detected by detecting the presence or absence of the label using a reagent that specifically

binds the delivery construct, or portion thereof. For example, an antibody specific for the delivery construct can be used to bind a delivery construct comprising a label distal to the cleavable linker in relation to the portion of the delivery construct bound by the antibody. Cleavage can then be assessed by detecting the presence of the label on molecules bound to the antibody. If cleavage has occurred, little or no label should be observed on the molecules bound to the antibody. By performing such experiments, enzymes that preferentially cleave at the basolateral membrane rather than the apical membrane can be identified, and, further, the ability of such enzymes to cleave the cleavable linker in a delivery construct can be confirmed.

[0209] Further, cleavage can also be tested using a fluorescence reporter assay as described in US Patent No. 6,759,207. Briefly, in such assays, the fluorescence reporter is contacted to the basolateral side of a monolayer of suitable epithelial cells under conditions that allow the cleaving enzyme to cleave the reporter. Cleavage of the reporter changes the structure of the fluorescence reporter, changing it from a non-fluorescent configuration to a fluorescent configuration. The amount of fluorescence observed indicates the activity of the cleaving enzyme present at the basolateral membrane.

[0210] Further, cleavage can also be tested using an intra-molecularly quenched molecular probe, such as those described in US Patent No. 6,592,847. Such probes generally comprise a fluorescent moiety that emits photons when excited with light of appropriate wavelength and a quencher moiety that absorbs such photons when in close proximity to the fluorescent moiety. Cleavage of the probe separates the quenching moiety from the fluorescent moiety, such that fluorescence can be detected, thereby indicating that cleavage has occurred. Thus, such probes can be used to identify and assess cleavage by particular cleaving enzymes by contacting the basolateral side of a monolayer of suitable epithelial cells with the probe under conditions that allow the cleaving enzyme to cleave the probe. The amount of fluorescence observed indicates the activity of the cleaving enzyme being tested.

6. EXAMPLES

[0211] The following examples merely illustrate the invention, and are not intended to limit the invention in any way.

6.1. Construction of a Delivery Construct

[0212] Five exemplary delivery construct expression vectors for delivering rat growth hormone (rGH) were constructed according to the following protocol. First, the rGH gene was amplified by PCR, incorporating restriction enzymes pairs of NdeI and EcoRI, PstI and PstI, AgeI and EcoRI, or PstI and EcoRI sites at two ends of the PCR products. After restriction enzyme digestion, the PCR products were cloned into pPE64-PstI-Δ553, which was digested with the corresponding restriction enzyme pairs. The resulting constructs were named as pPE-RGH(NdeI-EcoRI), pntPE-RGH(PstI), pntPE-RGH(AgeI-EcoRI), and pPE-RGH(PstI-EcoRI). These constructs thus comprise sequences encoding Domains I and II of ntPE (amino acids 26-372 as shown in Figure 3) and rGH (Accession No. P01244; *see Seeburg et al.*, 1977, *Nature* 270:486-494 and *Page et al.*, 1981, *Nucleic Acids Res.* 9:2087-2104), and are also tagged with a 6-His motif at the N-terminus of the polypeptide to facilitate purification. The final plasmids were verified by restriction enzyme digestions and DNA sequencing.

[0213] Expression vectors comprising cleavable linkers were constructed by introducing sequences encoding the appropriate amino acid sequence. To do so, oligonucleotides that encode sequences complementary to appropriate restriction sites and one of the following amino acid sequences were synthesized, then ligated into an expression vector prepared as described above between the ntPE sequences and the rGH sequences. For Delivery Construct 1, the cleavable linker sequence was RQPRGGL. For Delivery Construct 2, the cleavable linker sequence was GGLRQPR. For Delivery Construct 3, the cleavable linker sequence was RQPREGR. For Delivery Construct 4, the cleavable linker sequence was RQPRVGR. For Delivery Construct 5, the cleavable linker sequence was RQPRARR.

[0214] To separate rGH from PE protein in the event that the fusion protein is taken up by antigen presenting cells, a protease furin site was also inserted between the cleavable linker and rGH. To do so, constructs containing a sequence encoding the furin site with the five different cleavable linkers were made. Oligonucleotide sequences for the five cleavable linkers and a furin clip site are shown in Table 3 below. Each of the oligo duplexes was inserted into PstI site of pPE-RGH(PstI-EcoRI). The final constructs, named as pPE-RGH-F1, pPE-RGH-F2, pPE-RGH-F3, pPE-RGH-F4, pPE-RGH-F5 were confirmed by restriction enzyme digestion and DNA sequencing.

Table 3

Oligonucleotide pairs for introducing a furin cleavage site and protease cleavage site	
pPE-RGH-1	1 F: AACTGCAGCGCCAGCCTCGAGGAGGATTACTGCAGAA (SEQ ID NO:24) 1 R: TTCTGCAGTAATCCTCCTCGAGGCTGGCGCTGCAGTT (SEQ ID NO:25)
pPE-RGH-2	2 F: AACTGCAGGGAGGCTTACGCCAGCCTCGACTGCAGAA (SEQ ID NO:26) 2 R: TTCTGCAGTCGAGGCTGGCGTAAGCCTCCCTGCAGTT (SEQ ID NO:27)
pPE-RGH-3	3 F: AACTGCAGCGCCAGCCTCGAGAGGGCCGTCTGCAGAA (SEQ ID NO:28) 3 R: TTCTGCAGACGGCCCTCTCGAGGCTGGCGCTGCAGTT (SEQ ID NO:29)
pPE-RGH-4	4 F: AACTGCAGCGCCAGCCTCGAGTCGGCCGTCTGCAGAA (SEQ ID NO:30) 4 R: TTCTGCAGACGGCCGACTCGAGGCTGGCGCTGCAGTT (SEQ ID NO:31)
pPE-RGH-5	5 F: AACTGCAGCGCCAGCCTCGAGCACGTCGTCTGCAGAA (SEQ ID NO:32) 5 R: TTCTGCAGACGACGTGCTCGAGGCTGGCGCTGCAGTT (SEQ ID NO:33)

6.2. Expression of Delivery Constructs

[0215] *E. coli* BL21(DE3) pLysS competent cells (Novagen, Madison, WI) were transformed using a standard heat-shock method in the presence of the appropriate plasmid to generate ntPE-rat Growth Hormone (rGH) expression cells, selected on ampicillin-containing media, and isolated and grown in Luria-Bertani broth (Difco; Becton Dickinson, Franklin Lakes, N.J.) with antibiotic, then induced for protein expression by the addition of 1 mM isopropyl-D-thiogalactopyranoside (IPTG) at OD 0.6. Two hours following IPTG induction, cells were harvested by centrifugation at 5,000 rpm for 10 min. Inclusion bodies were isolated following cell lysis and proteins were solubilized in the buffer containing 100 mM

Tris_HCl (pH 8.0), 2 mM EDTA, 6 M guanidine HCl, and 65 mM dithiothreitol. Solubilized His ntPE-rGH is refolded in the presence of 0.1 M Tris, pH=7.4, 500 mM L-arginine, 0.9 mM GSSG, 2 mM EDTA. The refolded proteins were purified by Q sepharose Ion Exchange and Superdex 200 Gel Filtration chromatography (Amersham BioSciences, Inc., Sweden). The purity of proteins was assessed by SDS-PAGE and analytic HPLC (Agilent, Inc. Palo Alto, CA).

6.3. Characterization of a Delivery Construct

[0216] The following procedures can be used to assess proper refolding of a delivery construct. The protein refolding process is monitored by measuring, *e.g.*, Delivery Construct 1 binding activity with ntPE binding receptor, CD 91 receptors, and rGH binding proteins on a Biacore SPR instrument (Biacore, Sweden) according to the manufacturer's instructions. Proper refolding of other macromolecules in exemplary constructs can be tested in similar binding assays with appropriate binding agents. By testing such binding affinities, the skilled artisan can assess the proper folding of each portion of the delivery construct.

6.4. Delivery Construct Cleavage Assays

[0217] This example describes experiments performed to identify and verify enzymes that can be used to cleave the cleavable linkers of the delivery constructs described herein. First, Caco-2 (ATCC Accession No. HTB-37) cells in passage 21 were obtained from American Type Culture Collection (Manassas, VA). Human tracheal epithelial (HTE) cells were obtained from J. Whiddecombe of the Department of Physiology at the University of California, Davis Medical School. Caco-2 cells were routinely grown on 75 cm² plastic culture flasks (Becton Dickinson, Franklin Lakes, NJ) in DMEM containing 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a 5% CO₂/95% air atmosphere. HTE cells were grown as described in Yamaya *et al.*, 1992, *Am J Physiol.* 262(6 Pt 1):L713-24.

[0218] To identify suitable cleavable linkers, HTE or Caco-2 cells were seeded at a density of 5×10^4 cells/cm² onto 24-well collagen-coated polycarbonate transwell filters (Corning, Acton, MA) for 12-14 days. Confluent monolayers achieved a transepithelial resistance (TER) of $>500 \text{ ohm}\cdot\text{cm}^2$, as measured using an EVOM epithelial voltohmmeter and STX2 electrode (World Precision Instruments, Sarasota, FL). To determine specific enzyme activity, substrates specific for the tested peptidase (500 μM or 1 mM substrate in 250 μl DMEM without FBS or antibiotics) were added to either the apical (AP) or basolateral (BL) side of the monolayers. Peptidase substrates were obtained from Calbiochem, Inc.

(Division of EMD Biosciences, Inc., San Diego, CA). Cells were incubated for 2 hrs at 37°C in a 5% CO₂/95% air atmosphere. Both the apical and basolateral media was then measured for its specific enzyme activity according to the manufacturer's instruction. Cleavage was assessed by detecting fluorescence of the substrates, which reflects cleavage because it separates of the quenching agent from the fluorescent agent present on the substrate, which separation allows fluorescence to be detected.

[0219] Table 4 presents a summary of the results of these assays using HTE cells, while Table 5 presents a summary of the results of these assays using Caco-2 cells. For all results, baseline control values were subtracted from substrate values before percentages were determined and tests were performed at least in duplicate. The percentages presented in the tables represent the percent increase observed in assay in the apical or basolateral media, which depends on which side of the membrane exhibits higher peptidase activity. It should be noted that, even when substrate was added to the media on the apical side of the membrane, peptidase activity can be observed on the basolateral side of the membrane because of diffusion of the substrate across the membrane.

Table 4				
Peptidase tested in HTE cells	% AP>BL	% BL>AP	AP A405nm	BL A405nm
AP-500 uM Cathepsin B I	86%		0.37	0.20
BL-500 uM Cathepsin B I	64%		0.35	0.21
AP-500 uM Cathepsin G I	882%		0.04	0.004
BL-500 uM Cathepsin G I	6%		0.02	0.02
AP-500 uM Cathepsin G II	371%		0.02	0.01
BL-500 uM Cathepsin G II	0%	0%	0.02	0.02
AP-500 uM Cathepsin G III		11%	0.02	0.02
BL-500 uM Cathepsin G III		400%	0.01	0.03
AP-500 uM Chymotrypsin I	74%		0.04	0.02
BL-500 uM Chymotrypsin I		36%	0.03	0.04
AP-500 uM Elastase I	49%		0.05	0.03
BL-500 uM Elastase I		23%	0.02	0.03
AP-500 uM Elastase II	43%		0.29	0.20
BL-500 uM Elastase II	31%		0.18	0.13
AP-500 uM Elastase III	89%		0.04	0.02
BL-500 uM Elastase III	967%		0.03	0.003
AP-500 uM Elastase IV	84%		0.35	0.19
BL-500 uM Elastase IV	65%		0.23	0.14
AP-500 uM Elastase VIII	529%		0.16	0.03
BL-500 uM Elastase VIII	181%		0.09	0.03
AP-500 uM Papain	57%		0.02	0.02

BL-500 uM Papain	5%		0.03	0.03
AP-500 uM Subtilisin A I		9%	0.02	0.02
BL-500 uM Subtilisin A I		3000%	0.001	0.03
AP-500 uM Subtilisin A II	21%		0.02	0.02
BL-500 uM Subtilisin A II		55%	0.01	0.02
AP-500 uM Thrombin I	42%		0.15	0.11
BL-500 uM Thrombin I		15%	0.09	0.10
AP-500 uM Thrombin II	445%		0.40	0.07
BL-500 uM Thrombin II	741%		0.41	0.05
AP-500 uM Urokinase I	8%		0.11	0.10
BL-500 uM Urokinase I		4%	0.13	0.13
AP-1 mM Cathepsin B I	17%		0.18	0.15
BL-1 mM Cathepsin B I	42%		0.24	0.17
AP-1 mM Cathepsin G I	114%		0.05	0.02
BL-1 mM Cathepsin G I		47%	0.02	0.03
AP-1 mM Cathepsin G II	138%		0.05	0.02
BL-1 mM Cathepsin G II	19%		0.03	0.03
AP-1 mM Cathepsin G III	225%		0.07	0.02
BL-1 mM Cathepsin G III		54%	0.02	0.03
AP-1 mM Chymotrypsin I	35%		0.06	0.04
BL-1 mM Chymotrypsin I		90%	0.04	0.07
AP-1 mM Elastase I		108%	0.02	0.03
BL-1 mM Elastase I		864%	0.01	0.09
AP-1 mM Elastase II	62%		0.28	0.17
BL-1 mM Elastase II	42%		0.33	0.23
AP-1 mM Elastase III	318%		0.02	0.01
BL-1 mM Elastase III	131%		0.04	0.02
AP-1 mM Elastase IV	94%		0.41	0.21
BL-1 mM Elastase IV	61%		0.30	0.19
AP-1 mM Elastase VIII	233%		0.14	0.04
BL-1 mM Elastase VIII	41%		0.06	0.05
AP-1 mM Papain	424%		0.02	0.004
BL-1 mM Papain	141%		0.02	0.01
AP-1 mM Subtilisin A I	18%		0.03	0.03
BL-1 mM Subtilisin A I		290%	0.01	0.04
AP-1 mM Subtilisin A II	17%		0.03	0.02
BL-1 mM Subtilisin A II		318%	0.01	0.04
AP-1 mM Thrombin I	27%		0.17	0.14
BL-1 mM Thrombin I	28%		0.17	0.13
AP-1 mM Thrombin II	20%		0.12	0.10
BL-1 mM Thrombin II		13%	0.05	0.06
AP-1 mM Urokinase I		14%	0.19	0.21
BL-1 mM Urokinase I	4%		0.21	0.20

Table 5				
Peptidase tested in Caco-2 cells	% AP>BL	% BL>AP	AP A405nm	BL A405nm

AP-500 uM Cathepsin B I	150%		0.14	0.06
BL-500 uM Cathepsin B I	34%		0.17	0.12
AP-10 mM Cathepsin G I	195%		0.31	0.10
BL-10 mM Cathepsin G I		145%	0.42	1.03
AP-500 uM Cathepsin G III	35%		0.014	0.01
BL-500 uM Cathepsin G III	185%		0.03	0.01
AP-500 uM Cathepsin G I	232%		0.05	0.01
BL-500 uM Cathepsin G I		1709%	0.01	0.15
AP-500 uM Cathepsin G II	3900%		0.01	0.0003
BL-500 uM Cathepsin G II		1330%	0.003	0.04
AP-500 uM Chymotrypsin I		342%	0.01	0.04
BL-500 uM Chymotrypsin I		403%	0.03	0.16
AP-500 uM Elastase I	73%		0.01	0.01
BL-500 uM Elastase I	295%		0.04	0.01
AP- 500 uM Elastase II	59%		0.12	0.07
BL- 500 uM Elastase II		89%	0.01	0.02
AP-500 uM Elastase III	85%		0.01	0.07
BL-500 uM Elastase III		320%	0.003	0.01
AP- 500 uM Elastase IV	32%		0.11	0.08
BL- 500 uM Elastase IV		12%	0.02	0.02
AP-500 uM Elastase VIII		16%	0.02	0.02
BL-500 uM Elastase VIII		115%	0.01	0.02
AP-500 uM Papain		19%	0.018	0.02
BL-500 uM Papain	339%		0.07	0.02
AP-500 uM Subtilisin A I		***23%	-	0.05
BL-500 uM Subtilisin A I		***94%	-	0.20
AP-500 uM Subtilisin A II	N/A		-	-
BL-500 uM Subtilisin A II		***11%	-	0.02
AP-500 uM Thrombin I	81%		0.04	0.02
BL-500 uM Thrombin I		254%	0.01	0.04
AP-Thrombin II 500 uM	42%		0.08	0.06
BL-Thrombin II 500 uM	62%		0.09	0.06
AP-500 uM Urokinase I	111%		0.12	0.06
BL-500 uM Urokinase I		1044%	0.005	0.05
AP-1 mM Cathepsin B I	109%		0.27	0.13
BL-1 mM Cathepsin B I		58%	0.12	0.2
AP-20 mM Cathepsin G I		129%	0.10	0.23
BL-20 mM Cathepsin G I		540%	0.11	0.70
AP-1 mM Cathepsin G III	37%		0.01	0.01
BL-1 mM Cathepsin G III	103%		0.07	0.03
AP-1 mM Cathepsin G I	107%		0.08	0.04
BL-1 mM Cathepsin G I	144%		0.12	0.05
AP-1 mM Cathepsin G II		11%	0.05	0.06
BL-1 mM Cathepsin G II		7850%	0.00	0.04
AP-1 mM Chymotrypsin I	107%		0.08	0.04
BL-1 mM Chymotrypsin I		288%	0.02	0.07
AP-1 mM Elastase I	217%		0.03	0.001

BL-1 mM Elastase I		880%	0.003	0.02
AP-1 mM Elastase II	27%		0.17	0.14
BL-1 mM Elastase II		34%	0.02	0.03
AP-1 mM Elastase III	192%		0.02	0.01
BL-1 mM Elastase III	77%		0.02	0.01
AP-1 mM Elastase IV	42%		0.16	0.11
BL-1 mM Elastase IV		10%	0.04	0.05
AP-1 mM Elastase VIII	70%		0.05	0.03
BL-1 mM Elastase VIII	332%		0.11	0.03
AP-1 mM Papain	61%		0.02	0.01
BL-1 mM Papain		0%	0.005	0.005
AP-1 mM Subtilisin A I		***61%	-	0.13
BL-1 mM Subtilisin A I		***44%	-	0.09
AP-1 mM Subtilisin A II	N/A		-	-
BL-1 mM Subtilisin A II	N/A		-	-
AP-1 mM Thrombin I	420%		0.11	0.02
BL-1 mM Thrombin I		3400%	0.005	0.16
AP-Thrombin II 1 mM	163%		0.14	0.05
BL-Thrombin II 1 mM	29%		0.11	0.09
AP-1 mM Urokinase I	57%		0.17	0.11
BL-1 mM Urokinase I		230%	0.05	0.15

***denotes % over baseline control only

"-" denotes values below baseline control

6.5. Delivery of an Exemplary Macromolecule - Green Fluorescent Protein

[0220] The following example describes experiments performed to assess the transcytosis of an exemplary delivery construct for delivering green fluorescent protein ("GFP") across a mouse epithelial membrane. It is noted that this exemplary delivery construct does not comprise a cleavable linker; however, the presence or absence of the cleavable linker should not affect transcytosis of the delivery construct.

