TITLE: TISSUE-SPECIFIC ENDOTHELIAL MEMBRANE PROTEINS

Abstract: Methods and compositions for targeting of pharmaceuticals or other therapeutics to specific tissues using tissue-specific endothelial membrane proteins are provided. The compositions comprise a therapeutic complex composed of a ligand, a linker, and a therapeutic moiety, where the therapeutic moiety can enter the cell. The ligand can be an antibody or other molecule that binds to a tissue-specific protein on the endothelial membrane of a specific tissue. The ligand does not activate a receptor, but may activate endocytosis. The therapeutic moiety could be a drug, gene, antisense oligonucleotide, contrast agent, protein, toxin, or any type of molecule that acts on the specific tissue. The linker is preferably cleaved upon going to the interior of the cell. Alternatively, the linker may simply be the bond between the ligand and the therapeutic moiety. Alternatively, a lipophilic prodrug may be cleaved and may enter the cell due to its lipophilic properties.
TISSUE-SPECIFIC ENDO THELIAL MEMBRANE PROTEINS

Background of the Invention

Field of the Invention

[0002] This invention relates generally to targeting of pharmaceuticals or other therapeutics to specific tissues using tissue specific endothelial membrane proteins.

Description of the Related Art

[0003] When conventional pharmaceuticals are delivered to a patient they circulate throughout the entire body of the patient and act on most if not all tissues or cells of the body. This requires high doses for treatment and results in systemic toxicity and side effects.

[0004] Targeted delivery of therapeutic or diagnostic agents to specific organs, tissues or cells is much safer and more effective than such a non-specific treatment, because much smaller amounts of the drug are needed and there is considerably less chance for side-effects or toxicity.

[0005] Previous methods for the targeted delivery of pharmaceuticals include the use of implants (e.g., Elise (1999) PNAS USA 96:3104-3107), stents or catheters (e.g., Murphy (1992) Circulation 86:1596-1604), or vascular isolation of an organ (e.g., Vahrmeijer (1998) Semin. Surg. Oncol. 14:262-268). However, these techniques are invasive, traumatic and can cause extensive inflammatory responses and fibrocellular proliferation.

[0006] Most previous attempts at tissue-specific delivery depended on sites within the tissue that were inaccessible to the compounds due to the natural barrier of the vasculature. An alternative method for targeted delivery of compounds involves organ or tissue-specific molecules exposed on the luminal surface of the vasculature rather than on the tissue cells themselves. Use of these molecules would allow for a very specific reaction. The specificity is due to the fact that blood vessels must express these tissue-specific endothelial proteins because the vasculature forms a complex and dynamic system which adapts to the needs of the tissue in which it is immersed.

[0007] Previously, methods for identifying these organ or tissue-specific molecules, which were exposed and accessible on the luminal surface of the vasculature, did not result in the identification of usable molecules. This is because the endothelial membrane represents only a miniscule portion of the tissue mass of any organ. When organs are analyzed by conventional means, the endothelial membranes become dispersed throughout the entire tissue homogenate. This renders isolation of the endothelial membrane and its proteins for separate analysis essentially impossible. In addition, even if isolated and in culture, these membranes tend to lose their tissue-specific properties. In the event such molecules are isolated in a useful manner, methods must be conceived which allow for uses of these molecules related to the treatment of diseases in patients.
Summary of the Invention

[0008] There are several exemplary embodiments of the instant invention. One such embodiment includes a method for delivering a therapeutic agent to a specific tissue, comprising: administering a therapeutically effective amount of a therapeutic complex, said therapeutic complex comprising: a ligand which binds to a tissue-specific luminaly expressed protein, a therapeutic moiety, and a linker which links said therapeutic moiety to said ligand.

[0009] Another embodiment includes a brain-specific therapeutic complex which interacts with a targeted endothelial cell, comprising: a ligand which attaches said therapeutic complex to the luminal surface of a vascular endothelial cell membrane of the specific tissue, wherein said ligand binds to a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 3-5, 21, 22 or a homolog thereof; a linker; and a therapeutic moiety, wherein said linker links the ligand to the therapeutic moiety.

[0010] Another embodiment includes a method of determining the presence or concentration of folate binding protein (FBP) in a tissue or cell, comprising administering the above brain-specific therapeutic complex to said tissue or cell in vitro or in vivo, and identifying or quantitating the amount of the therapeutic complex which bound.

[0011] Another embodiment includes a pharmaceutical composition comprising the above brain-specific therapeutic complex and one or more pharmaceutically acceptable carriers.

[0012] Another embodiment includes a heart-specific therapeutic complex which interacts with a targeted endothelial cell, comprising: a ligand which attaches said therapeutic complex to the luminal surface of a vascular endothelial cell membrane of the specific tissue, wherein the ligand binds to a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 6-13, 23-29 or a homolog thereof; a linker; and a therapeutic moiety, wherein said linker links the ligand with the therapeutic moiety.

[0013] Another embodiment includes a method of determining the presence or concentration of CD36, cell adhesion regulator, sargaplycan epsilon, testis ecto-arginine ADP ribosyltransferase (NAR3), aquaporin 2 or cadherin-13 in a tissue or cell, comprising administering the above heart-specific therapeutic complex to said tissue or cell in vitro or in vivo, and identifying or quantitating the amount of the therapeutic complex which bound.

[0014] Another embodiment includes a pharmaceutical composition comprising the above heart-specific therapeutic complex and one or more pharmaceutically acceptable carriers.

[0015] Another embodiment includes a lung-specific therapeutic complex which interacts with a targeted endothelial cell, comprising: a ligand which attaches said therapeutic complex to the luminal surface of a vascular endothelial cell membrane of the specific tissue,
wherein the ligand binds to a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 14-17, 30-34 or a homolog thereof; a linker; and a therapeutic moiety, wherein said linker links the ligand with the therapeutic moiety.

[0016] Another embodiment includes a method of determining the presence or concentration of CD9, receptor for advanced glycosylation end products (RAGE), integrin alpha-X or CD81 in a tissue or cell, comprising administering the above lung-specific therapeutic complex to said tissue or cell \textit{in vitro or in vivo}, and identifying or quantitating the amount of the therapeutic complex which bound.

[0017] Another embodiment includes a pharmaceutical composition comprising the above lung-specific therapeutic complex and one or more pharmaceutically acceptable carriers.

[0018] Another embodiment includes a lung and heart-specific therapeutic complex which interacts with a targeted endothelial cell, comprising: a ligand which attaches said therapeutic complex to the luminal surface of a vascular endothelial cell membrane of the specific tissue, wherein said ligand binds to a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 18, 19, 35 or a homolog thereof; a linker; and a therapeutic moiety, wherein said linker links the ligand with the therapeutic moiety.