[0221] Briefly, a nt-PE-GFP construct was applied to the trachea of anesthetized female balb/c mice which were approximately 8 weeks of age. The mice were anesthetized with inhaled isoflurane and the trachea was exposed. A small hole was made on the trachea to allow application of our GFP material. In our experiments, 100 µg of GFP alone or ntPE-GFP was used, respectively. The GFP material was slowly dripped directly onto the exposed trachea in a 100 µl volume. After 15 minutes, the mice were euthanized by CO₂ asphyxiation. The trachea was removed and frozen in OCT (cat#25608-930-Tissue Tek) using biopsy cryomolds (cat#4565-Tissue Tek). The samples were sectioned onto slides and visualized by fluorescence microscopy (Nikon model Eclipse E400).

[0222] Micrographs of the epithelial sections are presented as Figures 1A-1C. Figure 1A shows the nt-PE-GFP construct adhering strongly to the apical surface of the trachea epithelium. Figure 1B shows transcytosis of the nt-PE-GFP construct across the trachea epithelium. Figure 1C shows release of the nt-PE-GFP construct from the basolateral side of the trachea epithelium. Figure 1D presents a micrograph of a negative control, a tracheal epithelial section from a mouse contacted with GFP alone. The tissues exposed for 15 minutes to obtain these micrographs.

[0223] The micrographs demonstrate that nt-PE-GFP interacts strongly with receptors on the apical surface of mouse tracheal epithelium, transcytoses across such epithelial tissue, and releases from the basolateral surface of the mouse tracheal epithelium.

[0224] In addition, plasma concentrations of the nt-PE-GFP construct were determined following administration of the delivery construct using an ELISA assay as described in Example 6.6.1, below. Serum samples were taken from anesthetized mice that had received intranasal administration of 100 µg of the nt-PE-GFP delivery construct every 30 minutes following administration. Figure 2 presents the results of this experiment, demonstrating that peak plasma levels of the delivery construct reached between 500-900 ng/ml, indicating that the delivery construct displayed approximately 22% bioavailability following intranasal administration.

6.6. Detection of Growth Hormone Protein in Tissue by Histological Examination

[0225] This example describes histological detection in tissues of a representative macromolecule for delivery, growth hormone. Following administration of a delivery construct, animals are euthanized by CO₂ asphyxiation and exanguinated by cardiac puncture. Specific tissues (lymph nodes, trachea, brain, spleen liver, GI tract) are removed, briefly rinsed in PBS to remove any residual blood and frozen in OCT. Sections (5 microns thick) are placed onto slides. Slides are fixed in acetone for 10 min and rinsed with PBS. Slides are incubated with 3% peroxidase for 5 min. Slides are then blocked with protein for an additional 5 min. Primary growth hormone antibody is incubated onto slides for 30 min at a 1:100 dilution followed by PBS washes. Biotin-labeled secondary antibody is then incubated for approximately 15 minutes followed by PBS washes. Streptavidin HRP label is incubated onto slides for 15 min followed by PBS washes. HRP Chromagen is applied for 5 min

followed by several rinses in distilled H₂O. Finally, the slides are counterstained with hematoxylin for 1 min, coverslipped, and examined for the presence of GH.

6.7. Transport and Cleavage of an Exemplary Delivery Construct in an *In Vitro* System

[0226] This example describes transport and cleavage of an exemplary delivery construct, Delivery Construct 2, comprising rat growth hormone (rGH) in an *in vitro* system using human tracheal epithelial cells.

6.7.1. Growth of Human Tracheal Epithelial Cells

[0227] Human tracheal epithelial (HTE) cells were isolated from tracheas as previously described and cultured on semi-permeable filter systems (0.45 μ m pore size; Corning, Acton, MA) coated with human placental collagen. See Yamaya *et al.*, 1992, *Am J Physiol*, 262:L713-24 and Sachs *et al.*, 2003, *In Vitro Cell Dev Biol Anim*, 39:56-62. Cell sheets were used at >10 days following plating, at which time they had a transepithelial resistance (TER) of >100 Ω ·ohms.cm² as measured with a "chopstick" voltohmmeter (Millicell ERS, Manassas, VA).

[0228] Caco-2 cells in passage 21 were obtained from American Type Culture Collection (Manassas, VA). Cells were routinely grown on 75 cm² plastic culture flasks (Becton Dickinson, Franklin Lakes, NJ) in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a 5% CO₂/95% air atmosphere. For the transport and cleavage studies, Caco-2 cells were seeded at a density of 5 X 10⁴ cells/cm² onto 24-well collagen-coated polycarbonate transwell filters (Corning, Acton MA) for 12-14 days. Confluent monolayers achieved a transepithelial resistance (TER) of >500 ohm·cm², as measured using the EVOM and STX2 electrode (World Precision Instruments).

6.7.2. Transport and Cleavage Assays

[0229] To determine transport and cleavage activity, Delivery Constructs 1 and 2 proteins (10 μ g in 100 μ l DMEM without phenol red, FBS or antibiotics) were added to the apical side of the epithelial monolayer. Cells were incubated for 4 hrs at 37°C in a 5% CO₂/95% air atmosphere. Both the apical and basolateral media was then assayed for its transport and cleavage activity by testing for the presence of rGH cleaved from the delivery construct by western blot analysis, as described below. As a control, 10 μ g of dextran fluorescein was also added to the apical wells in order to check for leakage.

[0230] Following the 4 hour incubation described above, apical and basolateral media samples were precipitated by the addition of trichloroacetic acid (TCA). The amount of TCA added to each sample was twenty percent relative to the sample volume. Each sample was vortexed and placed on ice for 30 minutes. After samples this incubation on ice, samples were centrifuged at 14,000RPM for 10 minutes. Next, the supernatants from each sample were aspirated and tubes containing the remaining pellet were left open to air dry. 4 μ l 0.2M NaOH was added to each pellet. Five minutes after the addition of NaOH, pellets were resuspended in 36 μ l 8M Urea.

[0231] Next, 10 μ l Sample Buffer containing DTT (Invitrogen NP0007, NP0009) was added to each sample. Samples were then placed on a 100°C heating block for 5 minutes. Half (19 μ l) of each sample was then loaded on to a 4%-12% Tris Bis Gel (Invitrogen NP3022BOX). For controls, recombinant rat GH (RDI R0125) and Delivery Construct 1 or Delivery Construct 2 proteins were also loaded directly into the gel. Electrophoresis was at 150V for 30 minutes. From the gel, samples were transferred onto nitrocellulose at 30V for 1 hour.

[0232] The Blocking Solution, Antibody Diluents, and Antibody Wash Solution from Invitrogen's WesternBreeze were used in subsequent steps. The nitrocellulose membrane was placed in blocking buffer and incubated at 4°C, overnight. The membrane was washed 3 times for 3 minutes each time. The membrane was then incubated at RT with 10 ml of 1:2000 rabbit anti growth hormone (RDI RDIRtGHabr). After one hour, the membrane was again rinsed 3 times for 3 minutes each. Membrane was incubated for 1 hour at room temperature with 10 ml of goat anti rabbit IgG AP (Pierce 31340) at 1:5000. The membrane was rinsed 3 times for 3 minutes per wash. 5 μ l of substrate (Pierce 34042) was added to the membrane. After color development reached desired intensity, reaction was halted by the removal of substrate and the addition of purified water. Finally, the membrane was washed in purified water for 30 minutes and air dried.

[0233] Results of the Western Blot analysis are presented in Figure 4. As seen in Figure 4, media from the basolateral side of the epithelial cell layer contained protein consistent with cleaved rGH separated from the remainder of Delivery Construct 2. In contrast, media from the apical side of the epithelial cell layer contained largely intact delivery construct. Thus, application of Delivery Construct 2 to the apical side of the human epithelial cell membrane resulted in both transport to the basolateral side of the membrane

and proper cleavage of the construct as shown by release of rGH detectable with anti-rGH antibody and of proper apparent molecular weight. Similar results were also observed for Delivery Construct 1 (data not shown).

6.8. Delivery of an Exemplary Macromolecule in an *In Vivo* System

[0234] This example describes use of exemplary Delivery Construct 2 in a mouse model, showing effective transport and cleavage of the delivery construct *in vivo* and the bioactivity of the macromolecule delivered by Delivery Construct 2, rGH.

6.8.1. Administration of a Delivery Construct Comprising Rat Growth Hormone

[0235] Using an animal feeding needle, 100 µg of Delivery Construct 2 (in 250 µl total volume) was orally delivered to female BALB/c mice, 5-6 weeks of age (Charles River Laboratories, Wilmington, MA). Delivery Construct 2 was diluted in 1 mg/ml bovine serum albumin (BSA) and phosphate buffered saline (PBS). As a positive control, control mice were subcutaneously (SC) injected on their dorsal side with 30 µg of recombinant rat growth hormone (rGH) diluted in PBS (100 µl total volume). At specific times after oral gavage and SC administration, mice were euthanized by CO₂ asphyxiation and exsanguinated. Whole blood and liver were collected and analyzed as described below. Because in the difference in molecular weights between Delivery Construct 2 and rGH, essentially the same number of rGH molecules were administered in both routes.

6.8.2. Pharmacokinetics of an Exemplary Macromolecule Administered with a Delivery Construct in an *In Vivo* System

[0236] To assess the pharmacokinetics of an exemplary macromolecule delivered with a delivery construct, ELISA assays were used to measure serum concentrations of rGH at defined timepoints following administration. The serum concentration data thus obtained was used to compare the pharmacokinetics of rGH administered with Delivery Construct 2 to those observed with conventional subcutaneous administration. The ELISA assays were performed as follows.

[0237] Costar 9018 E.I.A./R.I.A. 96-well plates were coated overnight with 200ng/well of goat anti-rGH (Diagnostics Systems Laboratories, Cat. No. R01235) in 0.2M NaHCO₃-Na₂CO₃, pH 9.4. Each 96-well plate was washed four times with PBS containing 0.05% Tween 20-0.01% thimerosal (wash buffer); blocked for 1 h with 200µl/well of PBS/Tween 20 containing 0.5% BSA-0.01% thimerosal (assay buffer). The standard curve was prepared

using recombinant rat GH (Diagnostics Systems Laboratories, Cat. No. R01205) diluted in assay buffer (PBST-0.5%BSA). The first point of the standard curve was prepared by adding 50 μ l recombinant rat GH to 10 ml assay buffer (1:200), vortexed, and moved 200 μ l to 800 μ l assay buffer (1:5). For each plate, 0.5 ml was moved to 0.5 ml assay buffer by doing 1:2 serial dilutions for all the subsequent points. The 10 points of the standard curve are: 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39 and 0.195 ng/well. Samples were diluted at 1:10 with assay buffer, loaded 100 μ l/well in triplicates onto a 96-well plate, and incubated overnight. Each 96-well plate was then washed four times with wash buffer, loaded 100 μ l/well of 2nd Ab (rabbit anti-rGH, Cell Sciences, Cat. No. PAAC1) at 1:300 in assay buffer (PBST-0.5%BSA) and incubate at RT for four hours. Each 96-well plate was then washed four times with wash buffer, loaded 100 μ l/well of 3rd Ab (goat anti-rabbit IgG- horseradish peroxidase (HRP), Pierce, Cat. No. 31460) at 1:2000 in assay buffer (PBST-0.5%BSA) and incubated at room temperature for two hours. All incubation and coating steps were performed at room temperature on a shaker at 6 RPM. The HRP substrate, TMB (3,3',5,5'-tetramethylbenzidine), used to quantify bound antibody, was measured at 450nm.

[0238] ELISA results are reported as the averages of the triplicate OD (450 nm) value of each sample. Rat GH concentrations were determined by the exceeding mean value plus three times the standard error of the mean (SEM) of the appropriate control value.

[0239] The results of the ELISA assays are presented in Figures 5-7. Figure 5 presents serum rGH concentrations in BALB/c mice which were dosed by subcutaneous (SC) injection of 30 μ g of non-glycosylated recombinant rat GH. Individual mice sera were tested at a dilution of 1:10, and the group average of rGH concentration were reported (n=4 mice per time point). Standard error of the mean (SEM) was indicated by the error bars. As shown in Figure 5, peak serum concentration of about 240 ng/ml rGH was observed 30 minutes after administration of subcutaneous rGH.

[0240] Figure 6 presents serum rGH concentrations in BALB/c mice which were dosed orally with 100 μ g of Delivery Construct 2. Individual mice sera were tested at a dilution of 1:10, and the group average of rGH concentration were reported (n=4 mice per time point). Standard error of the mean (SEM) was indicated by the error bars. As shown in Figure 5, peak serum concentration of about 280 ng/ml rGH was observed 20 minutes after administration of Delivery Construct 2.

[0241] Figure 7 presents a graphical representation comparing pharmacokinetics of rGH delivered subcutaneously and with Delivery Construct 2. The curve fitting comparison between rGH (SC) and Delivery Construct 2(Oral) was performed by evaluating ELISA data as described above using PK Solutions 2.0, Pharmacokinetics Data Analysis (Summit Research Services, Montrose, CO). As shown in Figure 7, Delivery Construct 2 yields substantially higher peak serum rGH concentrations than subcutaneous administrations of rGH. Further, the peak serum concentration is achieved faster with Delivery Construct 2 relative to subcutaneous rGH. The bioavailability of rGH delivered with Delivery Construct 2 observed was about 60% relative to rGH administered by subcutaneous injection.

6.8.3. Assays Demonstrating Activity of a Macromolecule Following Delivery with a Delivery Construct

[0242] This example describes analysis of the biological effects of an exemplary macromolecule, rGH, delivered with Delivery Construct 2 in an *in vivo* system. In brief, insulin-like growth factor I-binding protein 3 (IGF-I-BP3), growth hormone (GH) receptor and insulin-like growth factor I (IGF-I) expression levels were assessed in liver tissue from mice administered either Delivery Construct 2 or subcutaneous rGH as described above. These transcripts were analyzed because of the well-characterized effects of GH binding to its receptor on IGF-I-BP3 and GH receptor levels. In particular, functional activation of the GH receptor following binding by GH is known to result in upregulation of IGF-I-BP3 and downregulation of GH receptor expression. Of these, upregulation of IGF-I-BP3 mRNA expression is believed to be the most reliable indicator of GH receptor activation. *See, e.g., Sondergaard et al., 2003, Am J Physiol Endocrinol Metab 285:E427-32.*

[0243] Thus, Quantitative Real Time PCR was used to detect and quantify the amount of IGF-I-BP3, GH receptor, IGF-I, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in approximately 30 mg of mouse liver tissue prepared as described above. Collected liver tissue was stored at -70°C until further processing. Real-time detection of PCR was performed using the Applied Biosystems 7300 Real Time PCR system (Applied Biosystems, Foster City, CA). Total RNA from mouse liver was isolated according to the RNeasy Protect Mini Kit (Qiagen). Total RNA was used to generate cDNA for oligo dT oligodeoxynucleotide primer (T12-18) following the protocol for Omniscript Reverse Transcriptase (Qiagen). The primers used to amplify the cDNA were designed using Primer Express software (Applied Biosystems), synthesized by Operon (Alameda, CA), and are shown in Table 6:

Table 6

RT-PCR Primers	
IGF-I-BP3 (forward):	CGCAGAGAAATGGAGGACACA;
IGF-I-BP3 (reverse):	GGACGCCTCTGGGACTCA;
GH receptor (forward):	GTTGACGAAATAGTGCAACCTGAT;
GH receptor (reverse):	CACGAATCCCGGTCAAACCTAA;
IGF-I (forward):	GCTATGGCTCCAGCATTCG;
IGF-I (reverse):	GCTCCGGAAGCAACACTCA
GAPDH (forward):	GCAACAGGGTGGTGGACCT
GAPDH (reverse):	GGATAGGGCCTCTCTTGCTCA

[0244] Equal amounts of cDNA were used in duplicate and amplified with the SYBR Green I Master Mix (Applied Biosystems). The thermal cycling parameters were as follows: thermal activation for 10 min at 95°C, and 40 cycles of PCR (melting for 15 s at 95°C and annealing/extension for 1 min at 60°C). A standard curve was constructed with a dilution curve (1:10, 1:100, 1:500, 1:1,000, 1:2,000) of total RNA from a control mouse liver sample. A “no template control” was included with each PCR. Amplification efficiencies were validated and normalized against GAPDH. Correct PCR product size was confirmed by electrophoresis through a 1% agarose gel stained with ethidium bromide. Purity of the amplified PCR products was determined by a heat-dissociation protocol.