[0019] Another embodiment includes a method of determining the presence or concentration of vascular adhesion protein-1 (VAP-1) in a tissue or cell, comprising administering the above lung and heart-specific therapeutic complex to said tissue or cell \textit{in vitro or in vivo}, and identifying or quantitating the amount of the therapeutic complex which bound.

[0020] Another embodiment includes a pharmaceutical composition comprising the lung and heart-specific therapeutic complex and one or more pharmaceutically acceptable carriers.

[0021] Another embodiment includes a pancreas-specific therapeutic complex which interacts with a targeted endothelial cell, comprising: a ligand which attaches said therapeutic complex to the luminal surface of a vascular endothelial cell membrane of the specific tissue, wherein said ligand binds to a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 20, 36, 37 or a homolog thereof; a linker; and a therapeutic moiety, wherein said linker links the ligand with the therapeutic moiety.

[0022] Another embodiment includes a method of determining the presence or concentration of membrane dipeptidase-1 (MDP-1) in a tissue or cell, comprising administering the above pancreas-specific therapeutic complex to said tissue or cell \textit{in vitro or in vivo}, and identifying or quantitating the amount of the therapeutic complex which bound.

[0023] Another embodiment includes a pharmaceutical composition comprising the pancreas-specific therapeutic complex and one or more pharmaceutically acceptable carriers.
Another embodiment includes a method for the treatment of brain tumors comprising administering the above brain-specific therapeutic complex in an amount effective to reduce the number of cancer cells, wherein said therapeutic moiety is a chemotherapeutic agent.

Another embodiment includes a method for the treatment of heart disease comprising administering the above heart-specific therapeutic complex in an amount effective to ameliorate the disease, wherein said therapeutic moiety is a pharmaceutical agent.

Another embodiment includes a method for the treatment of lung cancer comprising administering the above lung-specific or lung and heart-specific therapeutic complex in an amount effective to reduce the number of cancer cells, wherein said therapeutic moiety is a chemotherapeutic agent.

Another embodiment includes a method for the treatment of pancreatic cancer comprising administering the above pancreas-specific therapeutic complex in an amount effective to reduce the number of cancer cells, wherein said therapeutic moiety is a chemotherapeutic agent.

Another embodiment includes a method for delivering a therapeutic agent to a specific tissue, comprising: administering a therapeutically effective amount of a therapeutic complex, said therapeutic complex comprising: a ligand which binds to a tissue-specific luminaly expressed protein, a therapeutic moiety, and a linker which links said therapeutic moiety to said ligand, wherein said tissue-specific luminaly expressed protein is selected from the group consisting of: folate binding protein, CD36, cell adhesion regulator, sarcoglycan epsilon, NAR3, aquaporin 2, cadherin-13, CD9, RAGE, integrin alpha-X, CD81, VAP-1, or MDP-1.

Another embodiment includes a method for brain-specific delivery of a substance in vivo or in vitro, comprising: providing a folate binding protein-binding agent, and administering said folate binding protein-binding agent in vivo or in vitro, wherein said substance is delivered to the brain or brain tissue as a result of the administration of the folate binding protein-binding agent.

Another embodiment includes a method of identifying a brain-specific ligand, comprising identifying a folate binding protein-binding agent.

Another embodiment includes a method for heart-specific delivery of a substance in vivo or in vitro, comprising: providing a CD36, cell adhesion regulator, sarcoglycan epsilon, NAR3, aquaporin 2, or cadherin-13-binding agent, and administering said CD36, cell adhesion regulator, sarcoglycan epsilon, NAR3, aquaporin 2, or cadherin-13-binding agent in vivo or in vitro, wherein said substance is delivered to the heart or heart tissue as a result of the administration of the CD36, cell adhesion regulator, sarcoglycan epsilon, NAR3, aquaporin 2, or cadherin-13-binding agent.
Another embodiment includes a method of identifying a heart-specific ligand, comprising identifying a CD36, cell adhesion regulator, sarcoglycan epsilon, NAR3, aquaporin 2, or cadherin-13-binding agent.

Another embodiment includes a method for lung-specific delivery of a substance in vivo or in vitro, comprising: providing a CD9, RAGE, integrin alpha-X, or CD81-binding agent, and administering said CD9, RAGE, integrin alpha-X, or CD81-binding agent in vivo or in vitro, wherein said substance is delivered to the lung or lung tissue as a result of the administration of the CD9, RAGE, integrin alpha-X, or CD81-binding agent.

Another embodiment includes a method of identifying a lung-specific ligand, comprising identifying a CD9, RAGE, integrin alpha-X, or CD81-binding agent.

Another embodiment includes a method for lung and heart-specific delivery of a substance in vivo or in vitro, comprising: providing a VAP-1-binding agent, and administering said VAP-1-binding agent in vivo or in vitro, wherein said substance is delivered to the lung and heart or lung and heart tissue as a result of the administration of the VAP-1-binding agent.

Another embodiment includes a method of identifying a lung and heart-specific ligand, comprising identifying a VAP-1-binding agent.

Another embodiment includes a method for pancreas-specific delivery of a substance in vivo or in vitro, comprising: providing a MDP-1-binding agent, and administering said MDP-1-binding agent in vivo or in vitro, wherein said substance is delivered to the pancreas or pancreas tissue as a result of the administration of the MDP-1-binding agent.

Another embodiment includes a method of identifying a pancreas-specific ligand, comprising identifying a MDP-1-binding agent.

Brief Description of the Drawings

FIG. 1 is a depiction of a typical therapeutic complex of the preferred embodiment interacting with an endothelial cell surface, tissue-specific molecule.

FIG. 2 depicts a photograph of an SDS polyacrylamide gel that shows an approximately 40 kDa polypeptide that is present in the sample of pig brain but which is not present in the other tissues.

FIG. 3 depicts a photograph of an SDS polyacrylamide gel that shows an approximately 85 kDa polypeptide that is present in the sample of pig brain but which is not present in the other tissues.

FIG. 4 depicts a photograph of an SDS polyacrylamide gel that shows an approximately 35 kDa polypeptide that is present in the sample of pig brain but which is not present in the other tissues.
FIG. 5 depicts a photograph of an SDS polyacrylamide gel that shows an approximately 80 kDa polypeptide that is present in the sample of pig heart but which is not present in the other tissues.

FIG. 6 depicts a photograph of an SDS polyacrylamide gel that shows two approximately 47 kDa polypeptides that are present in the sample of pig heart but which is not present in the other tissues.

FIG. 7A-C depict a photograph of SDS polyacrylamide gels that shows an approximately 55 kDa polypeptide that is present in the sample of pig heart but which is not present in the other tissues.

FIG. 8 depicts a photograph of an SDS polyacrylamide gel that shows an approximately 17 kDa polypeptide that is present in the sample of pig heart but which is not present in the other tissues.

FIG. 9 depicts a photograph of an SDS polyacrylamide gel that shows an approximately 125 kDa polypeptide that is present in the sample of pig heart but which is not present in the other tissues.

FIG. 10 depicts a photograph of an SDS polyacrylamide gel that shows an approximately 100 kDa polypeptide that is present in the sample of pig lung and heart but which is not present in the other tissues.

FIG. 11 depicts a photograph of an SDS polyacrylamide gel that shows an approximately 25 kDa polypeptide that is present in the sample of pig lung but which is not present in the other tissues.

FIG. 12A-D depict photographs of two-dimensional gels that show an approximately 48 kDa polypeptide that is present in the sample of lung but which is not present in the other tissues.

FIG. 13A-D depict photographs of two-dimensional gels that show an approximately 125 kDa polypeptide that is present in the sample of lung but which is not present in the other tissues.

FIG. 14A-D depict photographs of two-dimensional gels that show an approximately 45 kDa polypeptide that is present in the sample of pig pancreas but which is not present in the other tissues.

FIG. 15A-D show the immunohistochemistry of tissue sections from a rat which was injected with either an antibody specific for CD71 (OX-26) or a control (albumin specific) antibody. FIG. 15A shows brain from a rat injected with biotin-labeled OX-26, FIG. 15B shows brain from a rat injected with biotin-labeled monoclonal antibody specific for albumin, FIG.
15C shows lung from a rat injected with biotin-labeled OX-26, FIG. 15D shows lung from a rat injected with biotin-labeled monoclonal antibody specific for albumin.

[0054] FIG. 16A-E are a series of immunohistograms showing various tissue sections taken from a rat that was injected with a biotin-labeled monoclonal antibody specific for folate binding protein.

[0055] FIG. 17A-F are a series of immunohistograms showing various tissue sections taken from a rat that was injected with gentamicin that was linked to a monoclonal antibody specific for folate binding protein.

Detailed Description of the Preferred Embodiment

[0056] One embodiment described herein supplies both compositions and methods of use of therapeutic compounds for delivery to a specific tissue whether or not such tissue is in a diseased state. Specifically, the invention utilizes tissue-specific luminally exposed proteins on endothelial cells so that the tissue-specific therapeutic complexes described herein will localize to a specific tissue due to binding of these complexes to luminally-exposed endothelial proteins. This embodiment allows for localization and concentration of a pharmaceutical agent to a specific tissue, thus increasing the therapeutic index of that pharmaceutical agent. This localization decreases the chances of side effects due to the agent and may allow one to use a lower concentration of the agent to achieve the same effect. Localization to a luminally-exposed tissue specific endothelial protein affords the added advantage that a single ligand can be used to treat a variety of diseases involving that tissue. In other words, a disease specific ligand for each disease state of a tissue need not be generated; as sufficient amounts of one or more therapeutic complexes will bind to the effected tissue which is expressing a protein normally found on the luminal endothelial cells of that tissue or organ. This feature allows the use of a single ligand to produce therapeutic complexes to treat any disease associated with the tissue. The tissue-specific molecule may be identified by the method of U.S. Patent Application No. 09/528,742, filed March 20, 2000, or any other method of identification. The method disclosed in U.S. patent application No. 09/528,742 permits the in vivo isolation of all proteins that are exposed on the inner surface of blood vessels from different tissues. All other proteins that make up the tissues (which are the vast majority) are discarded in the process. The resulting set of luminally exposed vascular proteins can then be separated and analyzed biochemically to identify each protein individually. By comparing the set of proteins expressed in each tissue, proteins are identified that are specific to a given tissue. Proteins of interest are then sequenced. The ligand upon binding to the target protein, or the protein that is tissue-specifically luminally expressed, preferably does not activate a specific signal transduction pathway in the cell it binds to, but may activate the process of transcytosis or pinocytosis.
Endothelial cell tissue-specific proteins are accessible to the blood, and thus, they can act at site-specific targets used to localize therapeutic complexes to a specific tissue. Blood vessels express these tissue-specific endothelial proteins because the vasculature forms a complex and dynamic system which adapts to the needs of the tissue in which it is immersed. Many of these proteins are constitutively expressed, meaning that their levels of expression are not significantly changed in different disease states, making them ideal targets for the delivery of pharmaceuticals whether or not the tissue or organ containing the tissue is in the diseased state. In addition, many of these proteins are involved in transcytosis, the process of transporting materials from within the blood vessels into the tissue.

**DEFINITIONS**

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

The term "target protein" as used herein is a tissue-specific, luminaly exposed vascular protein.

The term “therapeutic complex” is any type of molecule which includes a ligand specific for a target protein and one or more therapeutic moieties and a linker. However, it is to be understood that a therapeutic complex may also comprise an enzyme or some other inducer of cleavage which allows a prodrug to be converted into the corresponding pharmaceutical agent.

The term “ligand” as used herein is a molecule that specifically binds to the target protein. These can be peptides, antibodies or parts of antibodies, as well as non-protein moieties.

The term “linker” when used in conjunction with a therapeutic complex is any bond, small molecule, or other vehicle which allows the ligand and the therapeutic moiety to be targeted to the same area, tissue, or cell. The linker binds or otherwise holds together the ligand and the therapeutic moiety for binding to the target protein.

The term “therapeutic moiety” as used herein is any type of substance which can be used to effect a certain outcome. The outcome can be positive or negative, alternatively, the outcome can simply be diagnostic. The outcome may also be more subtle such as simply changing the molecular expression in a cell. The therapeutic moiety may also be an enzyme which allows conversion of a prodrug into the corresponding pharmaceutical agent.

The term "tissue-specific" refers to a molecule that is preferentially expressed on a specific tissue or cell-type, allowing a majority of the therapeutic complex (typically greater than 50%) to bind to that tissue after administration. The molecule may be found at a considerably higher concentration in one or a few tissues than in the others. For example, a tissue-specific
molecule may be highly upregulated in the lung compared to other tissues but can be dosed to be
even more specific based on the statistical distribution of binding throughout the vasculature.
Proper, often lower, dosing of the therapeutic complex would be given such that the amounts that
appear randomly at non-targeted tissue would render little or no side effects.