[0245] The results of this analysis are shown in Figures 8-10. Figure 8 shows expression levels of IGF-I-BP3 mRNA in the liver of female BALB/c mice treated with 30 µg recombinant rGH by subcutaneous injection or with 100 µg of Delivery Construct 2 by oral gavage. Total RNA extracted from the liver was subjected to quantitative RT-PCR using primers specific for IGF-I-BP3, as described above. Values were normalized to glyceraldehyde-3 phosphate dehydrogenase (GAPDH) and expressed as % of control. As shown in Figure 8, administration of subcutaneous rGH resulted in an about 250% increase in IGF-1-BP3 mRNA expression levels in the liver at 60 minutes following administration. In contrast, Delivery Construct 2 caused an almost 400% increase in IGF-1-BP3 mRNA expression levels in the liver 30 minutes following administration. Thus, oral administration of Delivery Construct 2 effectively delivered rGH to the bloodstream of the test mice, thereby demonstrating that a delivery construct can effectively deliver an active macromolecule across a mucous membrane in an *in vivo* system. Moreover, Delivery Construct 2 delivered more active rGH to the liver than subcutaneous administration, and the effects of

administration of rGH were observed substantially faster than possible with subcutaneous administration of rGH.

[0246] Figure 9 shows expression levels of growth hormone (GH) receptor mRNA in the liver of female BALB/c mice treated with recombinant rat growth hormone (rGH) by subcutaneous injection (30 μ g) or Delivery Construct 2 by oral gavage (100 μ g). Total RNA extracted from the liver was subjected to quantitative RT-PCR using primers specific for GH receptor, shown above. Values were normalized to glyceraldehyde-3 phosphate dehydrogenase (GAPDH) and expressed as % of control. As shown in Figure 9, administration of subcutaneous rGH resulted in a reduction in GH receptor mRNA expression levels in the liver at 60 minutes following administration to about 65% of those observed prior to administration. In contrast, Delivery Construct 2 caused such mRNA levels to decrease to about 15% of those observed prior to administration. Thus, these results confirm that oral administration of Delivery Construct 2 is effective to deliver rGH to the bloodstream of a subject, and further, that Delivery Construct 2 delivers significantly more active rGH to mouse liver than conventional subcutaneous administration of rGH as shown by the enhanced downregulation of GH receptor mRNA expression.

[0247] Figure 10 shows expression levels of insulin-like growth factor I (IGF-I) mRNA in the liver of female BALB/c mice treated with recombinant rat growth hormone (rGH) by subcutaneous injection (30 μ g) or Delivery Construct 2 by oral gavage (100 μ g). Total RNA extracted from the liver was subjected to quantitative RT-PCR using primers specific for IGF-I, shown above. Values were normalized to glyceraldehyde-3 phosphate dehydrogenase (GAPDH) and expressed as % of control. As shown in Figure 10, administration of either subcutaneous rGH or Delivery Construct 2 resulted in a reduction in IGF-1 mRNA expression levels in the liver at 30 minutes following administration to about 20% of those observed prior to administration. Thus, both subcutaneous rGH and orally-administered Delivery Construct 2 yielded the same effects, demonstrating that Delivery Construct 2 can deliver active rGH to the bloodstream of a

6.9. Reduced Immunogenicity of Macromolecules Administered with a Delivery Construct

[0248] This example shows that an exemplary macromolecule, rGH, administered orally with Delivery Construct 2, is less immunogenic than rGH administered subcutaneously.

[0249] To assess the relative immunogenicity in mice for rGH administered a delivery construct relative to subcutaneous administration, the serum titer of anti-rGH IgG antibodies from oral administration of 3, 10, or 30 µg Delivery Construct 2 or 3 or 10 µg subcutaneous rGH was determined in an ELISA assay. To do so, 100 µl 1 ng/ µl recombinant rGH diluted in coating buffer (0.2 M NaHCO₃-Na₂CO₃, pH 9.4) was added to Costar EIA/RIA plates, then incubated at room temperature for 16-24 hours. Next, the plates were washed 4 times with 300 µl wash buffer (phosphate-buffered saline). The plates were then blocked with 200 µl blocking buffer (0.5% BSA in phosphate-buffered saline) and incubated at room temperature for 1 hour. Next, the plates were again washed four times with 300 µl wash buffer.

[0250] After preparation of the plates, 100 µl diluted samples, standard positive control, or assay buffer as negative control was added to the appropriate well and incubated for one hour. Mouse serum samples and positive control (anti-rGH IgG) were diluted 1:20 in assay buffer (0.5% BSA in phosphate-buffered saline). The plates were then washed four times with 300 µl wash buffer. Next, 100 µl secondary antibody (goat anti-mouse IgG conjugated to horseradish peroxidase, 0.4 mg/ml, Pierce #31430, diluted at 1:6000 in assay buffer and incubated at room temperature for one hour. Next, the plates were again washed four times with 300 µl wash buffer. Next, 100 µl 3,3',5,5'-Tetramethylbenzidine substrate (Sigma) was added to each well and incubated for 2-10 minutes, depending on color development. 100 µl /well 1M H₂SO₄ was then added to stop the reaction and absorbance read at 450 nm. All assays were performed in triplicate and the results averaged.

[0251] Representative results of the ELISAs are shown in Figures 11A-D. The graphs presented in these figures demonstrate that 3, 10, and 30 µg Delivery Construct 2 administered orally elicited a lower titer of anti-rGH IgG antibodies than either 3 or 10 µg subcutaneous rGH. In particular, subcutaneous administration of 10 µg rGH caused a substantial anti-rGH IgG response in all eight mice, while the eight mice administered 3, 10, or 30 µg Delivery Construct 2 by oral gavage had minimal anti-rGH IgG responses. Further, these observations were consistent whether the sera were diluted 1:25 (Figures 11A and 11C) or 1:200 (Figures 11B and 11D). Finally, it should be noted that each mouse administered 3, 10, or 30 µg Delivery Construct 2 experienced a minimal immune response against rGH, as shown by the tight clustering of the data points in Figures 11C and D. Thus, these results demonstrate that oral administration of Delivery Construct 2 not only delivers more active

rGH to the liver than possible with subcutaneous injection, but further, the active rGH is less immunogenic when administered orally with Delivery Construct 2 compared to subcutaneous administration.

6.10. Exemplary Delivery Construct for Delivery of Human Growth Hormone

[0252] This example describes construction of an exemplary delivery construct for delivering human growth hormone, termed Delivery Construct 6. Techniques similar to those described in Example 6.1, above, were used to construct a plasmid used to express Delivery Construct 6. The nucleotide sequence of the portion of this plasmid that encodes the exemplary delivery construct is presented as Figure 12, while the amino acid sequence of the delivery construct is presented as Figure 13.

6.11. Exemplary Delivery Construct for Delivering Interferon Alpha

[0253] This example describes construction of an exemplary delivery construct for delivering IFN α (in this case, IFN α -2b), termed Delivery Construct 7. Techniques similar to those described in Example 6.1, above, were used to construct a plasmid used to express Delivery Construct 7. The nucleotide sequence of the portion of this plasmid that encodes the exemplary Delivery Construct 7 is presented as Figure 14, while the amino acid sequence of Delivery Construct 7 is presented as Figure 15.

6.12. Delivery of Interferon Alpha in an *In Vivo* System

[0254] This example demonstrates the use of Delivery Construct 7 to deliver IFN α -2b to the bloodstream of a subject in a mouse model system.

[0255] Using an animal feeding needle, 100 μ g of Delivery Construct 7 (in 250 μ l total volume) was orally administered to female BALB/c mice, 5-6 weeks of age (Charles River Laboratories, Wilmington, MA). Delivery Construct 7 was diluted in 1 mg/ml bovine serum albumin (BSA) and phosphate buffered saline (PBS). At specific times after oral gavage and SC administration, mice were euthanized by CO₂ asphyxiation and exsanguinated. Whole blood was collected and analyzed as described below.

[0256] ELISA assays were used to measure serum concentrations of IFN α -2b in one mouse immediately following administration and in three mice at 15, 30, and 75 minutes following administration. The ELISA assays were performed using R&D Systems Serum ELISA Kit No.41110-1 according to the manufacturer's instructions.

[0257] ELISA results are reported as the averages of the triplicate OD (450 nm) value of each sample. The results of the ELISA assays are presented in Figure 16. As shown in Figure 16, IFN α -2b was detected at low (about 3 ng/ml) concentration 15 minutes after administration. 30 minutes following administration, serum concentration of IFN α -2b was about 43 ng/ml. 45 minutes following administration, serum concentration of IFN α -2b had fallen to about 13 ng/ml.

6.13. Exemplary Delivery Construct for Delivering Proinsulin

[0258] This example describes construction of an exemplary delivery construct for delivering proinsulin, termed Delivery Construct 8. Techniques similar to those described in Example 6.1, above, are used to construct a plasmid used to express Delivery Construct 8. The amino acid sequence of Delivery Construct 8 is presented as Figure 17.

6.14. Exemplary Delivery Construct for Delivering Insulin

[0259] This example describes construction of an exemplary delivery construct for delivering insulin, termed Delivery Construct 9. Techniques similar to those described in Example 6.1, above, are used to construct a plasmid used to express Delivery Construct 9, with certain modifications.

[0260] In particular, the scheme used to express Delivery Construct 9 is modified because insulin comprises two separate amino acid chains. In this example, the B-chain of insulin is expressed together with the remainder of the Delivery Construct, constructed according to the general scheme presented in Example 6.1. Amino acids corresponding to the A-chain are made either synthetically (*e.g.*, chemically synthesizing the A-chain peptide from amino acids) or recombinantly (*e.g.*, expressed in a suitable recombinant system such as, for example, *E. coli*, yeast, *etc.*) The two polypeptides are then combined under conditions that permit association of the A-chain and B-chain. Then, disulfide bonds are made between the two chains of insulin as found in native insulin by application of mildly oxidizing conditions. The amino acid sequence of the two amino acid chains of Delivery Construct 9 is presented as Figure 17.

[0261] The present invention provides, *inter alia*, delivery constructs and methods of inducing an immune response in a subject. While many specific examples have been provided, the above description is intended to illustrate rather than limit the invention. Many variations of the invention will become apparent to those skilled in the art upon review of this

specification. The scope of the invention should, therefore, be determined not with reference to the above description, but instead should be determined with reference to the appended claims along with their full scope of equivalents.

[0262] All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent document were so individually denoted. Citation of these documents is not an admission that any particular reference is "prior art" to this invention.

Table 7

Human Peptidases by Class				
Aspartic-type peptidases	Cysteine-type peptidases	Metallopeptidases	Serine-type peptidases	Threonine-type peptidases
BAE1_HUMAN (P56817)	BLMH_HUMAN (Q13867)	AMPB_HUMAN (Q9H4A4)	ACRL_HUMAN (P58840)	PS7L_HUMAN (Q8TAA3)
BAE2_HUMAN (Q9Y5Z0)	CATB_HUMAN (P07858)	AMPE_HUMAN (Q07075)	ACRO_HUMAN (P10323)	PSA1_HUMAN (P25786)
CATD_HUMAN (P07339)	CATC_HUMAN (P53634)	AMPN_HUMAN (P15144)	APOA_HUMAN (P08519)	PSA2_HUMAN (P25787)
CATE_HUMAN (P14091)	CATF_HUMAN (Q9UBX1)	ART1_HUMAN (Q9NZ08)	BSS4_HUMAN (Q9GZN4)	PSA3_HUMAN (P25788)
NAP1_HUMAN (O96009)	CATH_HUMAN (P09668)	LCAP_HUMAN (Q9UIQ6)	CIR_HUMAN (P00736)	PSA4_HUMAN (P25789)
PEPA_HUMAN (P00790)	CATK_HUMAN (P43235)	LKHA_HUMAN (P09960)	CIS_HUMAN (P09871)	PSA5_HUMAN (P28066)
PEPC_HUMAN (P20142)	CATL_HUMAN (P07711)	PSA_HUMAN (P55786)	CAP7_HUMAN (P20160)	PSA6_HUMAN (P60900)
RENI_HUMAN (P00797)	CATO_HUMAN (P43234)	RNPL_HUMAN (Q9HAU8)	CATG_HUMAN (P08311)	PSA7_HUMAN (O14818)
VPRT_HUMAN (P10265)	CATS_HUMAN (P25774)	THDE_HUMAN (Q9UKU6)	CFAB_HUMAN (P00751)	PSB1_HUMAN (P20618)
Other Peptidases	CATW_HUMAN (P56202)	ACET_HUMAN (P22966)	CFAD_HUMAN (P00746)	PSB2_HUMAN (P49721)
	CATZ_HUMAN (Q9UBR2)	ACE_HUMAN (P12821)	CFAI_HUMAN (P05156)	PSB3_HUMAN (P49720)
FAC2_HUMAN (Q9Y256)				

CSL2_HUMAN (O60911)	MEPD_HUMAN (P52888)	CLCR_HUMAN (Q99895)	PSB4_HUMAN (P28070)
TNAG_HUMAN (Q9UJW2)	NEUL_HUMAN (Q9BYT8)	CO2_HUMAN (P06681)	PSB5_HUMAN (P28074)
CAN1_HUMAN (P07384)	PMIP_HUMAN (Q99797)	CORI_HUMAN (Q9Y5Q5)	PSB6_HUMAN (P28072)
CAN2_HUMAN (P17655)	MM01_HUMAN (P03956)	CRAR_HUMAN (P48740)	PSB7_HUMAN (Q99436)
CAN3_HUMAN (P20807)	MM02_HUMAN (P08253)	CTRB_HUMAN (P17538)	PSB8_HUMAN (P28062)
CAN5_HUMAN (O15484)	MM03_HUMAN (P08254)	CTRL_HUMAN (P40313)	PSB9_HUMAN (P28065)
CAN6_HUMAN (Q9Y6Q1)	MM07_HUMAN (P09237)	DES1_HUMAN (Q9UL52)	PSBA_HUMAN (P40306)
CAN7_HUMAN (Q9Y6W3)	MM08_HUMAN (P22894)	EL1_HUMAN (Q9UN11)	
CAN9_HUMAN (O14815)	MM09_HUMAN (P14780)	EL2A_HUMAN (P08217)	
CANA_HUMAN (Q9HC96)	MM10_HUMAN (P09238)	EL2B_HUMAN (P08218)	
CANB_HUMAN (Q9UMQ6)	MM11_HUMAN (P24347)	EL3A_HUMAN (P09093)	
UBL1_HUMAN (P09936)	MM12_HUMAN (P39900)	EL3B_HUMAN (P08861)	
UBL3_HUMAN (P15374)	MM13_HUMAN (P45452)	ELNE_HUMAN (P08246)	
UBL5_HUMAN (Q9Y5K5)	MM14_HUMAN (P50281)	ENTK_HUMAN (P98073)	
GPI8_HUMAN (Q92643)	MM15_HUMAN (P51511)	FA10_HUMAN (P00742)	
LGMN_HUMAN (Q99538)	MM16_HUMAN (P51512)	FA11_HUMAN (P03951)	
CFLA_HUMAN (O15519)	MM17_HUMAN (Q9ULZ9)	FA12_HUMAN (P00748)	
IIBC_HUMAN (P29466)	MM19_HUMAN (Q99542)	FA7_HUMAN (P08709)	
ICE2_HUMAN (P42575)	MM20_HUMAN (O60882)	FA9_HUMAN (P00740)	

ICE3_HUMAN (P42574)	MM21_HUMAN (Q8N119)	GRAA_HUMAN (P12544)
ICE4_HUMAN (P49662)	MM24_HUMAN (Q9Y5R2)	GRAB_HUMAN (P10144)
ICE5_HUMAN (P51878)	MM25_HUMAN (Q9NPA2)	GRAH_HUMAN (P20718)
ICE6_HUMAN (P55212)	MM26_HUMAN (Q9NRE1)	GRAK_HUMAN (P49863)
ICE7_HUMAN (P55210)	MM28_HUMAN (Q9HZ39)	GRAM_HUMAN (P51124)
ICE8_HUMAN (Q14790)	BMP1_HUMAN (P13497)	HATT_HUMAN (O60235)
ICE9_HUMAN (P55211)	MEPA_HUMAN (Q16819)	HEPS_HUMAN (P05981)
ICEA_HUMAN (Q92851)	MEPB_HUMAN (Q16820)	HGFA_HUMAN (Q04756)
ICEE_HUMAN (P31944)	AD02_HUMAN (Q99965)	HGFL_HUMAN (P26927)
MLT1_HUMAN (Q9UDY8)	AD07_HUMAN (Q9HZU9)	HGF_HUMAN (P14210)
PGPI_HUMAN (Q9NXJ5)	AD08_HUMAN (P78325)	HPTR_HUMAN (P00739)
FAFX_HUMAN (Q93008)	AD09_HUMAN (Q13443)	HPT_HUMAN (P00738)
FAFY_HUMAN (O00507)	AD10_HUMAN (O14672)	KAL_HUMAN (P03952)
UB10_HUMAN (Q14694)	AD11_HUMAN (O75078)	KLK1_HUMAN (P06870)
UB11_HUMAN (P51784)	AD12_HUMAN (O43184)	KLK2_HUMAN (P20151)
UB12_HUMAN (O75317)	AD15_HUMAN (Q13444)	KLK3_HUMAN (P07288)
UB13_HUMAN (Q92995)	AD17_HUMAN (P78536)	KLK4_HUMAN (Q9Y5K2)
UB14_HUMAN (P54578)	AD18_HUMAN (Q9Y3Q7)	KLK5_HUMAN (Q9Y337)
UB15_HUMAN (Q9Y4E8)	AD19_HUMAN (Q9H013)	KLK6_HUMAN (Q92876)

UB16_HUMAN (Q9Y5T5)	AD20_HUMAN (O43506)	KLK7_HUMAN (P49862)
UB18_HUMAN (Q9UMW8)	AD21_HUMAN (Q9UKJ8)	KLK8_HUMAN (O60259)
UB19_HUMAN (O94966)	AD22_HUMAN (Q9P0K1)	KLK9_HUMAN (Q9UKQ9)
UB20_HUMAN (Q9Y2K6)	AD28_HUMAN (Q9UKQ2)	KLKA_HUMAN (O43240)
UB21_HUMAN (Q9UK80)	AD29_HUMAN (Q9UKF5)	KLKB_HUMAN (Q9UBX7)
UB22_HUMAN (Q9UPT9)	AD30_HUMAN (Q9UKF2)	KLKC_HUMAN (Q9UKR0)
UB24_HUMAN (Q9UPU5)	AD33_HUMAN (Q9BZ11)	KLKD_HUMAN (Q9UKR3)
UB25_HUMAN (Q9UHP3)	AT10_HUMAN (Q9H324)	KLKE_HUMAN (Q9P0G3)
UB26_HUMAN (Q9BXU7)	AT12_HUMAN (P58397)	KLKF_HUMAN (Q9H2R5)
UB28_HUMAN (Q96RU2)	AT14_HUMAN (Q8WXS8)	LCLP_HUMAN (P34168)
UB29_HUMAN (Q9HBJ7)	AT15_HUMAN (Q8TE58)	MAS2_HUMAN (O00187)
UB32_HUMAN (Q8NFA0)	AT16_HUMAN (Q8TE57)	MCT1_HUMAN (P23946)
UB33_HUMAN (Q8TEY7)	AT17_HUMAN (Q8TE56)	NETR_HUMAN (P56730)
UB35_HUMAN (Q9P2H5)	AT18_HUMAN (Q8TE60)	PLMN_HUMAN (P00747)
UB36_HUMAN (Q9P275)	AT19_HUMAN (Q8TE59)	PR27_HUMAN (Q9BQR3)
UB37_HUMAN (Q86T82)	AT20_HUMAN (P59510)	PRN3_HUMAN (P24158)
UB38_HUMAN (Q8NB14)	ATS1_HUMAN (Q9UHI8)	PRTC_HUMAN (P04070)
UB40_HUMAN (Q9NVE5)	ATS2_HUMAN (O95450)	PRTZ_HUMAN (P22891)
UB42_HUMAN (Q9H9J4)	ATS3_HUMAN (O15072)	PS23_HUMAN (O95084)

UB44_HUMAN (Q9H0E7)	ATS4_HUMAN (O75173)	PSS8_HUMAN (Q16651)
UB46_HUMAN (P62068)	ATS5_HUMAN (Q9UNA0)	ST14_HUMAN (Q9Y5Y6)
UBP1_HUMAN (O94782)	ATS6_HUMAN (Q9UKP5)	TEST_HUMAN (Q9Y6M0)
UBP2_HUMAN (O75604)	ATS7_HUMAN (Q9UKP4)	THRB_HUMAN (P00734)
UBP3_HUMAN (Q9Y6I4)	ATS8_HUMAN (Q9UP79)	TMS2_HUMAN (O15393)
UBP4_HUMAN (Q13107)	ATS9_HUMAN (Q9P2N4)	TMS3_HUMAN (P57727)
UBP5_HUMAN (P45974)	ECE1_HUMAN (P42892)	TMS4_HUMAN (Q9NRS4)
UBP6_HUMAN (P35125)	ECE2_HUMAN (O60344)	TMS5_HUMAN (Q9H3S3)
UBP7_HUMAN (Q93009)	ECEL_HUMAN (Q95672)	TMS6_HUMAN (Q8IU80)
UBP8_HUMAN (P40818)	KELL_HUMAN (P23276)	TPA_HUMAN (P00750)
GGH_HUMAN (Q92820)	NEP_HUMAN (P08473)	TRB1_HUMAN (Q15661)
SEN1_HUMAN (Q9P0U3)	PEX_HUMAN (P78562)	TRB2_HUMAN (P20231)
SEN3_HUMAN (Q9H4L4)	CBP1_HUMAN (P15085)	TRY1_HUMAN (P07477)
SEN5_HUMAN (Q96HI0)	CBP2_HUMAN (P48052)	TRY2_HUMAN (P07478)
SEN6_HUMAN (Q9GZR1)	CBP4_HUMAN (Q9UI42)	TRY3_HUMAN (P35030)
SEN7_HUMAN (Q9BQF6)	CBP5_HUMAN (Q8WXQ8)	TRYA_HUMAN (P15157)
SEN8_HUMAN (Q96LD8)	CBP6_HUMAN (Q8N4T0)	TRYD_HUMAN (Q9BZJ3)
SNP2_HUMAN (Q9HC62)	CBPB_HUMAN (P15086)	TRYG_HUMAN (Q9NRR2)
ESP1_HUMAN (Q14674)	CBPC_HUMAN (P15088)	TS50_HUMAN (Q9UI38)

			CBPD_HUMAN (O75976)	UROK_HUMAN (P00749)
			CBPE_HUMAN (P16870)	HRA1_HUMAN (Q92743)
			CBPM_HUMAN (P14384)	HRA2_HUMAN (O43464)
			CBPN_HUMAN (P15169)	HRA3_HUMAN (P83110)
			CPX2_HUMAN (Q8N436)	HRA4_HUMAN (P83105)
			CPXM_HUMAN (Q96SM3)	FURI_HUMAN (P09958)
			IDE_HUMAN (P14735)	MSIP_HUMAN (Q14703)
			MPPA_HUMAN (Q10713)	NEC1_HUMAN (P29120)
			MPPB_HUMAN (O75439)	NEC2_HUMAN (P16519)
			NRDC_HUMAN (O43847)	PCK5_HUMAN (Q92824)
			UCR1_HUMAN (P31930)	PCK6_HUMAN (P29122)
			UCR2_HUMAN (P22695)	PCK7_HUMAN (Q16549)
			AMPL_HUMAN (P28838)	PCK9_HUMAN (Q8NBP7)
			PEL1_HUMAN (Q8NDH3)	TPP2_HUMAN (P29144)
			DNPE_HUMAN (Q9ULA0)	PPCE_HUMAN (P48147)
			MDP1_HUMAN (P16444)	DPP4_HUMAN (P27487)
			CGL1_HUMAN (Q96KP4)	DPP6_HUMAN (P42658)
			CGL2_HUMAN (Q96KN2)	SEPR_HUMAN (Q12884)
			ACY1_HUMAN (Q03154)	ACPH_HUMAN (P13798)

			GCP_HUMAN (Q9NPF4) AMP1_HUMAN (P53582) PEPD_HUMAN (P12955) XPP2_HUMAN (O43895) AMP2_HUMAN (P50579) P2G4_HUMAN (Q9UQ80) FOH1_HUMAN (Q04609) NLD2_HUMAN (Q9Y3Q0) NLDL_HUMAN (Q9UQQ1) TFR1_HUMAN (P02786) TFR2_HUMAN (Q9UP52) AF31_HUMAN (O43931) AF32_HUMAN (Q9Y4W6) SPG7_HUMAN (Q9UQ90) YME1_HUMAN (Q96TA2) PAPA_HUMAN (Q13219) FAC1_HUMAN (O75844) DPP3_HUMAN (Q9NY33) MS2P_HUMAN (O43462)	CPVL_HUMAN (Q9H3G5) PRTP_HUMAN (P10619) RISC_HUMAN (Q9HB40) CLPP_HUMAN (Q16740) LONM_HUMAN (P36776) SPC3_HUMAN (Q9BY50) SPC4_HUMAN (P21378) DPP2_HUMAN (Q9UHL4) PCP_HUMAN (P42785) TSSP_HUMAN (Q9NQE7) HYEP_HUMAN (P07099) TPPI_HUMAN (O14773) RHB1_HUMAN (O75783) RHB2_HUMAN (Q9NX52) RHB4_HUMAN (P58872)	
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What is claimed is:

1. A delivery construct, comprising:

- a)- a receptor binding domain,
- b)- a transcytosis domain,
- c)- a macromolecule to be delivered to a subject, and
- d)- a cleavable linker,

wherein cleavage at said cleavable linker separates said macromolecule from the remainder of said construct, and wherein said cleavable linker is cleavable by an enzyme that i) exhibits greater activity at a basal-lateral membrane of a polarized epithelial cell of said subject than at an apical membrane of the polarized epithelial cell, or ii) exhibits greater activity in the plasma of said subject than at an apical membrane of the polarized epithelial cell of the subject.

- 2. The delivery construct of Claim 1, further comprising a second cleavable linker.
- 3. The delivery construct of Claim 1, wherein said cleavable linker comprises an amino acid sequence that is selected from the group consisting of Ala-Ala-Pro-Phe (SEQ ID NO.:4), Gly-Gly-Phe (SEQ ID NO.:5), Ala-Ala-Pro-Val (SEQ ID NO.:6), Gly-Gly-Leu (SEQ ID NO.:7), Ala-Ala-Leu (SEQ ID NO.:8), Phe-Val-Arg (SEQ ID NO.:9), Val-Gly-Arg (SEQ ID NO.:10).
- 4. The delivery construct of Claim 1, wherein said enzyme that is present at a basal-lateral membrane of a polarized epithelial cell is selected from the group consisting of Cathepsin GI, Chymotrypsin I, Elastase I, Subtilisin AI, Subtilisin AII, Thrombin I, and Urokinase I.
- 5. The delivery construct of Claim 1, wherein said receptor binding domain is selected from the group consisting of receptor binding domains from *Pseudomonas* exotoxin A, cholera toxin, botulinum toxin, diptheria toxin, shiga toxin, or shiga-like toxin; monoclonal antibodies; polyclonal antibodies; single-chain antibodies; TGF α ; EGF; IGF-I; IGF-II; IGF-III; IL-1; IL-2; IL-3; IL-6; MIP-1a; MIP-1b; MCAF; and IL-8.

6. The delivery construct of Claim 1, wherein said receptor binding domain binds to a cell-surface receptor that is selected from the group consisting of α 2-macroglobulin receptor, epidermal growth factor receptor, transferrin receptor, chemokine receptor, CD25, CD11B, CD11C, CD80, CD86, TNF α receptor, TOLL receptor, M-CSF receptor, GM-CSF receptor, scavenger receptor, and VEGF receptor.
7. The delivery construct of Claim 5, wherein said receptor binding domain of *Pseudomonas* exotoxin A is Domain Ia of *Pseudomonas* exotoxin A.
8. The delivery construct of Claim 7, wherein said receptor binding domain of *Pseudomonas* exotoxin A has an amino acid sequence that is SEQ ID NO.:1.
9. The delivery construct of Claim 1, wherein said transcytosis domain is selected from the group consisting of transcytosis domains from *Pseudomonas* exotoxin A, botulinum toxin, diphtheria toxin, pertussis toxin, cholera toxin, heat-labile *E. coli* enterotoxin, shiga toxin, and shiga-like toxin.
10. The delivery construct of Claim 9, wherein said transcytosis domain is *Pseudomonas* exotoxin A transcytosis domain.
11. The delivery construct of Claim 10, wherein said *Pseudomonas* exotoxin A transcytosis domain has an amino acid sequence that is SEQ ID NO.:2.
12. The delivery construct of Claim 1, wherein said macromolecule is selected from the group of a nucleic acid, a peptide, a polypeptide, a protein, and a lipid.
13. The delivery construct of Claim 12, wherein said polypeptide is selected from the group consisting of polypeptide hormones, cytokines, chemokines, growth factors, and clotting factors.
14. The delivery construct of Claim 13, wherein said polypeptide is selected from the group consisting of IGF-I, IGF-II, IGF-III, EGF, IFN- α , IFN- β , IFN- γ , G-CSF, GM-CSF, IL-1, IL-2, IL-3, IL-6, IL-8, IL-12, EPO, growth hormone, factor VII, vasopressin, calcitonin, parathyroid hormone, luteinizing hormone-releasing factor, tissue plasminogen activators, proinsulin, insulin, glucocorticoid, amylin, adrenocorticototropin, enkephalin, and glucagon-like peptide 1.

15. The delivery construct of Claim 14, wherein said polypeptide is human growth hormone.
16. The delivery construct of Claim 12, wherein said protein is human insulin.
17. The delivery construct of Claim 12, wherein said protein is human IFN- α .
18. The delivery construct of Claim 12, wherein said protein is human IFN- α 2b.
19. The delivery construct of Claim 12, wherein said protein is human proinsulin.
20. The delivery construct of Claim 12, further comprising a second macromolecule that is selected from the group of a nucleic acid, a peptide, a polypeptide, a protein, a lipid, or a small organic molecule and a second cleavable linker, wherein cleavage at said second cleavable linker separates said second macromolecule from the remainder of said construct.
21. The delivery construct of Claim 17, wherein said macromolecule is a first polypeptide and said second macromolecule is a second polypeptide.
22. The delivery construct of Claim 21, wherein said first polypeptide and said second polypeptide associate to form a multimer.
23. The delivery construct of Claim 22, wherein said multimer is a dimer, tetramer, or octamer.
24. The delivery construct of Claim 23, wherein said dimer is an antibody.
25. A polynucleotide that encodes a delivery construct, said delivery construct comprising:
 - a)- a receptor binding domain,
 - b)- a transcytosis domain,
 - c)- a macromolecule to be delivered to a subject, and
 - d)- a cleavable linker,

wherein cleavage at said cleavable linker separates said macromolecule from the remainder of said construct, and wherein said cleavable linker is cleavable by an enzyme that i) exhibits greater activity at a basal-lateral membrane of a polarized epithelial cell of said subject than at an apical membrane of the polarized epithelial cell, or ii) exhibits greater activity in the plasma of said subject than at an apical membrane of the polarized epithelial cell of the subject.

26. A polynucleotide that hybridizes under stringent hybridization conditions to the polynucleotide of Claim 25.
27. The polynucleotide of Claim 25, wherein said delivery construct further comprises a second cleavable linker.
28. The polynucleotide of Claim 25, wherein said cleavable linker comprises an amino acid sequence that is selected from the group consisting of Ala-Ala-Pro-Phe (SEQ ID NO.:4), Gly-Gly-Phe (SEQ ID NO.:5), Ala-Ala-Pro-Val (SEQ ID NO.:6), Gly-Gly-Leu (SEQ ID NO.:7), Ala-Ala-Leu (SEQ ID NO.:8), Phe-Val-Arg (SEQ ID NO.:9), Val-Gly-Arg (SEQ ID NO.:10).
29. The polynucleotide of Claim 25, wherein said enzyme that is present at a basal-lateral membrane of a polarized epithelial cell is selected from the group consisting of Cathepsin GI, Chymotrypsin I, Elastase I, Subtilisin AI, Subtilisin AII, Thrombin I, and Urokinase I.
30. The polynucleotide of Claim 25, wherein said receptor binding domain is selected from the group consisting of receptor binding domains from *Pseudomonas* exotoxin A, cholera toxin, botulinum toxin, diphtheria toxin, shiga toxin, or shiga-like toxin; monoclonal antibodies; polyclonal antibodies; single-chain antibodies; TGF α ; EGF; IGF-I; IGF-II; IGF-III; IL-1; IL-2; IL-3; IL-6; MIP-1a; MIP-1b; MCAF; and IL-8.
31. The polynucleotide of Claim 25, wherein said receptor binding domain binds to a cell-surface receptor that is selected from the group consisting of α 2-macroglobulin receptor, EGFR, IGFR, transferrin receptor, chemokine receptor, CD25, CD11B, CD11C, CD80, CD86, TNF α receptor, TOLL receptor, M-CSF receptor, GM-CSF receptor, scavenger receptor, and VEGF receptor.