[0065] The term "luminal surface" as used herein means the surface of any perfusible
space, e.g., the luminally-exposed surface of cells lining a perfusible space, e.g., endothelial cells in
a vascular space (e.g., the lumen of an artery, vein, capillary, sinus, and the like).

[0066] The term "perfusible space" means any tissue or organ space that can be
perfused with a cell-impermeable reagent-containing buffer of the invention, e.g., any vascular or
lymphatic lumen, the CSF space, lumens of ducts, vitreous-aqueous humor space of the eye, fascial
planes, and the like, including spaces only present under disease, inflammatory or other conditions,
e.g., cysts, tumors, and the like.

[0067] The term "avidin" as used herein means any biotin-binding compound such as
avidin, streptavidin, any modified or mutant avidin produced by laboratory techniques which is
capable of binding biotin or a functional equivalent of biotin, any compound designed to function
6,022,951.

[0068] The term "biotin" as used herein means biotin, any modified biotin, and also
includes biotin analogs and equivalents thereof, e.g., biotin methyl ester, desthiobiotin,
diaminobiotin or 2-iminobiotin. See, e.g., Hofmann (1982) Biochemistry 21:978-984; Reznik
208.

[0069] The term "cell membrane impermeable reagent" as used herein means a
reagent that cannot enter or pass through the lipid bilayer of a cell membrane; e.g., the cell
membrane impermeable reagents of the invention, when perfused into tissue spaces, will only bind
to molecules exposed to the lumen of the space (assuming the membranes of the cells lining the
lumen are intact).

[0070] As used herein, "isolated," when referring to a molecule or composition, such
as, e.g., an isolated cell membrane impermeable reagent or tissue- or organ-specific molecule,
means that the molecule or composition is separated from at least one other compound, such as a
protein, DNA, RNA, lipid, carbohydrate, or other contaminants with which it is associated in vivo
or in its naturally occurring state. Thus, a tissue- or organ-specific molecule is considered isolated
when it has been isolated from any other component with which it is naturally associated. An
isolated composition can, however, also be substantially pure. An isolated composition can be in a homogeneous state. It can be in a dry or an aqueous solution. Purity and homogeneity can be determined, e.g., using any analytical chemistry technique, as described herein.

[0071] As used herein, when referring to a polynucleotide, “full-length” means a polynucleotide sequence that comprises an entire polypeptide coding region that is flanked by at least one start codon and at least one stop codon and encodes a full-length polypeptide. When referring to a polypeptide “full-length” means a protein having the amino acid sequence of a protein that is functional when expressed in its native state in vivo or an unprocessed precursor thereof. Although the sequence of the full-length polypeptide may correspond to the sequence of the functional protein, the full-length polypeptide need not be functional.

[0072] By “homologous polypeptide” is meant a polypeptide having at least 99%, 95%, at least 90%, at least 85%, at least 80%, at least 70%, at least 60%, at least 50%, at least 40% or at least 25% amino acid identity or similarity to a tissue-specific luminally-exposed polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs.: 3-37. Identity or similarity may be determined using the FASTA version 3.078 algorithm with the default parameters. Alternatively, protein identity or similarity may be identified using BLASTP with the default parameters, BLASTX with the default parameters, or TBLASTN with the default parameters. (Altschul, S.F. et al. Gapped BLAST and PSI-BLAST: A New Generation of Protein Database Search Programs, Nucleic Acid Res. 25: 3389-3402 (1997)). Homologous polypeptide also includes a polypeptide having at least 99%, 95%, at least 90%, at least 85%, at least 80%, at least 70%, at least 60%, at least 50%, at least 40% or at least 25% amino acid identity or similarity to a fragment comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids of a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs.: 3-37.

[0073] As used herein, the terms “protein” and “polypeptide” are interchangeable. Additionally, the terms “luminally exposed” and “luminally expressed” are used synonymously.

GENERAL TECHNIQUES

[0074] The embodiments described herein can be practiced in conjunction with any method or protocol known in the art and described in the scientific and patent literature. The various compositions (e.g., natural or synthetic compounds, polypeptides, peptides, nucleic acids, antibodies, toxins, and the like) used in the embodiment described herein can be isolated from a variety of sources, genetically engineered, amplified, and/or expressed recombinantly. Alternatively, these compositions can be synthesized in vitro by well-known chemical synthesis techniques, as described in, e.g., Organic Synthesis, collective volumes, Gilman et al. (Eds) John
The therapeutic complexes of the present invention bind to the target proteins, for example from the pancreas, lung, heart, and brain, to specifically deliver a therapeutic moiety to the tissue or organ of choice. The therapeutic complexes are composed of at least one ligand, a linker, and at least one therapeutic moiety (see FIG. 1). However, the attachment of the three types of components of the therapeutic complex can be envisioned to have a large number of different embodiments. The therapeutic moiety can be one or more of any type of molecule which is used in a therapeutic or diagnostic way. For example, the therapeutic moiety can be an antibiotic which needs to be taken up by a specific tissue. The therapeutic complex can be envisioned to concentrate and target the antibiotic to the tissue where it is needed, thus increasing the therapeutic index of that antibiotic. Alternatively, the therapeutic moiety can be for diagnostic purposes. Further examples of the use of therapeutic complexes in the specific embodiments of the present invention will be outlined in more detail in the section entitled "Type of Therapeutic Complex Interactions".

The ligand is a molecule which specifically binds to the target protein, in this case, the luminaly-expressed tissue-specific proteins. In one embodiment, the ligand is some type of antibody or part thereof which specifically binds to a luminaly expressed, tissue-specific molecule. Usually, the ligand recognizes an epitope which does not participate in the binding of a natural ligand. The ligand of the luminaly-expressed tissue-specific endothelial protein can be identified by any technique known to one of skill in the art, for example, using a two-hybrid technique, a combinatorial library, or producing an antibody molecule. The ligand may be a protein, RNA, DNA, small molecule or any other type of molecule which specifically binds to target proteins.

The target protein may be an integral membrane protein (such as a receptor) or may be a ligand itself. Should the tissue-specific molecule be a ligand which binds to a luminaly expressed protein, the ligand, or a fragment thereof which exhibits the lumen and tissue-specificity, is used in the construction of the therapeutic complex of the invention. Alternatively, antibodies, antibody fragments, or antibody complexes specific to, or with similar binding characteristics to, the luminaly exposed ligand molecule may be used in the construction of the therapeutic complex of the invention.