32. The polynucleotide of Claim 30, wherein said receptor binding domain of *Pseudomonas* exotoxin A is Domain Ia of *Pseudomonas* exotoxin A.
33. The polynucleotide of Claim 31, wherein said receptor binding domain of *Pseudomonas* exotoxin A has an amino acid sequence that is SEQ ID NO.:1.
34. The polynucleotide of Claim 25, wherein said transcytosis domain is selected from the group consisting of transcytosis domains from *Pseudomonas* exotoxin A, botulinum toxin, diptheria toxin, pertussis toxin, cholera toxin, heat-labile *E. coli* enterotoxin, shiga toxin, and shiga-like toxin.
35. The polynucleotide of Claim 34, wherein said transcytosis domain is *Pseudomonas* exotoxin A transcytosis domain.
36. The polynucleotide of Claim 35, wherein said *Pseudomonas* exotoxin A transcytosis domain has an amino acid sequence that is SEQ ID NO.: 2.
37. The polynucleotide of Claim 25, wherein said macromolecule is selected from the group of a peptide, a polypeptide, and a protein.
38. The polynucleotide of Claim 37, wherein said polypeptide is selected from the group consisting of polypeptide hormones, cytokines, chemokines, growth factors, and clotting factors.
39. The polynucleotide of Claim 38, wherein said polypeptide is selected from the group consisting of IGF-I, IGF-II, IGF-III, EGF, IFN- α , IFN- α 2b, IFN- β , IFN- γ , G-CSF, GM-CSF, IL-1, IL-2, IL-3, IL-6, IL-8, IL-12, EPO, growth hormone, factor VII, vasopressin, calcitonin, parathyroid hormone, luteinizing hormone-releasing factor, tissue plasminogen activators, proinsulin, insulin, glucocorticoid, amylin, adrenocorticotropin, enkephalin, and glucagon-like peptide 1.
40. The polynucleotide of Claim 39, wherein said polypeptide is human growth hormone.
41. The polynucleotide of Claim 39, wherein said protein is human insulin.
42. A polynucleotide that encodes a delivery construct, said polynucleotide comprising:
 - a)- a nucleic acid sequence encoding a receptor binding domain,

- b)- a nucleic acid sequence encoding a transcytosis domain,
- c)- a nucleic acid sequence encoding a cleavable linker, and
- d)- a nucleic acid sequence comprising a polylinker insertion site,

wherein cleavage at said cleavable linker separates said macromolecule from the remainder of said construct, and wherein said cleavable linker is cleavable by an enzyme that i) exhibits greater activity at a basal-lateral membrane of a polarized epithelial cell of said subject than at an apical membrane of the polarized epithelial cell, or ii) exhibits greater activity in the plasma of said subject than at an apical membrane of the polarized epithelial cell of the subject.

- 43. An expression vector comprising the polynucleotide of Claim 25.
- 44. A cell comprising the expression vector of Claim 43.
- 45. A composition comprising a delivery construct, said delivery construct comprising:
 - a)- a receptor binding domain,
 - b)- a transcytosis domain,
 - c)- a macromolecule to be delivered to a subject, and
 - d)- a cleavable linker,

wherein cleavage at said cleavable linker separates said macromolecule from the remainder of said construct, and wherein said cleavable linker is cleavable by an enzyme that i) exhibits greater activity at a basal-lateral membrane of a polarized epithelial cell of said subject than at an apical membrane of the polarized epithelial cell, or ii) exhibits greater activity in the plasma of said subject than at an apical membrane of the polarized epithelial cell of the subject.

- 46. The composition of Claim 45, wherein said composition further comprises a pharmaceutically acceptable diluent, excipient, vehicle, or carrier.
- 47. The composition of Claim 45, wherein said composition is formulated for nasal or oral administration.

48. A method for delivering a macromolecule to a subject, comprising contacting an apical surface of a polarized epithelial cell of the subject with a delivery construct, wherein said delivery construct comprises a receptor binding domain, a transcytosis domain, a cleavable linker, and the macromolecule, wherein the transcytosis domain transcytoses the macromolecule to and through the basal-lateral membrane of said epithelial cell, wherein cleavage at said cleavable linker separates said macromolecule from the remainder of said construct, and wherein said cleavable linker is cleavable by an enzyme that i) exhibits greater activity at a basal-lateral membrane of a polarized epithelial cell of said subject than at an apical membrane of the polarized epithelial cell, or ii) exhibits greater activity in the plasma of said subject than at an apical membrane of the polarized epithelial cell of the subject, and wherein cleavage at the cleavable linker separates the macromolecule from the remainder of the delivery construct, thereby delivering the macromolecule to the subject.
49. The method of Claim 48, wherein said receptor binding domain is selected from the group consisting of receptor binding domains from *Pseudomonas* exotoxin A, cholera toxin, diptheria toxin, shiga toxin, or shiga-like toxin; monoclonal antibodies; polyclonal antibodies; single-chain antibodies; TGF α ; EGF; IGF-I; IGF-II; IGF-III; IL-1; IL-2; IL-3; IL-6; MIP-1a; MIP-1b; MCAF; and IL-8.
50. The method of Claim 48, wherein said receptor binding domain binds to a cell surface receptor selected from the group consisting of α 2-macroglobulin receptor, EGFR, IGFR, transferrin receptor, chemokine receptor, CD25, CD11B, CD11C, CD80, CD86, TNF α receptor, TOLL receptor, M-CSF receptor, GM-CSF receptor, scavenger receptor, and VEGF receptor.
51. The method of Claim 48, wherein said transcytosis domain is selected from the group consisting of transcytosis domains from *Pseudomonas* exotoxin A, botulinum toxin, diptheria toxin, pertussis toxin, cholera toxin, heat-labile *E. coli* enterotoxin, shiga toxin, and shiga-like toxin.
52. The method of Claim 48, wherein said macromolecule is selected from the group consisting of a peptide, a polypeptide, a protein, a nucleic acid, and a lipid.
53. The method of Claim 48, wherein said enzyme that is present at a basal-lateral membrane of a polarized epithelial cell is selected from the group consisting of

Cathepsin GI, Chymotrypsin I, Elastase I, Subtilisin AI, Subtilisin AII, Thrombin I, and Urokinase I.

54. The method of Claim 48, wherein said cleavable linker comprises an amino acid sequence that is selected from the group consisting of Ala-Ala-Pro-Phe (SEQ ID NO.:4), Gly-Gly-Phe (SEQ ID NO.:5), Ala-Ala-Pro-Val (SEQ ID NO.:6), Gly-Gly-Leu (SEQ ID NO.:7), Ala-Ala-Leu (SEQ ID NO.:8), Phe-Val-Arg (SEQ ID NO.:9), Val-Gly-Arg (SEQ ID NO.:10).
55. The method of Claim 48, wherein the epithelial cell is selected from the group consisting of nasal epithelial cells, oral epithelial cells, intestinal epithelial cells, rectal epithelial cells, vaginal epithelial cells, and pulmonary epithelial cells.
56. The method of Claim 48, wherein said mammal is a human.
57. The method of Claim 48, wherein said delivery construct contacts the apical membrane of the epithelial cell.
58. A method for delivering a macromolecule to the bloodstream of a subject, comprising contacting the delivery construct of Claim 1 to an apical surface of a polarized epithelial cell of the subject, such that the macromolecule is delivered to the bloodstream of the subject, wherein a lower titer of antibodies specific for the macromolecule is induced in the serum of the subject than is induced by subcutaneously administering the macromolecule to a subject separately from the remainder of the delivery construct.
59. The method of Claim 58, wherein the macromolecule is selected from the group consisting of a peptide, a polypeptide, a protein, a nucleic acid, and a lipid.
60. The method of Claim 58, wherein the macromolecule is selected from the group consisting of polypeptide hormones, cytokines, chemokines, growth factors, and clotting factors.
61. The method of Claim 58, wherein the macromolecule is selected from the group consisting of IGF-I, IGF-II, IGF-III, EGF, IFN- α , IFN- β , IFN- γ , G-CSF, GM-CSF, IL-1, IL-2, IL-3, IL-6, IL-8, IL-12, EPO, growth hormone, factor VII, vasopressin, calcitonin, parathyroid hormone, luteinizing hormone-releasing factor, tissue

plasminogen activators, proinsulin, insulin, glucocorticoid, amylin, adrenocorticotropin, enkephalin, and glucagon-like peptide 1.

62. The method of Claim 58, wherein the macromolecule is human growth hormone.
63. The method of Claim 58, wherein the macromolecule is human insulin.
64. The method of Claim 58, wherein the subject is a mouse, dog, goat, or human.
65. The method of Claim 58, wherein the titer of antibodies specific for the macromolecule induced in the serum of the subject by the macromolecule delivered by with the delivery construct is less than about 75% of the titer of antibodies induced by subcutaneously administering the macromolecule to a subject separately from the remainder of the delivery construct.
66. The method of Claim 58, wherein the titer of antibodies specific for the macromolecule induced in the serum of the subject by the macromolecule delivered by the delivery construct is less than about 50% of the titer of antibodies induced by subcutaneously administering the macromolecule to a subject separately from the remainder of the delivery construct.
67. The method of Claim 58, wherein the titer of antibodies specific for the macromolecule induced in the serum of the subject by the macromolecule delivered by the delivery construct is less than about 25% of the titer of antibodies induced by subcutaneously administering the macromolecule to a subject separately from the remainder of the delivery construct.
68. The method of Claim 58, wherein the titer of antibodies specific for the macromolecule induced in the serum of the subject by the macromolecule delivered by the delivery construct is less than about 10% of the titer of antibodies induced by subcutaneously administering the macromolecule to a subject separately from the remainder of the delivery construct.

Figures 1A-D

Fig. 1A

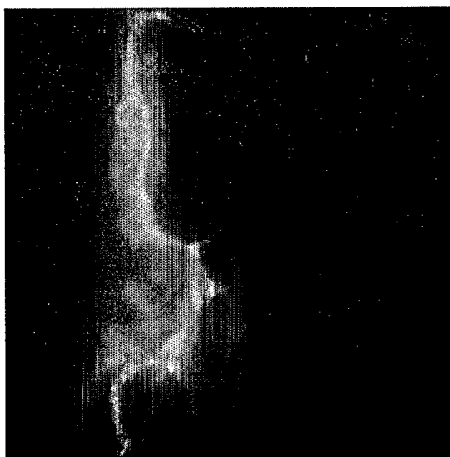


Fig. 1B



Fig. 1C

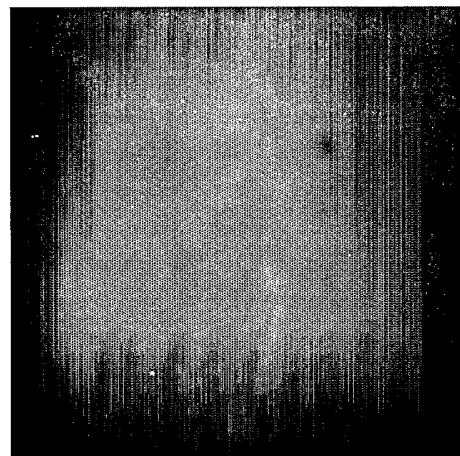


Fig. 1D

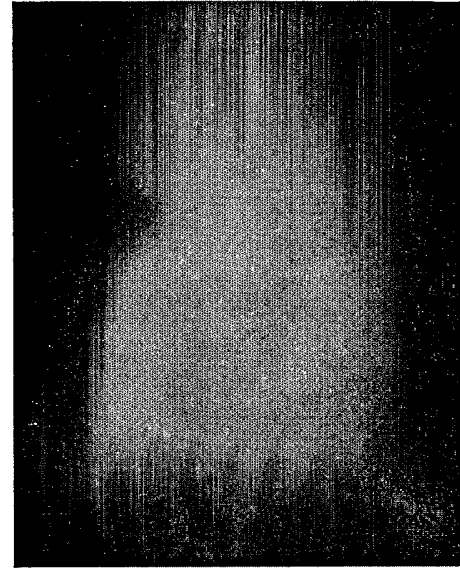


Figure 2

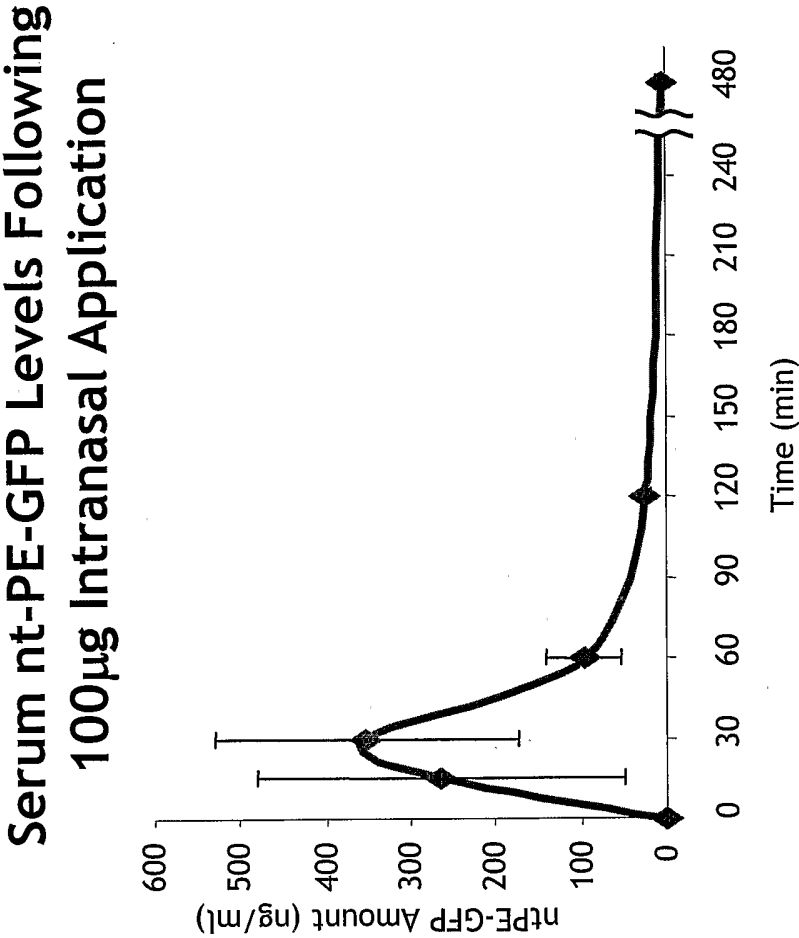


Figure 3*Pseudomonas Aeruginosa* Exotoxin A Amino Acid Sequence

Start of Domain I ↓

1 mhliphwipl vaslgllagg ssasa aeeaf dlwnecakac vldlkdgvr~~s~~ srmsvdpaia
 61 dtngqgv~~l~~hy smvleggnda lklaidnals itsdgl~~t~~irl eggvep~~n~~kpv rysytrqarg
 121 swslnwlvp~~i~~ ghekp~~s~~nikv fihelnagnq lshmspiyti emgdellakl ardatffvra
 181 hesnemqptl aishagvs~~v~~ maqtqprrek rwsewasgkv lcldp~~l~~dgv ynylaqqrcn

Start of Domain II ↓

241 lddtwegkiy rvlagnpakh dldikptvis hrlhfpeggs laaltahqac hlpletftrh

↓Furin clip site

301 ~~rqpr~~~~g~~weqle qcgypvqrlv alylaarlsw nqv~~d~~qvirna laspgsggd~~l~~ geaireqpeq

Start of Domain III ↓

361 arlaltlaaa eserfvrggt gndeagaana dvvsltcpva agecagpads gdallernyp
 421 tgaefl~~g~~dg~~g~~ dv~~s~~fstrgtq nwtverllqa hrqleergyv fvgyhg~~t~~file aaqsivfggv
 481 rarsq~~l~~dai wr~~g~~fyiagdp alaygyaq~~d~~ epdarg~~r~~irn gallrvyvpr sslpgfy~~r~~ts
 541 ltlaapeaag ev~~E~~rlighpl plrl~~d~~aitgp eeeggrleti lgwplaertv vipsaiptdp
 601 rnv~~g~~gdldps sipdkeqais alpdyasqpg kppredlk