Should the tissue-specific luminaly exposed protein (target protein) be a receptor, natural ligands can be identified by one of skill in the art in a number of different ways. For example, a two-hybrid technique can be used. Alternatively, high-throughput screening can be
used to identify peptides which can act as ligands. Other methods of identifying ligand are known to one of skill in the art.

[0079] In one embodiment, the ligand of the therapeutic complex uses a different epitope than the natural ligand of the receptor target protein, so that there is no competition for binding sites.

[0080] In another embodiment, the ligand is an antibody molecule and preferably the antibody molecule has a higher specificity or binds to the tissue-specific luminally exposed receptor target protein in such a way that it will not be necessary to compete with the natural ligand.

[0081] Antibodies and fragments can be made by standard methods (See, for example, E. Harlow et al., Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1988). However, the isolation, identification, and molecular construction of antibodies has been developed to such an extent that the choices are almost inexhaustible. Therefore, examples of antibody parts, and complexes will be provided with the understanding that this can only represent a sampling of what is available.

[0082] The smallest fragment to bear the antigen binding site is the Fv portion of an antibody, a 26 kDa heterodimer consisting of the amino-terminal variable domains of the heavy and light chains. (Bird et al. (1988) Science 242:423-426). The antigen binding moiety can be located in a whole antibody, antibody fragment, or subfragment. Antibodies can be whole immunoglobulin (IgG) of any class, e.g., IgG, IgM, IgA, IgD, IgE, chimeric antibodies or hybrid antibodies with dual or multiple antigen or epitope specificities, or fragments, such as F(ab')2, Fab', Fab and the like, including hybrid fragments. Any immunoglobulin or any natural, synthetic, or genetically engineered protein that acts like an antibody by binding to luminally-exposed molecules can be used to target the therapeutic complex.

[0083] Preparations of polyclonal antibodies can be made using standard methods which are well known in the art. Antibodies can include antiserum preparations from a variety of commonly used animals, e.g., goats, primates, donkeys, swine, rabbits, horses, hens, guinea pigs, rats, or mice, and even human antisera after appropriate selection and purification. Animal antisera are raised by inoculating the animals with immunogenic epitopes of the tissue-specific luminally-exposed molecules isolated by the methods disclosed herein. The animals are then bled and the serum or an immunoglobulin-containing serum fraction is recovered.

[0084] Hybridoma-derived monoclonal antibodies (human, monkey, rat, mouse, or the like) are also suitable for use in the present invention and have the advantage of high specificity. They are readily prepared by what are now generally considered conventional procedures for the immunization of mammals with preparations such as, the immunogenic epitopes of the tissue-specific luminally-exposed molecules isolated by the methods disclosed herein, fusion of immune
lymph or spleen cells with an immortal myeloma cell line, and isolation of specific hybridoma clones. More unconventional methods of preparing monoclonal antibodies are not excluded, such as interspecies fusions and genetic engineering manipulations of hypervariable regions, as it is primarily the specificity of the antibodies for the tissue-specific luminally-exposed molecules that affects their utility in the present invention.

[0085] In one embodiment, the antibody is a single chain Fv region. Antibody molecules have two generally recognized regions, in each of the heavy and light chains. These regions are the so-called “variable” region which is responsible for binding to the specific antigen in question, and the so-called “constant” region which is responsible for biological effector responses such as complement binding, binding to neutrophils and macrophages, etc. The constant regions are not necessary for antigen binding. The constant regions have been separated from the antibody molecule, and variable binding regions have been obtained. Therefore, the constant regions are clearly not necessary for the binding action of the antibody molecule when it is acting as the ligand portion of the therapeutic complex.

[0086] The variable regions of an antibody are composed of a light chain and a heavy chain. Light and heavy chain variable regions have been cloned and expressed in foreign hosts, while maintaining their binding ability. Therefore, it is possible to generate a single chain structure from the multiple chain aggregate (the antibody), such that the single chain structure will retain the three-dimensional architecture of the multiple chain aggregate.

[0087] Fv fragments which are single polypeptide chain binding proteins having the characteristic binding ability of multi-chain variable regions of antibody molecules, can be used for the ligand of the present invention. These ligands are produced, for example, following the methods of Ladner et al., US 5,260,203, issued November 9, 1993, using a computer based system and method to determine chemical structures. These chemical structures are used for converting two naturally aggregated but chemically separated light and heavy polypeptide chains from an antibody variable region into a single polypeptide chain which will fold into a three dimensional structure very similar to the original structure of the two polypeptide chains. The two regions may be linked using an amino acid sequence as a bridge.

[0088] The single polypeptide chain obtained from this method can then be used to prepare a genetic sequence coding therefor. The genetic sequence can then be replicated in appropriate hosts, further linked to control regions, and transformed into expression hosts, wherein it can be expressed. The resulting single polypeptide chain binding protein, upon refolding, has the binding characteristics of the aggregate of the original two (heavy and light) polypeptide chains of the variable region of the antibody.
In a further embodiment, the antibodies are multivalent forms of single-chain antigen-binding proteins. Multivalent forms of single-chain antigen-binding proteins have significant utility beyond that of the monovalent single-chain antigen-binding proteins. A multivalent antigen-binding protein has more than one antigen-binding site which results in an enhanced binding affinity. The multivalent antibodies can be produced using the method disclosed in Whitlow et al., U.S. Pat. No. 5,869,620, issued February 9, 1999. The method involves producing a multivalent antigen-binding protein by linking at least two single-chain molecules, each single chain molecule having two binding portions of the variable region of an antibody heavy or light chain linked into a single chain protein. In this way the antibodies can have binding sites for different parts of an antigen or have binding sites for multiple antigens.

In one embodiment, the antibody is an oligomer. The oligomer is produced as in PCT/EP97/05897, filed October 24, 1997, by first isolating a specific ligand from a phage-displayed library. Oligomers overcome the problem of the isolation of mostly low affinity ligands from these libraries, by oligomerizing the low-affinity ligands to produce high affinity oligomers. The oligomers are constructed by producing a fusion protein with the ligand fused to a semi-rigid hinge and a coiled coil domain from Cartilage Oligomeric Matrix Protein (COMP). When the fusion protein is expressed in a host cell, it self assembles into oligomers.

Preferably, the oligomers are peptabodies (Terskilkh et al., Biochemistry 94:1663-1668 (1997)). Peptabodies can be exemplified as IgM antibodies which are pentameric with each binding site having low-affinity binding, but able to bind in a high affinity manner as a complex. Peptabodies are made using phage-displayed random peptide libraries. A short peptide ligand from the library is fused via a semi-rigid hinge at the N-terminus of the COMP (cartilage oligomeric matrix protein) pentamerization domain. The fusion protein is expressed in bacteria where it assembles into a pentameric antibody which shows high affinity for its target. Depending on the affinity of the ligand, an antibody with very high affinity can be produced.

Preferably the antibody, antibody part or antibody complex of the present invention is produced in humans or is "humanized" (i.e. non-immunogenic in a human) by recombinant or other technology. Such antibodies are the equivalents of the monoclonal and polyclonal antibodies disclosed herein, but are less immunogenic, and are better tolerated by the patient.

Humanized antibodies may be produced, for example, by replacing an immunogenic portion of an antibody with a corresponding, but non-immunogenic portion (i.e. chimeric antibodies) (See, for example, Robinson, et al., International Patent Publication No. PCT/US86/02269; Akira, et al., European Patent Application No. 184,187; Taniguchi, European Patent Application No. 171,496; Morrison, et al., European Patent Application No. 173,494;


[0095] Other types of antibodies can be generated and used to construct the therapeutic complexes of the invention. For example, chimeric antibodies which comprise portions derived from two different species, such as a human constant region and a murine variable or binding region, can be constructed. The portions derived from two different species can be joined together chemically by conventional techniques or can be prepared as single contiguous proteins using genetic engineering techniques. DNA encoding the proteins of both the light chain and heavy chain portions of the chimeric antibody can be expressed as contiguous proteins. Chimeric antibodies can be constructed as disclosed in International Publication Number WO 93/03151. Binding proteins can also be prepared which are derived from immunoglobulins and which are multivalent and multispecific, such as the "diabodies" described in International Publication Number WO 94/13804.

[0096] Antibodies can be purified by methods well known in the art. For example, antibodies can be affinity purified by passing the antibodies over a column to which a tissue-specific luminality-exposed molecule is bound. The bound antibodies can then be eluted from the column, using a buffer with a high salt concentration.

[0097] Small molecules are any non-biopolymeric DNA, RNA, organic, or inorganic molecules such as macrocycles, alkene isomers, and many of what is typically thought of as drugs in the pharmaceutical industry. These molecules are often identified through combinatorial processes. In particular, a ligand can be identified using a process called "docking", an approach to rational drug design which seeks to predict the structure and binding free energy of a ligand-receptor complex given only the structures of the free ligand and receptor. Typically, these small molecules are used to bind to a specific protein and elicit an effect. However, it is envisioned in this context that they would simply be used to bind a specific protein and thus localize the attached drug to the required organs.
Ligands can also be produced, for example, using a library of expression vectors which contain stochastically generated polynucleotide sequences. Host cells containing the expression vectors are cultured so as to produce polypeptides encoded by the polynucleotide sequences. The polypeptides can then be screened for the ability to bind to a tissue-specific luminally-exposed molecule of interest by using protein binding assays known in the art, such as electrophoresis through a non-denaturing gel, column chromatography, the yeast two-hybrid assay, and the like. This method of generating binding molecules is taught in U.S. Pat. No. 5,763,192. Computer-aided molecular design can also be used to generate ligands. (See, Caflisch, A. (1996) J. Comput. Aided Mol. Des. 10:372-96).

The “linker” as used herein is any bond, small molecule, or other vehicle which allows the ligand and the therapeutic moiety to be targeted to the same area, tissue, or cell. Preferably, the linker is cleavable.

In one embodiment the linker is a chemical bond between one or more ligands and one or more therapeutic moieties. Thus, the bond may be covalent or ionic. An example of a therapeutic complex where the linker is a chemical bond would be a fusion protein. In one embodiment, the chemical bond is acid sensitive and the pH sensitive bond is cleaved upon going from the blood stream (pH 7.5) to the transcytotic vesicle or the interior of the cell (pH about 6.0). Alternatively, the bond may not be acid sensitive, but may be cleavable by a specific enzyme or chemical which is subsequently added or naturally found in the microenvironment of the targeted site. Alternatively, the bond may be a bond that is cleaved under reducing conditions, for example a disulfide bond. Alternatively, the bond may not be cleavable.

Any kind of acid cleavable or acid sensitive linker may be used. Examples of acid cleavable bonds include, but are not limited to: a class of organic acids known as cis-polycarboxylic alkenes. This class of molecule contains at least three carboxylic acid groups (COOH) attached to a carbon chain that contains at least one double bond. These molecules as well as how they are made and used is disclosed in Shen, et al. U.S. Patent No. 4,631,190. Alternatively, molecules such as amino-sulfhydryl cross-linking reagents which are cleavable under mildly acidic conditions may be used. These molecules are disclosed in Blattler et al., U.S. Patent No. 4,569,789.

Alternatively, the acid cleavable linker may be a time-release bond, such as a biodegradable, hydrolyzable bond. Typical biodegradable carrier bonds include esters, amides or urethane bonds, so that typical carriers are polyesters, polyamides, polyurethanes and other condensation polymers having a molecular weight between about 5,000 and 1,000,000. Examples of these carriers/bonds are shown in Peterson, et al., U.S. Patent No. 4,356,166. Other acid
cleavable linkers may be found in U.S. patent Nos. 4,569,789 and 4,631,190 or Blattner et al. in Biochemistry 24: 1517-1524 (1984). The linkers are cleaved by natural acidic conditions, or alternatively, acid conditions can be induced at a target site as explained in Abrams et al., U.S. Patent No. 4,171,563.

[0103] Examples of linking reagents which contain cleavable disulfide bonds (reducible bonds) include, but are not limited to "DPDPB", 1,4- di-[3’-(2’-pyridyl)diithio]propionamido]butane; "SADP", (N-succinimidyl(4-azidophenyl)1,3’-dithiopropionate); "Sulfos –SADP" (Sulfo succinimidyl (4-azidophenyl)diithio)propionate; "DSP" – Dithio bis (succinimidylpropionate); "DTSSP" – 3,3’ – Dithio bis (sulfo succinimidylpropionate); "DTBP" - dimethyl 3,3’-dithiobispropionimidate – 2 HCl, all available from Pierce Chemicals (Rockford, Illinois).

[0104] Examples of linking reagents cleavable by oxidation are "DST" - disuccinimidyl tartarate; and "Sulfo-DST" - disuccinimidyl tartarate. Again, these linkers are available from Pierce Chemicals.