Figure 4

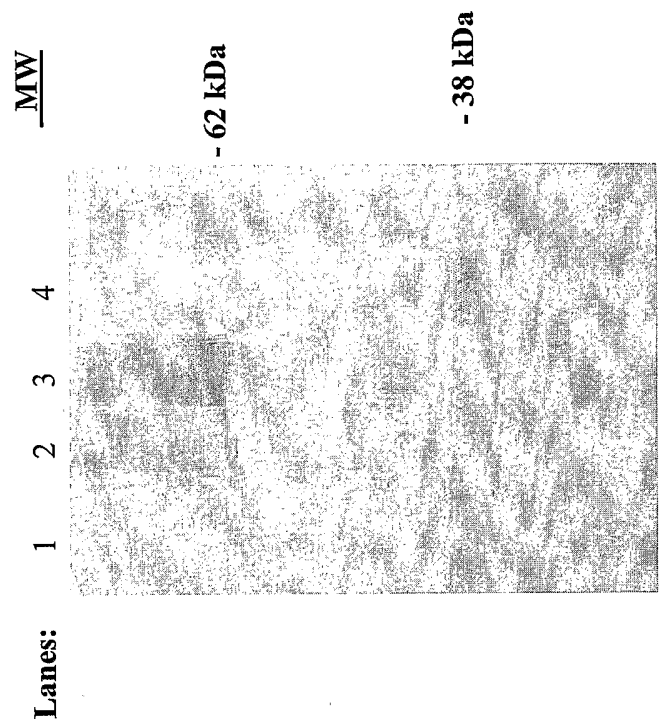


Figure 5

Serum Concentrations following SC Delivery of 30 ug of rGH

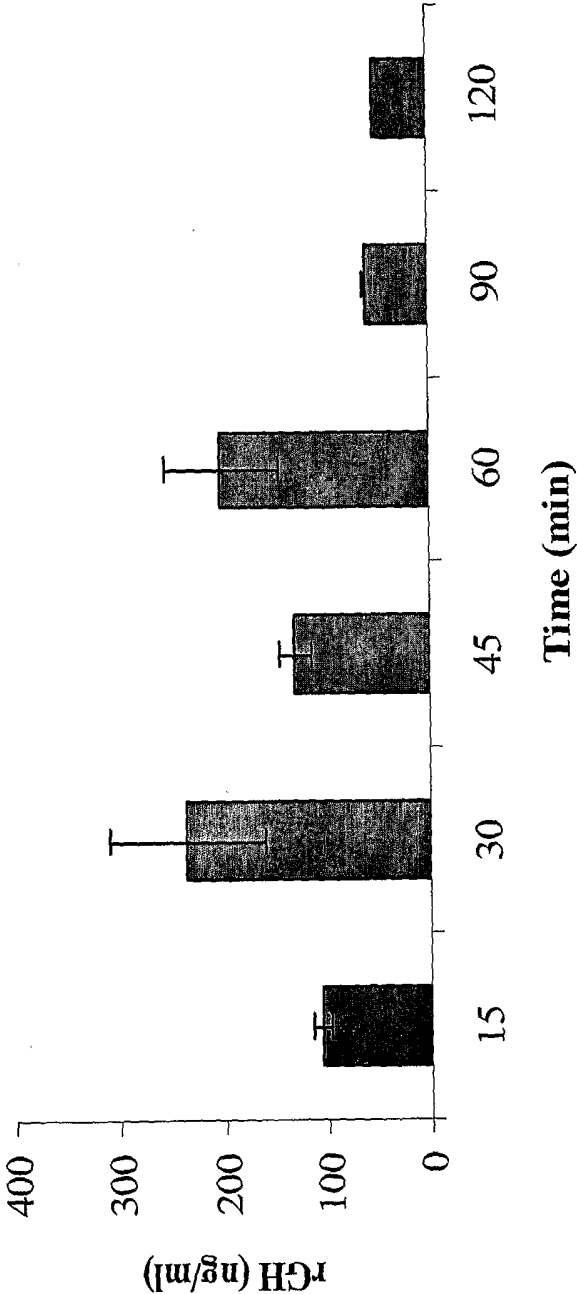


Figure 6

Serum Concentrations following Oral
Delivery of 100ug of Delivery Construct 2

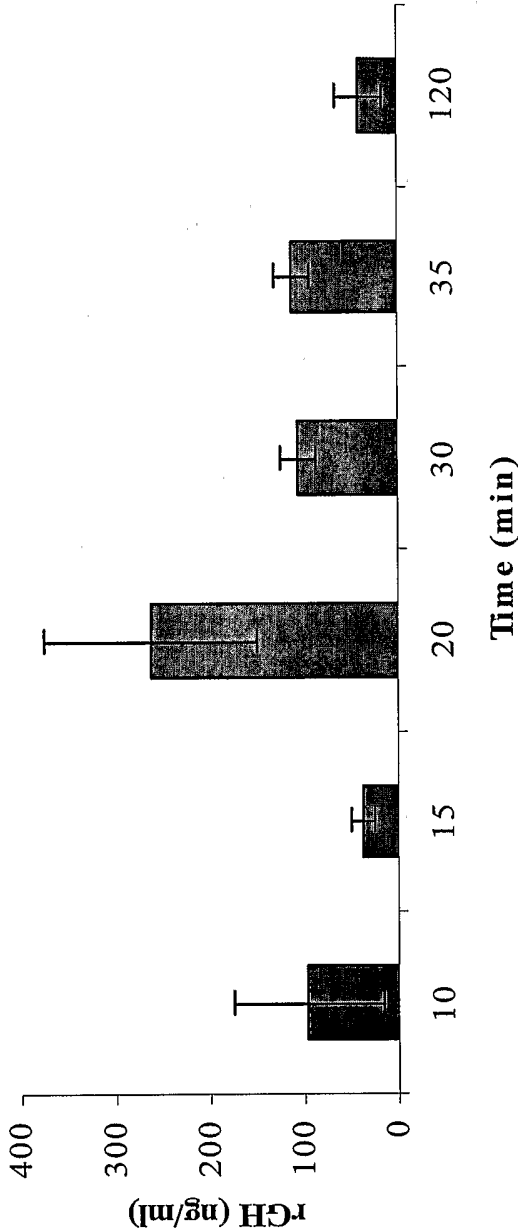


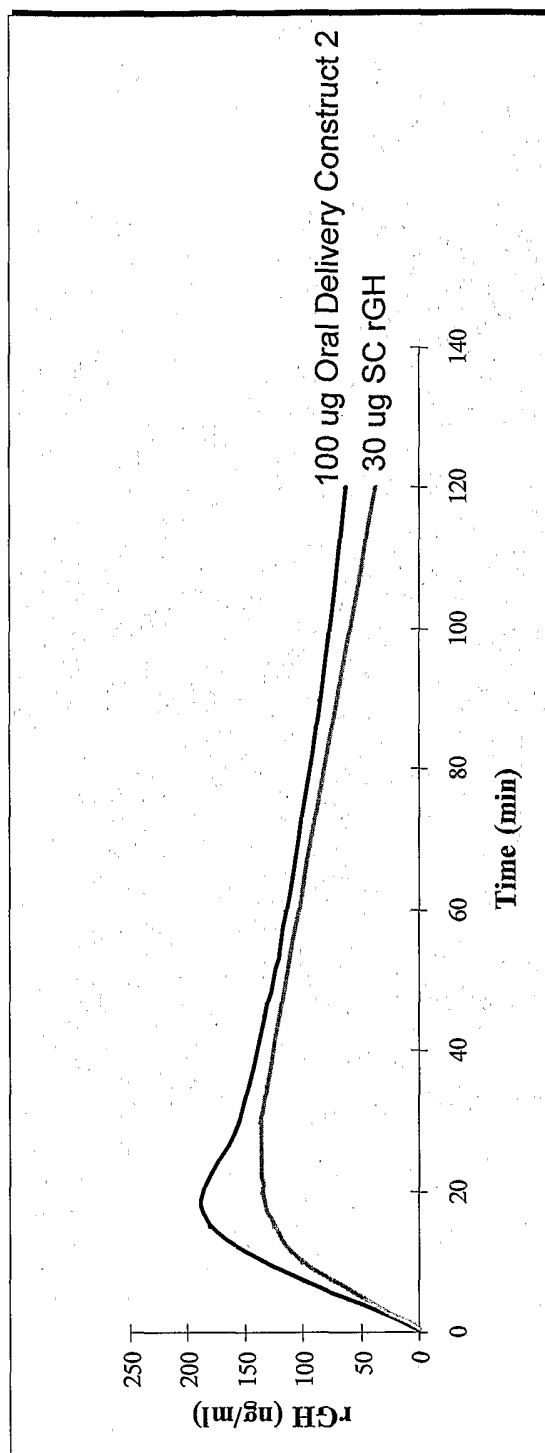
Figure 7

Figure 8

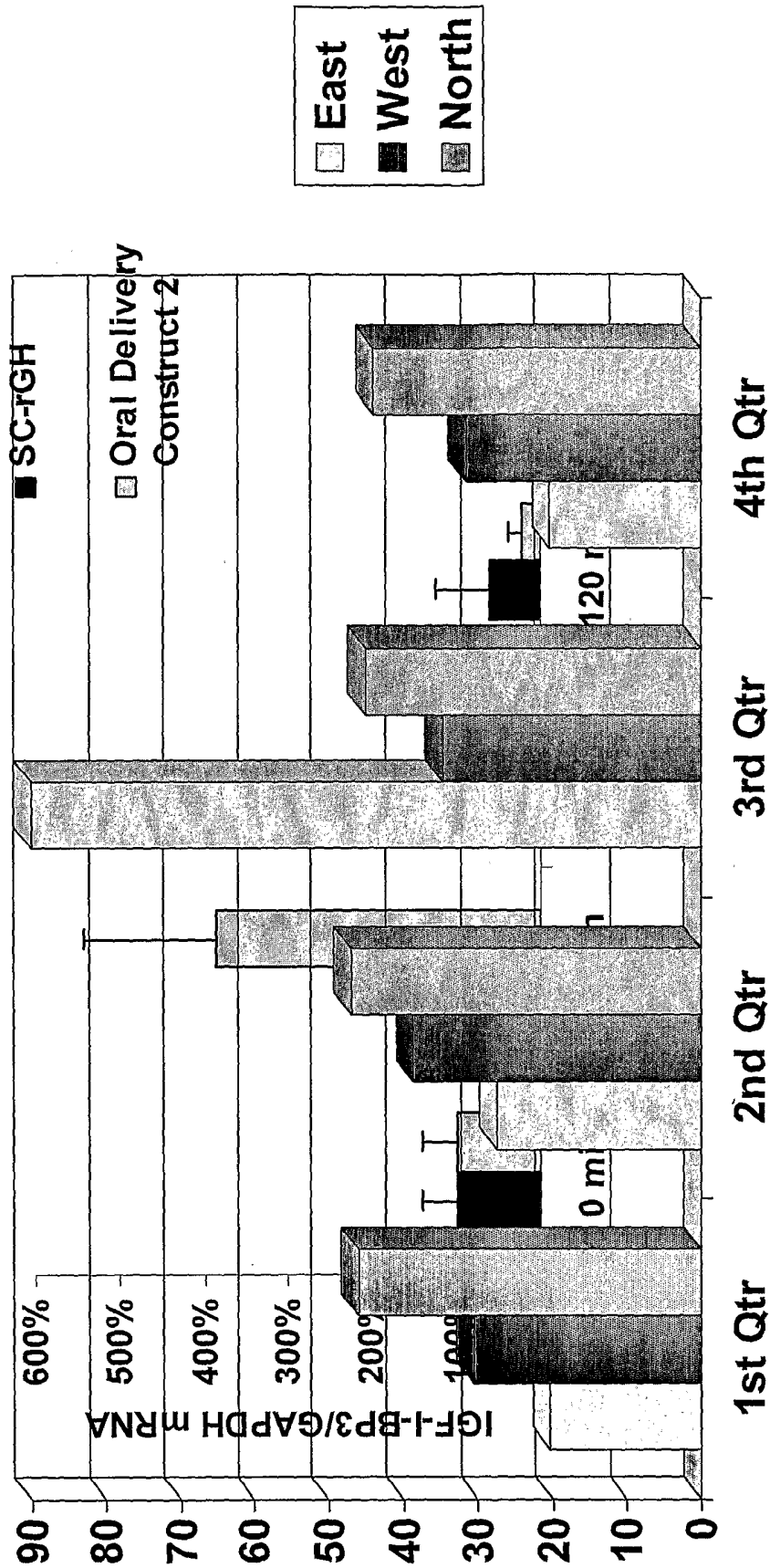


Figure 9

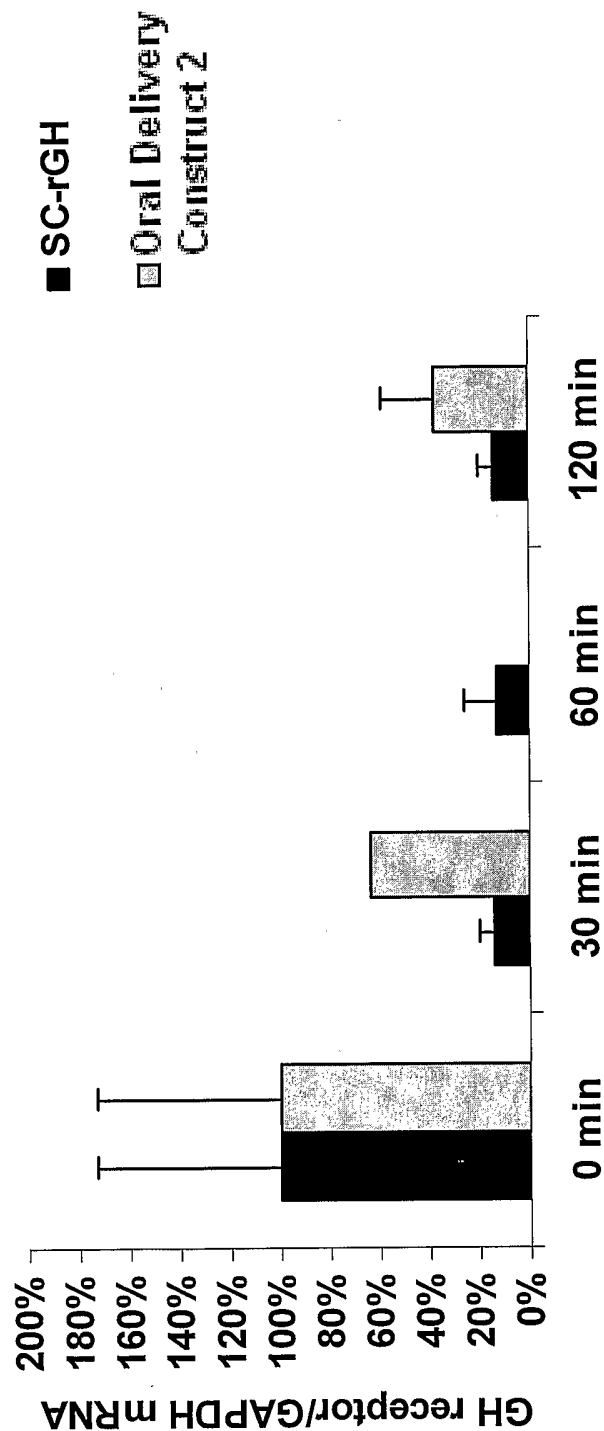
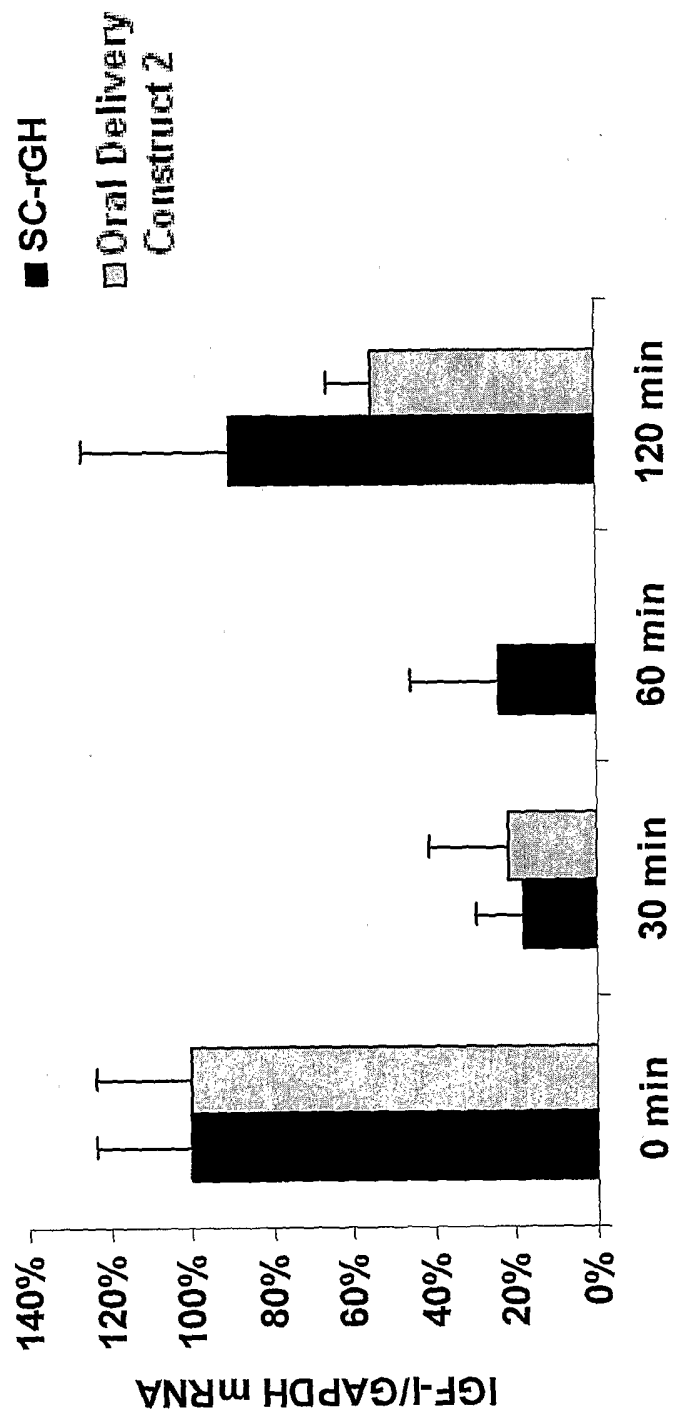