[0105] Examples of non-cleavable linkers are "Sulfo-LC-SMPT" - (sulfo succinimidyl 6-[alpha-methyl-alpha-(2-pyridylthio)toluamido]hexanoate; "SMPT"; "ABH" - Azidobenzoyl hydrazide; "NHS-ASA" - N-Hydroxysuccinimidy1-4-azidosalicylic acid; "SASD" - Sulfo succinimidyl 2-(p-azidosalicylamido)ethyl - 1,3-dithiopropionate; "APDP" - N-(4-(p-azidosalicylamido)butyl) - 3’(2’-pyridylditthio) propionamide; "BASED" - Bis-[beta - (4-azidosalicylamido)ethyl] disulfide; "HSAB" - N-hydroxysuccinimidyl - 4 azidobenzoate; "APG" - p-Azidophenyl glyoxal monohydrate; "SANPAH" - N-Succinimidyl - 6(4’-azido-2’-nitrophenyl - amino)hexanoate; "Sulfo – SANPAH" -Sulfo succinimidyl 6-(4’-azido-2’-nitrophenylamino)hexanoate; "ANB-NOS" - N-5-Azido-2-nitrobenzoyloxyoxysuccinimide; "SAND" - Sulfo succinimidyl-2-(m-azido-o-mnitrobenzamido)-ethyl-1,3’- dithiopropionate; "PNP-DTP" - p-nitrophenyl-2-diazo-3,3,3-trifluoropropionate; "SMCC" - Succinimidyl 4-(N-maleimidomethyl)cyclohexane – 1- carboxylate; "Sulfo-SMCC" - Sulfo succinimidyl 4-(N-maleimidomethyl)cyclohexane – 1- carboxylate; "MBS" - m-Maleimidobenzyol-N-hydroxysuccinimide ester; "sulfo-MBS" - m-Maleimidobenzoyl-N-hydroxysulfosuccinimide ester; "SIAB" - N-Succinimidyl(4-iodoacetyl)aminobenzoate; "Sulfo-SIAB" - N-Sulfosuccinimidyl(4-iodoacetyl)aminobenzoate; "SMBP" - Succinimidyl 4-(p-maleimidophenyl)butyrate; "Sulfo-SMPB" - Sulfo succinimidyl 4-(p-maleimidophenyl)butyrate; "DSS" - Disuccinimidyl ester; "BSSS" - bis(sulfo succinimidyl) ester; "BMH" - Bis maleimidoexane; "DFDNB" - 1,5-difluoro-2,4-dinitrobenzene; "DMA" - dimethyl adipimidate 2 HCl; "DMP" - Dimethyl pimelimidate – 2HCl; "DMS" - dimethyl succinimidate – 2- HCl; "SPDP" - N-succinimidyl-3-(2-pyridylthio)propionate; "Sulfo –HSAB" - Sulfo succinimidyl 4-(p-azidophenyl)butyrate; "Sulfo-
SAPB" - Sulfosuccinimidyl 4-(p-azidophenylbutyrate); "ASIB" - 1-9p-azidosalicylamido)-4-
(iodoacetamido)butane; "ASBA" - 4-(p-Azidosalicylamido)butylamine. All of these linkers are
available from Pierce Chemicals.

[0106] In another embodiment the linker is a small molecule such as a peptide linker.
In one embodiment the peptide linker is not cleavable. In a further embodiment the peptide linker
is cleavable by base, under reducing conditions, or by a specific enzyme. In one embodiment, the
enzyme is indigenous. Alternatively, the small peptide may be cleavable by an non-indigenous
enzyme which is administered after or in addition to the therapeutic complex. Alternatively, the
small peptide may be cleaved under reducing conditions, for example, when the peptide contains a
disulfide bond. Alternatively, the small peptide may be pH sensitive. Examples of peptide linkers
include: poly(L-Gly), (Poly L-Glycine linkers); poly(L-Glu), (Poly L-Glutamine linkers); poly(L-
Lys), (Poly L-Lysine linkers). In one embodiment, the peptide linker has the formula (amino acid)_n,
where n is an integer between 2 and 100, preferably wherein the peptide comprises a
polymer of one or more amino acids.

[0107] In a further embodiment, the peptide linker is cleavable by proteinase such as
one having the sequence Gly-(D)Phe-Pro-Arg-Gly-Phe-Pro-Ala-Gly-Gly (SEQ ID NO: 1) (Suzuki,
et al. 1998, J. Biomed. Mater. Res. Oct;42(1):112-6). This embodiment has been shown to be
advantageous for the treatment of bacterial infections, particularly Pseudomonas aeruginosa.
Gentamicin or an alternate antibiotic is cleaved only when the wounds are infected by
Pseudomonas aeruginosa because there is significantly higher activity of thrombin-like proteinase
enzymes then in non-infected tissue.

[0108] In a further embodiment the linker is a cleavable linker comprising,
poly(ethylene glycol) (PEG) and a dipeptide, L-alanyl-L-valine (Ala-Val), cleavable by the enzyme
thermolysin. This linker is advantageous because thermolysin-like enzyme has been reported to be
expressed at the site of many tumors. Alternatively, a 12 residue spacer Thr-Arg-His-Arg-Gln-Pro-
Arg-Gly-Trp-Glu-Gln-Leu (SEQ ID NO: 2) may be used which contains the recognition site for the

[0109] The chemical and peptide linkers can be bonded between the ligand and the
therapeutic moiety by techniques known in the art for conjugate synthesis, i.e. using genetic
engineering, or chemically. The conjugate synthesis can be accomplished chemically via the
appropriate antibody by classical coupling reactions of proteins to other moieties at appropriate
functional groups. Examples of the functional groups present in proteins and utilized normally for
chemical coupling reactions are outlined as follows. The carbohydrate structures may be oxidized
to aldehyde groups that in turn are reacted with a compound containing the group H2NNH-R
(wherein R is the compound) to the formation of a C=N-H-NH-R group. The thiol group (cysteines
in proteins) may be reacted with a compound containing a thiol-reactive group to the formation of a thioether group or disulfide group. The free amino group (at the amino terminus of a protein or on a lysine) in amino acid residues may be reacted with a compound containing an electrophilic group, such as an activated carboxy group, to the formation of an amide group. Free carboxy groups in amino acid residues may be transformed to a reactive carboxy group and then reacted with a compound containing an amino group to the formation of an amide group.

[0110] The linker may alternatively be a liposome. Many methods for the preparation of liposomes are well known in the art. For example, the reverse phase evaporation method, freeze-thaw methods, extrusion methods, and dehydration-rehydration methods. (see Storm, et al. *PSTT* 1:19-31 (1998)).