Figure 10



Figures 11A and 11B

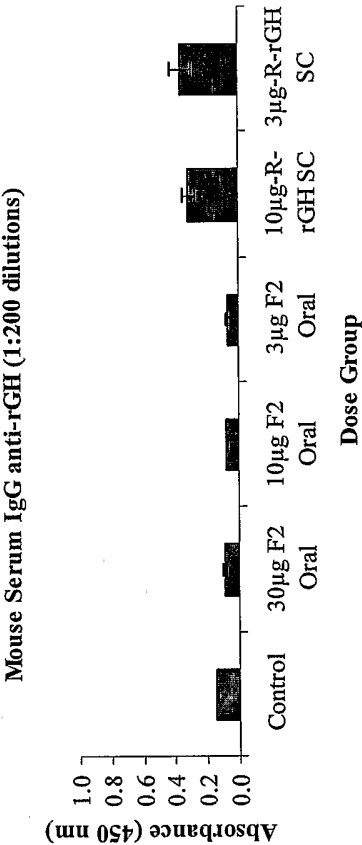


Figure 11B

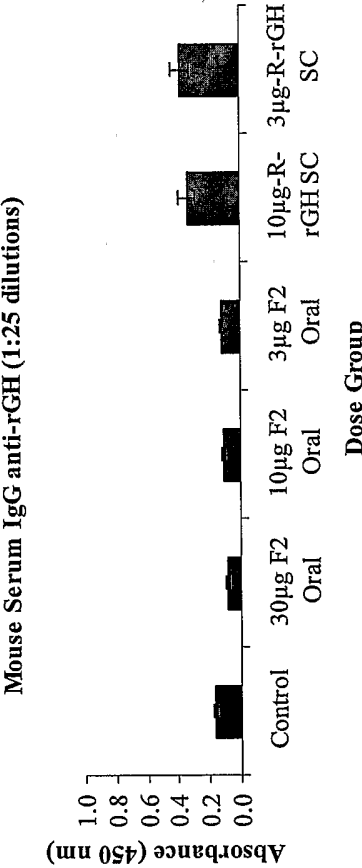


Figure 11A

Figures 11C and 11D

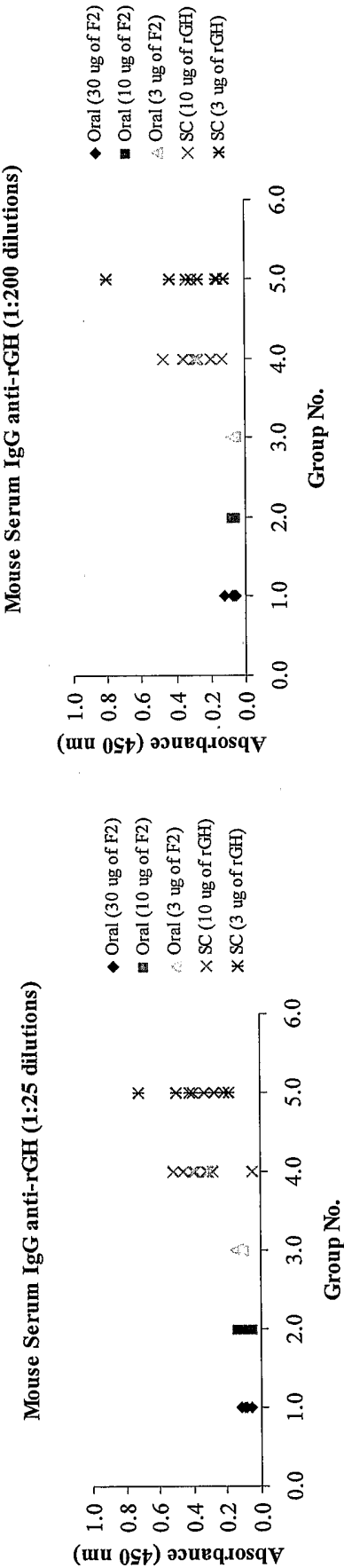


Figure 11A

Figure 11B

Figure 12

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cagggcggtgc tgcactactc catggtcctg gagggcgggca acgacgcgct caagctggcc 180
atcgacaacg ccctcagcat caccagcgac ggcctgacca tccgcctcga agggcggtc 240
gagccgaaca agccggtgcg ctacagctac acgcgccagg cgcgcggcag ttggtcgctg 300
aactggctgg taccgatcgg ccacgagaag ccctcgaaca tcaagggtgt catccacgaa 360
ctgaacgccg gcaaccagct cagccacatg tcgccgatct acaccatcga gatgggcgac 420
gagttgctgg cgaagctggc gcgcgatgcc accttcttcg tcagggcgca cgagagcaac 480
gagatgcagc cgacgctcgc catcagccat gccgggggtca gcgtgggtcat ggcccagacc 540
cagccgcgcc gggaaaagcg ctggagcgaa tggggccagcg gcaagggtgt gtgcctgctc 600
gaccgcgtgg acggggtcta caactacctc gccagcaac gctgcaacct cgacgatacc 660
tggaagggca agatctaccg ggtgctcgcc ggcaaccgga cgaagcatga cctggacatc 720
aaaccacggt tcatcagtca tcgcctgcac tttcccgagg gcggcagcct ggccgcgctg 780
accgcgcacc aggcttgcca cctgccgctg gagactttca cccgtcatcg ccagccgcgc 840
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cgtctggaag acggttcccc gcgtaccggt cagatcttca aacagaccta ctccaaattc 1560
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cgtaaagaca tggacaaagt tgaaaccttc ctgcgtatcg ttcagtgccg ttccgttgaa 1680
ggttcctgcg gtttctaa
1698

```

Figure 13A

Met 1	Ala	Glu	Glu	Ala	Phe	Asp	Leu	Trp	Asn	Glu	Cys	Ala	Lys	Ala	Cys
				5					10					15	
Val	Leu	Asp	Leu	Lys	Asp	Gly	Val	Arg	Ser	Ser	Arg	Met	Ser	Val	Asp
			20					25					30		
Pro	Ala	Ile	Ala	Asp	Thr	Asn	Gly	Gln	Gly	Val	Leu	His	Tyr	Ser	Met
		35					40					45			
Val	Leu	Glu	Gly	Gly	Asn	Asp	Ala	Leu	Lys	Leu	Ala	Ile	Asp	Asn	Ala
	50					55					60				
Leu	Ser	Ile	Thr	Ser	Asp	Gly	Leu	Thr	Ile	Arg	Leu	Glu	Gly	Gly	Val
65					70					75					80
Glu	Pro	Asn	Lys	Pro	Val	Arg	Tyr	Ser	Tyr	Thr	Arg	Gln	Ala	Arg	Gly
				85					90					95	
Ser	Trp	Ser	Leu	Asn	Trp	Leu	Val	Pro	Ile	Gly	His	Glu	Lys	Pro	Ser
			100					105					110		
Asn	Ile	Lys	Val	Phe	Ile	His	Glu	Leu	Asn	Ala	Gly	Asn	Gln	Leu	Ser
		115					120					125			
His	Met	Ser	Pro	Ile	Tyr	Thr	Ile	Glu	Met	Gly	Asp	Glu	Leu	Leu	Ala
	130					135					140				
Lys	Leu	Ala	Arg	Asp	Ala	Thr	Phe	Phe	Val	Arg	Ala	His	Glu	Ser	Asn
145				150						155					160
Glu	Met	Gln	Pro	Thr	Leu	Ala	Ile	Ser	His	Ala	Gly	Val	Ser	Val	Val
				165					170					175	
Met	Ala	Gln	Thr	Gln	Pro	Arg	Arg	Glu	Lys	Arg	Trp	Ser	Glu	Trp	Ala
			180					185					190		
Ser	Gly	Lys	Val	Leu	Cys	Leu	Leu	Asp	Pro	Leu	Asp	Gly	Val	Tyr	Asn
		195					200					205			
Tyr	Leu	Ala	Gln	Gln	Arg	Cys	Asn	Leu	Asp	Asp	Thr	Trp	Glu	Gly	Lys
	210					215					220				
Ile	Tyr	Arg	Val	Leu	Ala	Gly	Asn	Pro	Ala	Lys	His	Asp	Leu	Asp	Ile
225				230						235					240
Lys	Pro	Thr	Val	Ile	Ser	His	Arg	Leu	His	Phe	Pro	Glu	Gly	Gly	Ser
				245					250					255	
Leu	Ala	Ala	Leu	Thr	Ala	His	Gln	Ala	Cys	His	Leu	Pro	Leu	Glu	Thr
			260					265					270		
Phe	Thr	Arg	His	Arg	Gln	Pro	Arg	Gly	Trp	Glu	Gln	Leu	Glu	Gln	Cys
		275					280					285			
Gly	Tyr	Pro	Val	Gln	Arg	Leu	Val	Ala	Leu	Tyr	Leu	Ala	Ala	Arg	Leu
	290					295					300				
Ser	Trp	Asn	Gln	Val	Asp	Gln	Val	Ile	Arg	Asn	Ala	Leu	Ala	Ser	Pro
305				310						315					320
Gly	Ser	Gly	Gly	Asp	Leu	Gly	Glu	Ala	Ile	Arg	Glu	Gln	Pro	Glu	Gln
				325					330					335	
Ala	Arg	Leu	Ala	Leu	Thr	Leu	Ala	Ala	Ala	Glu	Ser	Glu	Arg	Phe	Val
			340					345					350		
Arg	Gln	Gly	Thr	Gly	Asn	Asp	Glu	Ala	Gly	Ala	Ala	Asn	Leu	Gln	Gly
	355						360					365			
Gly	Leu	Arg	Gln	Pro	Arg	Phe	Pro	Thr	Ile	Pro	Leu	Ser	Arg	Leu	Phe
	370					375					380				

Figure 13B

Asp	Asn	Ala	Met	Leu	Arg	Ala	His	Arg	Leu	His	Gln	Leu	Ala	Phe	Asp
385					390					395					400
Thr	Tyr	Gln	Glu	Phe	Glu	Glu	Ala	Tyr	Ile	Pro	Lys	Glu	Gln	Lys	Tyr
				405					410					415	
Ser	Phe	Leu	Gln	Asn	Pro	Gln	Thr	Ser	Leu	Cys	Phe	Ser	Glu	Ser	Ile
			420					425					430		
Pro	Thr	Pro	Ser	Asn	Arg	Glu	Glu	Thr	Gln	Gln	Lys	Ser	Asn	Leu	Glu
		435					440					445			
Leu	Leu	Arg	Ile	Ser	Leu	Leu	Leu	Ile	Gln	Ser	Trp	Leu	Glu	Pro	Val
450						455					460				
Gln	Phe	Leu	Arg	Ser	Val	Phe	Ala	Asn	Ser	Leu	Val	Tyr	Gly	Ala	Ser
465					470					475					480
Asp	Ser	Asn	Val	Tyr	Asp	Leu	Leu	Lys	Asp	Leu	Glu	Glu	Gly	Ile	Gln
				485					490					495	
Thr	Leu	Met	Gly	Arg	Leu	Glu	Asp	Gly	Ser	Pro	Arg	Thr	Gly	Gln	Ile
			500					505						510	
Phe	Lys	Gln	Thr	Tyr	Ser	Lys	Phe	Asp	Thr	Asn	Ser	His	Asn	Asp	Asp
		515					520					525			
Ala	Leu	Leu	Lys	Asn	Tyr	Gly	Leu	Leu	Tyr	Cys	Phe	Arg	Lys	Asp	Met
	530					535					540				
Asp	Lys	Val	Glu	Thr	Phe	Leu	Arg	Ile	Val	Gln	Cys	Arg	Ser	Val	Glu
545					550					555					560
Gly	Ser	Cys	Gly	Phe											
				565											

Figure 14

```

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cagggcggtgc tgcactactc catggtcctg gagggcgcca acgacgcgct caagctggcc 180
atcgacaacg ccctcagcat caccagcgac ggctgacca tccgcctcga agggcggtc 240
gagccgaaca agccggtgcg ctacagctac acgcgccagg cgcgcgccag ttggtcgctg 300
aactggctgg taccgatcgg ccacgagaag cctcgaaca tcaaggtgtt catccacgaa 360
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gaccgcgtgg acggggtcta caactacctc gccagcaac gctgcaacct cgacgatacc 660
tggaaggca agatctaccg ggtgctcgcc ggcaaccgga cgaagcatga cctggacatc 720
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```


Figure 15B

Ser	Arg	Arg	Thr	Leu	Met	Leu	Leu	Ala	Gln	Met	Arg	Arg	Ile	Ser	Leu
385					390					395					400
Phe	Ser	Cys	Leu	Lys	Asp	Arg	His	Asp	Phe	Gly	Phe	Pro	Gln	Glu	Glu
				405					410					415	
Phe	Gly	Asn	Gln	Phe	Gln	Lys	Ala	Glu	Thr	Ile	Pro	Val	Leu	His	Glu
			420					425					430		
Met	Ile	Gln	Gln	Ile	Phe	Asn	Leu	Phe	Ser	Thr	Lys	Asp	Ser	Ser	Ala
		435					440					445			
Ala	Trp	Asp	Glu	Thr	Leu	Leu	Asp	Lys	Phe	Tyr	Thr	Glu	Leu	Tyr	Gln
	450					455				460					
Gln	Leu	Asn	Asp	Leu	Glu	Ala	Cys	Val	Ile	Gln	Gly	Val	Gly	Val	Thr
465					470					475					480
Glu	Thr	Pro	Leu	Met	Lys	Glu	Asp	Ser	Ile	Leu	Ala	Val	Arg	Lys	Tyr
				485					490					495	
Phe	Gln	Arg	Ile	Thr	Leu	Tyr	Leu	Lys	Glu	Lys	Lys	Tyr	Ser	Pro	Cys
			500					505					510		
Ala	Trp	Glu	Val	Val	Arg	Ala	Glu	Ile	Met	Arg	Ser	Phe	Ser	Leu	Ser
	515						520					525			
Thr	Asn	Leu	Gln	Glu	Ser	Leu	Arg	Ser	Lys	Glu					
	530					535									

Figure 16
Oral Delivery of 100 ug Delivery Construct 8 in BALB/c Mice

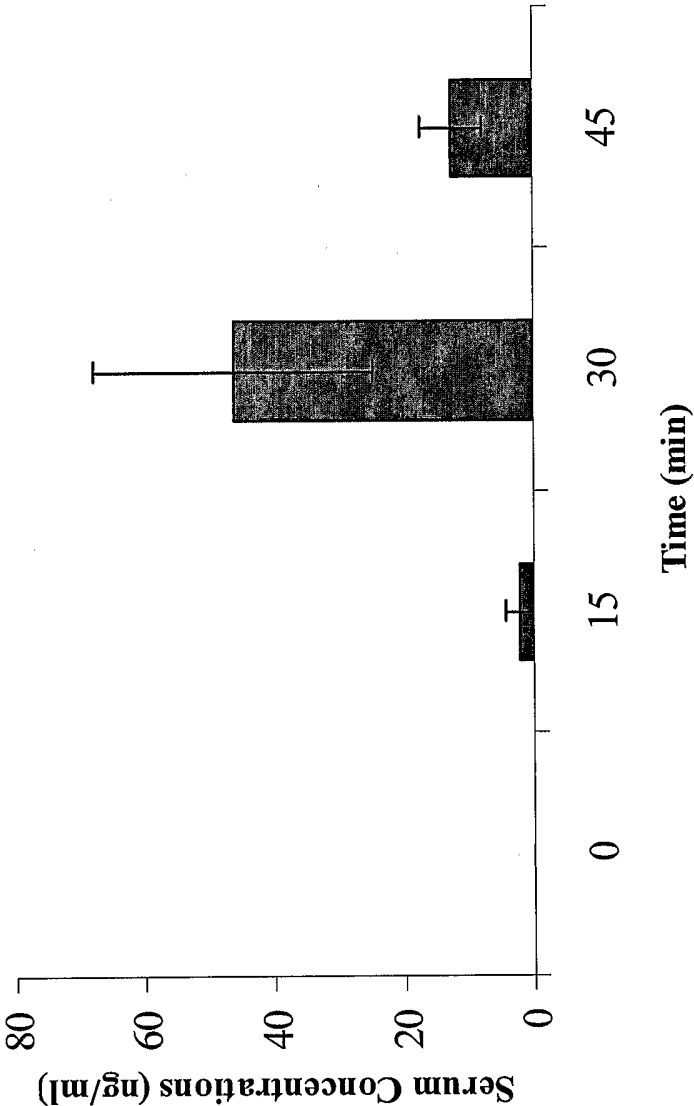


Figure 17A

Met	Ala	Glu	Glu	Ala	Phe	Asp	Leu	Trp	Asn	Glu	Cys	Ala	Lys	Ala	Cys
1				5					10					15	
Val	Leu	Asp	Leu	Lys	Asp	Gly	Val	Arg	Ser	Ser	Arg	Met	Ser	Val	Asp
			20					25					30		
Pro	Ala	Ile	Ala	Asp	Thr	Asn	Gly	Gln	Gly	Val	Leu	His	Tyr	Ser	Met
			35				40					45			
Val	Leu	Glu	Gly	Gly	Asn	Asp	Ala	Leu	Lys	Leu	Ala	Ile	Asp	Asn	Ala
	50					55					60				
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Glu	Pro	Asn	Lys	Pro	Val	Arg	Tyr	Ser	Tyr	Thr	Arg	Gln	Ala	Arg	Gly
				85					90					95	
Ser	Trp	Ser	Leu	Asn	Trp	Leu	Val	Pro	Ile	Gly	His	Glu	Lys	Pro	Ser
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Asn	Ile	Lys	Val	Phe	Ile	His	Glu	Leu	Asn	Ala	Gly	Asn	Gln	Leu	Ser
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His	Met	Ser	Pro	Ile	Tyr	Thr	Ile	Glu	Met	Gly	Asp	Glu	Leu	Leu	Ala
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Lys	Leu	Ala	Arg	Asp	Ala	Thr	Phe	Phe	Val	Arg	Ala	His	Glu	Ser	Asn
145					150					155					160
Glu	Met	Gln	Pro	Thr	Leu	Ala	Ile	Ser	His	Ala	Gly	Val	Ser	Val	Val
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Met	Ala	Gln	Thr	Gln	Pro	Arg	Arg	Glu	Lys	Arg	Trp	Ser	Glu	Trp	Ala
			180					185					190		
Ser	Gly	Lys	Val	Leu	Cys	Leu	Leu	Asp	Pro	Leu	Asp	Gly	Val	Tyr	Asn
		195					200					205			
Tyr	Leu	Ala	Gln	Gln	Arg	Cys	Asn	Leu	Asp	Asp	Thr	Trp	Glu	Gly	Lys
	210					215					220				
Ile	Tyr	Arg	Val	Leu	Ala	Gly	Asn	Pro	Ala	Lys	His	Asp	Leu	Asp	Ile
225					230					235					240
Lys	Pro	Thr	Val	Ile	Ser	His	Arg	Leu	His	Phe	Pro	Glu	Gly	Gly	Ser
				245					250					255	
Leu	Ala	Ala	Leu	Thr	Ala	His	Gln	Ala	Cys	His	Leu	Pro	Leu	Glu	Thr
			260					265					270		
Phe	Thr	Arg	His	Arg	Gln	Pro	Arg	Gly	Trp	Glu	Gln	Leu	Glu	Gln	Cys
		275					280					285			
Gly	Tyr	Pro	Val	Gln	Arg	Leu	Val	Ala	Leu	Tyr	Leu	Ala	Ala	Arg	Leu
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Ser	Trp	Asn	Gln	Val	Asp	Gln	Val	Ile	Arg	Asn	Ala	Leu	Ala	Ser	Pro
305					310					315					320
Gly	Ser	Gly	Gly	Asp	Leu	Gly	Glu	Ala	Ile	Arg	Glu	Gln	Pro	Glu	Gln
				325					330					335	
Ala	Arg	Leu	Ala	Leu	Thr	Leu	Ala	Ala	Ala	Glu	Ser	Glu	Arg	Phe	Val
			340					345					350		
Arg	Gln	Gly													

Figure 17B

Leu	Val	Glu	Ala	Leu	Tyr	Leu	Val	Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr
385					390					395					400
Thr	Pro	Lys	Thr	Arg	Arg	Glu	Ala	Glu	Asp	Leu	Gln	Val	Gly	Gln	Val
				405					410					415	
Glu	Leu	Gly	Gly	Gly	Pro	Gly	Ala	Gly	Ser	Leu	Gln	Pro	Leu	Ala	Leu
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Glu	Gly	Ser	Leu	Gln	Lys	Arg	Gly	Ile	Val	Glu	Gln	Cys	Cys	Thr	Ser
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Ile	Cys	Ser	Leu	Tyr	Gln	Leu	Glu	Asn	Tyr	Cys	Asn				
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Figure 18B

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SEQUENCE LISTING

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<120> METHODS AND COMPOSITIONS FOR NEEDLELESS
DELIVERY OF MACROMOLECULES

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Trp Asn Glu Cys Ala Lys Ala Cys Val Leu Asp Leu Lys Asp Gly Val
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Arg Ser Ser Arg Met Ser Val Asp Pro Ala Ile Ala Asp Thr Asn Gly
              50              55              60
Gln Gly Val Leu His Tyr Ser Met Val Leu Glu Gly Gly Asn Asp Ala
65              70              75              80
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Thr Ile Arg Leu Glu Gly Gly Val Glu Pro Asn Lys Pro Val Arg Tyr
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Ser Tyr Thr Arg Gln Ala Arg Gly Ser Trp Ser Leu Asn Trp Leu Val
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Pro Ile Gly His Glu Lys Pro Ser Asn Ile Lys Val Phe Ile His Glu
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Leu Asn Ala Gly Asn Gln Leu Ser His Met Ser Pro Ile Tyr Thr Ile
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Glu Met Gly Asp Glu Leu Leu Ala Lys Leu Ala Arg Asp Ala Thr Phe
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Phe Val Arg Ala His Glu Ser Asn Glu Met Gln Pro Thr Leu Ala Ile
              180             185             190
Ser His Ala Gly Val Ser Val Val Met Ala Gln Thr Gln Pro Arg Arg
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Glu Lys Arg Trp Ser Glu Trp Ala Ser Gly Lys Val Leu Cys Leu Leu
              210             215             220
Asp Pro Leu Asp Gly Val Tyr Asn Tyr Leu Ala Gln Gln Arg Cys Asn
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Arg His Arg Gln Pro Arg Gly Trp Glu Gln Leu Glu Gln Cys Gly Tyr
              35              40              45

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 65 70 75 80
 Gly Gly Asp Leu Gly Glu Ala Ile Arg Glu Gln Pro Glu Gln Ala Arg
 85 90 95
 Leu Ala Leu Thr Leu Ala Ala Ala Glu Ser Glu Arg Phe Val Arg Gln
 100 105 110
 Gly Thr Gly Asn Asp Glu Ala Gly Ala Ala Asn Ala Asp Val Val Ser
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 35          40          45
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Leu Ser Ile Thr Ser Asp Gly Leu Thr Ile Arg Leu Glu Gly Gly Val
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Glu Pro Asn Lys Pro Val Arg Tyr Ser Tyr Thr Arg Gln Ala Arg Gly
 85          90          95
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Ser Gly Lys Val Leu Cys Leu Leu Asp Pro Leu Asp Gly Val Tyr Asn
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Tyr Leu Ala Gln Gln Arg Cys Asn Leu Asp Asp Thr Trp Glu Gly Lys
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tttagctgcc tgaaggatag gcacgatttt ggctttcctc aagaggagtt tggcaaccag 1260
tttcagaagg ctgagaccat ccctgtgctg cagcagatga tccagcagat ctttaacctg 1320
tttagcacca aggatagcag cgctgcttgg gatgagaccc tgctggataa gttttacacc 1380
gagctgtacc agcagctgaa cgatctggag gcttgcgtga tccagggcgt gggcgctgacc 1440
gagacccctc tgatgaagga ggatagcatc ctggctgtga ggaagtactt tcagaggatc 1500
accctgtacc tgaaggagaa gaagtacagc ccctgcgctt gggaagtcgt gagggctgag 1560
atcatgagga gctttagcct gaggaccaac ctgcaagaga gcttgaggtc taaggagtaa 1620

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<210> 37

<211> 539

<212> PRT

<213> Polypeptide

<400> 37

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Met Ala Glu Glu Ala Phe Asp Leu Trp Asn Glu Cys Ala Lys Ala Cys
 1          5          10          15
Val Leu Asp Leu Lys Asp Gly Val Arg Ser Ser Arg Met Ser Val Asp
          20          25          30
Pro Ala Ile Ala Asp Thr Asn Gly Gln Gly Val Leu His Tyr Ser Met
          35          40          45
Val Leu Glu Gly Gly Asn Asp Ala Leu Lys Leu Ala Ile Asp Asn Ala
          50          55          60
Leu Ser Ile Thr Ser Asp Gly Leu Thr Ile Arg Leu Glu Gly Gly Val
65          70          75          80
Glu Pro Asn Lys Pro Val Arg Tyr Ser Tyr Thr Arg Gln Ala Arg Gly
          85          90          95
Ser Trp Ser Leu Asn Trp Leu Val Pro Ile Gly His Glu Lys Pro Ser
          100          105          110
Asn Ile Lys Val Phe Ile His Glu Leu Asn Ala Gly Asn Gln Leu Ser
          115          120          125
His Met Ser Pro Ile Tyr Thr Ile Glu Met Gly Asp Glu Leu Leu Ala
          130          135          140
Lys Leu Ala Arg Asp Ala Thr Phe Phe Val Arg Ala His Glu Ser Asn
          145          150          155          160
Glu Met Gln Pro Thr Leu Ala Ile Ser His Ala Gly Val Ser Val Val
          165          170          175
Met Ala Gln Thr Gln Pro Arg Arg Glu Lys Arg Trp Ser Glu Trp Ala
          180          185          190
Ser Gly Lys Val Leu Cys Leu Leu Asp Pro Leu Asp Gly Val Tyr Asn
          195          200          205
Tyr Leu Ala Gln Gln Arg Cys Asn Leu Asp Asp Thr Trp Glu Gly Lys
          210          215          220
Ile Tyr Arg Val Leu Ala Gly Asn Pro Ala Lys His Asp Leu Asp Ile
          225          230          235          240
Lys Pro Thr Val Ile Ser His Arg Leu His Phe Pro Glu Gly Gly Ser
          245          250          255
Leu Ala Ala Leu Thr Ala His Gln Ala Cys His Leu Pro Leu Glu Thr
          260          265          270
Phe Thr Arg His Arg Gln Pro Arg Gly Trp Glu Gln Leu Glu Gln Cys
          275          280          285
Gly Tyr Pro Val Gln Arg Leu Val Ala Leu Tyr Leu Ala Ala Arg Leu
          290          295          300
Ser Trp Asn Gln Val Asp Gln Val Ile Arg Asn Ala Leu Ala Ser Pro
          305          310          315          320
Gly Ser Gly Gly Asp Leu Gly Glu Ala Ile Arg Glu Gln Pro Glu Gln
          325          330          335

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Ala Arg Leu Ala Leu Thr Leu Ala Ala Ala Glu Ser Glu Arg Phe Val
 340 345 350
 Arg Gln Gly Thr Gly Asn Asp Glu Ala Gly Ala Ala Asn Leu Gln Gly
 355 360 365
 Gly Leu Arg Gln Pro Arg Cys Asp Leu Pro Gln Thr His Ser Leu Gly
 370 375 380
 Ser Arg Arg Thr Leu Met Leu Leu Ala Gln Met Arg Arg Ile Ser Leu
 385 390 395 400
 Phe Ser Cys Leu Lys Asp Arg His Asp Phe Gly Phe Pro Gln Glu Glu
 405 410 415
 Phe Gly Asn Gln Phe Gln Lys Ala Glu Thr Ile Pro Val Leu His Glu
 420 425 430
 Met Ile Gln Gln Ile Phe Asn Leu Phe Ser Thr Lys Asp Ser Ser Ala
 435 440 445
 Ala Trp Asp Glu Thr Leu Leu Asp Lys Phe Tyr Thr Glu Leu Tyr Gln
 450 455 460
 Gln Leu Asn Asp Leu Glu Ala Cys Val Ile Gln Gly Val Gly Val Thr
 465 470 475 480
 Glu Thr Pro Leu Met Lys Glu Asp Ser Ile Leu Ala Val Arg Lys Tyr
 485 490 495
 Phe Gln Arg Ile Thr Leu Tyr Leu Lys Glu Lys Lys Tyr Ser Pro Cys
 500 505 510
 Ala Trp Glu Val Val Arg Ala Glu Ile Met Arg Ser Phe Ser Leu Ser
 515 520 525
 Thr Asn Leu Gln Glu Ser Leu Arg Ser Lys Glu
 530 535

<210> 38

<211> 460

<212> PRT

<213> Polypeptide

<400> 38

Met Ala Glu Glu Ala Phe Asp Leu Trp Asn Glu Cys Ala Lys Ala Cys
 1 5 10 15
 Val Leu Asp Leu Lys Asp Gly Val Arg Ser Ser Arg Met Ser Val Asp
 20 25 30
 Pro Ala Ile Ala Asp Thr Asn Gly Gln Gly Val Leu His Tyr Ser Met
 35 40 45
 Val Leu Glu Gly Gly Asn Asp Ala Leu Lys Leu Ala Ile Asp Asn Ala
 50 55 60
 Leu Ser Ile Thr Ser Asp Gly Leu Thr Ile Arg Leu Glu Gly Gly Val
 65 70 75 80
 Glu Pro Asn Lys Pro Val Arg Tyr Ser Tyr Thr Arg Gln Ala Arg Gly
 85 90 95
 Ser Trp Ser Leu Asn Trp Leu Val Pro Ile Gly His Glu Lys Pro Ser
 100 105 110
 Asn Ile Lys Val Phe Ile His Glu Leu Asn Ala Gly Asn Gln Leu Ser
 115 120 125
 His Met Ser Pro Ile Tyr Thr Ile Glu Met Gly Asp Glu Leu Leu Ala
 130 135 140
 Lys Leu Ala Arg Asp Ala Thr Phe Phe Val Arg Ala His Glu Ser Asn
 145 150 155 160
 Glu Met Gln Pro Thr Leu Ala Ile Ser His Ala Gly Val Ser Val Val
 165 170 175
 Met Ala Gln Thr Gln Pro Arg Arg Glu Lys Arg Trp Ser Glu Trp Ala
 180 185 190
 Ser Gly Lys Val Leu Cys Leu Leu Asp Pro Leu Asp Gly Val Tyr Asn
 195 200 205
 Tyr Leu Ala Gln Gln Arg Cys Asn Leu Asp Asp Thr Trp Glu Gly Lys
 210 215 220
 Ile Tyr Arg Val Leu Ala Gly Asn Pro Ala Lys His Asp Leu Asp Ile
 225 230 235 240

Lys Pro Thr Val Ile Ser His Arg Leu His Phe Pro Glu Gly Gly Ser
 245 250 255
 Leu Ala Ala Leu Thr Ala His Gln Ala Cys His Leu Pro Leu Glu Thr
 260 265 270
 Phe Thr Arg His Arg Gln Pro Arg Gly Trp Glu Gln Leu Glu Gln Cys
 275 280 285
 Gly Tyr Pro Val Gln Arg Leu Val Ala Leu Tyr Leu Ala Ala Arg Leu
 290 295 300
 Ser Trp Asn Gln Val Asp Gln Val Ile Arg Asn Ala Leu Ala Ser Pro
 305 310 315 320
 Gly Ser Gly Gly Asp Leu Gly Glu Ala Ile Arg Glu Gln Pro Glu Gln
 325 330 335
 Ala Arg Leu Ala Leu Thr Leu Ala Ala Glu Ser Glu Arg Phe Val
 340 345 350
 Arg Gln Gly Thr Gly Asn Asp Glu Ala Gly Ala Ala Asn Leu Gln Gly
 355 360 365
 Gly Leu Arg Gln Pro Arg Phe Val Asn Gln His Leu Cys Gly Ser His
 370 375 380
 Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr
 385 390 395 400
 Thr Pro Lys Thr Arg Arg Glu Ala Glu Asp Leu Gln Val Gly Gln Val
 405 410 415
 Glu Leu Gly Gly Gly Pro Gly Ala Gly Ser Leu Gln Pro Leu Ala Leu
 420 425 430
 Glu Gly Ser Leu Gln Lys Arg Gly Ile Val Glu Gln Cys Cys Thr Ser
 435 440 445
 Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Cys Asn
 450 455 460

<210> 39

<211> 404

<212> PRT

<213> Polypeptide

<400> 39

Met Ala Glu Glu Ala Phe Asp Leu Trp Asn Glu Cys Ala Lys Ala Cys
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 Val Leu Asp Leu Lys Asp Gly Val Arg Ser Ser Arg Met Ser Val Asp
 20 25 30
 Pro Ala Ile Ala Asp Thr Asn Gly Gln Gly Val Leu His Tyr Ser Met
 35 40 45
 Val Leu Glu Gly Gly Asn Asp Ala Leu Lys Leu Ala Ile Asp Asn Ala
 50 55 60
 Leu Ser Ile Thr Ser Asp Gly Leu Thr Ile Arg Leu Glu Gly Gly Val
 65 70 75 80
 Glu Pro Asn Lys Pro Val Arg Tyr Ser Tyr Thr Arg Gln Ala Arg Gly
 85 90 95
 Ser Trp Ser Leu Asn Trp Leu Val Pro Ile Gly His Glu Lys Pro Ser
 100 105 110
 Asn Ile Lys Val Phe Ile His Glu Leu Asn Ala Gly Asn Gln Leu Ser
 115 120 125
 His Met Ser Pro Ile Tyr Thr Ile Glu Met Gly Asp Glu Leu Leu Ala
 130 135 140
 Lys Leu Ala Arg Asp Ala Thr Phe Phe Val Arg Ala His Glu Ser Asn
 145 150 155 160
 Glu Met Gln Pro Thr Leu Ala Ile Ser His Ala Gly Val Ser Val Val
 165 170 175
 Met Ala Gln Thr Gln Pro Arg Arg Glu Lys Arg Trp Ser Glu Trp Ala
 180 185 190
 Ser Gly Lys Val Leu Cys Leu Leu Asp Pro Leu Asp Gly Val Tyr Asn
 195 200 205
 Tyr Leu Ala Gln Gln Arg Cys Asn Leu Asp Asp Thr Trp Glu Gly Lys
 210 215 220

12/12

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Ile Tyr Arg Val Leu Ala Gly Asn Pro Ala Lys His Asp Leu Asp Ile
225                230                235                240
Lys Pro Thr Val Ile Ser His Arg Leu His Phe Pro Glu Gly Gly Ser
                245                250                255
Leu Ala Ala Leu Thr Ala His Gln Ala Cys His Leu Pro Leu Glu Thr
                260                265                270
Phe Thr Arg His Arg Gln Pro Arg Gly Trp Glu Gln Leu Glu Gln Cys
                275                280                285
Gly Tyr Pro Val Gln Arg Leu Val Ala Leu Tyr Leu Ala Ala Arg Leu
                290                295                300
Ser Trp Asn Gln Val Asp Gln Val Ile Arg Asn Ala Leu Ala Ser Pro
305                310                315                320
Gly Ser Gly Gly Asp Leu Gly Glu Ala Ile Arg Glu Gln Pro Glu Gln
                325                330                335
Ala Arg Leu Ala Leu Thr Leu Ala Ala Glu Ser Glu Arg Phe Val
                340                345                350
Arg Gln Gly Thr Gly Asn Asp Glu Ala Gly Ala Ala Asn Leu Gln Gly
                355                360                365
Gly Leu Arg Gln Pro Arg Phe Val Asn Gln His Leu Cys Gly Ser His
                370                375                380
Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr
385                390                395                400
Thr Pro Lys Thr

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<210> 40

<211> 21

<212> PRT

<213> Polypeptide

<400> 40

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Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu
1           5           10           15
Glu Asn Tyr Cys Asn
                20

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