[0111] The liposomes may be produced in a solution containing the therapeutic moiety so that the substance is encapsulated during polymerization. Alternatively, the liposomes can be polymerized first, and the biologically active substance can be added later by resuspending the polymerized liposomes in a solution of a biologically active substance and treating with sonication to affect encapsulation of the therapeutic moiety. The liposomes can be polymerized in the presence of the ligand such that the ligand becomes a part of the phospholipid bilayer. In one embodiment, the liposome contains the therapeutic moiety on the inside and the ligand on the outside.

[0112] The liposomes contemplated in the present invention can comprise a variety of structures. For example, the liposomes can be multilamellar large vesicles (MLV), oligolamellar vesicles (OLV), unilamellar vesicles (UV), small unilamellar vesicles (SUV), medium sized unilamellar vesicles (MUV), large unilamellar vesicles (LUV), giant unilamellar vesicles (GUV), or multivesicular vesicles (MVV). Each of these liposome structures are well known in the art (see Storm, et al. *PSTT* 1:19-31 (1998)).

[0113] In one embodiment, the liposome is a "micromachine" that evulses pharmaceuticals for example by the application of specific frequency radio waves. In another embodiment, the liposomes can be degraded such that they will release the therapeutic moiety in the targeted cell, for example, the liposomes may be acid or alkaline sensitive, or degraded in the presence of a low or high pH, such that the therapeutic moiety is released within the cell. Alternatively, the liposomes may be uncharged so that they will be taken up by the targeted cell. The liposomes may also be pH sensitive or sensitive to reducing conditions.

[0114] One type of liposome which may be advantageously used in the present invention is that identified in Langer et al., US Patent No. 6,004,534, issued December 21, 1999. In this application a method of producing modified liposomes which are prepared by polymerization of double and triple bond-containing monomeric phospholipids is disclosed. These
liposomes have surprisingly enhanced stability against the harsh environment of the gastrointestinal tract. Thus, they have utility for oral and/or mucosal delivery of the therapeutic moiety. It has also been shown that the liposomes may be absorbed into the systemic circulation and lymphatic circulation. The liposomes are generally prepared by polymerization (i.e., radical initiation or radiation) of double and triple bond-containing monomeric phospholipids.

[0115] In other embodiments of the present invention, the linker can also be a liposome having a long blood circulation time. Such liposomes are well known in the art, (see United States Patent Numbers, 5,013,556; 5,225,212; 5,213,804; 5,356,633; and 5,843,473. Liposomes having long blood circulation time are characterized by having a portion of their phospholipids derivatized with polyethylene glycol (PEG) or other similar polymer. In some embodiments, the end of the PEG molecule distal to the phospholipid may be activated so as to be chemically reactive. Such a reactive PEG molecule can be used to link a ligand to the liposome. One example of a reactive PEG molecule is the maleimide derivative of PEG described in United States Patent Number 5,527,528).

[0116] In yet other embodiments, the linker may be a microcapsule, a nanoparticle, a magnetic particle, and the like (Kumar, J. Pharm. Sci., May-Aug 3(2):234-258, 2000; and Gill et al., Trends Biotechnol. Nov;18(11):469-79, 2000), with the lipophilic therapeutic moiety on or in the container, and the container functioning as the linker in the therapeutic complex.

[0117] Alternatively, the linker may be a photocleavable linker. For example, a 1-2-(nitrophenyl)-ethyl moiety can be cleaved using 300 to 360 nm light (see Pierce catalog no. 21332ZZ). It can be envisioned that the photocleavable linker would allow activation and action of the drug in an even more specific area, for example a particular part of the organ. The light could be localized using a catheter into the vessel. Alternatively, light may be used to localize treatment to a specific part of the digestive tract and the light may be manipulated through a natural orifice to the area. Alternatively, the light can be surgically manipulated to the area.

[0118] Alternatively, the linker may not be cleavable, but the therapeutic moiety or ligand is. An example of this is when the therapeutic moiety is a prodrug and the enzyme which cleaves the prodrug is administered with the therapeutic complex. Alternatively, the enzyme is part of the therapeutic complex or indigenous and the prodrug is administered separately. Preferably, the enzyme or prodrug which is administered separately is administered within about 48 hours of the first administration. Alternatively, the prodrug or enzyme which is administered separately may be administered between about 1 min and 24 hours, alternatively between about 2 min and 8 hours. The prodrug or enzyme which is administered separately, may be readministered at a later date and may continue to be administered until the effect of the drug is not longer needed or until the enzymatic cleavage of all of the drug is effected.
THERAPEUTIC MOIETIES

[0119] The "therapeutic moiety" could be any chemical, molecule, or complex which effects a desired result. Examples include but are not limited to: conventional pharmaceutical agents such as antibiotics, anti-neoplastic agents, immunosuppressive agents, hormones, and the like, one or more genes, antisense oligonucleotides, contrast agents, proteins, toxins, radioactive molecules or atoms, surfactant proteins, or clotting proteins. The therapeutic moiety may be lipophilic, a quality which will help it enter the targeted cell.

[0120] The contrast agents may be any type of contrast agent known to one of skill in the art. The most common contrast agents basically fall into one of four groups: X-ray reagents, radiography reagents, magnetic resonance imaging agents, and ultrasound agents. The X-ray reagents include ionic, iodine-containing reagents as well as non-ionic agents such as Omniquin (Nycomed) and Ultravist (Schering). Radiographic agents include radioisotopes as disclosed below. Magnetic Resonance Imaging reagents include magnetic agents such a Gadolinium and iron-oxide chelates. Ultrasound agents include microbubbles of gas and a number of bubble-releasing formulations.

[0121] The radionuclides may be diagnostic or therapeutic. Examples of radionuclides that are generally medically useful include: Y, Ln, Cu, Lu, Tc, Re, Co, Fe and the like such as $^{90}Y$, $^{111}In$, $^{67}Cu$, $^{77}Lu$, $^{99m}Tc$ and the like, preferably trivalent cations, such as $^{90}Y$ and $^{111}ln$.

[0122] Radionuclides that are suitable for imaging organs and tissues in vivo via diagnostic gamma scintillation photometry include the following: $\gamma$-emitting radionuclides: $^{111}In$, $^{113m}In$, $^{67}Ga$, $^{68}Ga$, $^{99m}Tc$, $^{51}Cr$, $^{197}Hg$, $^{203}Hg$, $^{169}Yb$, $^{85}Sr$, and $^{87}Sr$. The preparation of chelated radionuclides that are suitable for binding by Fab' fragments is taught in U.S. Pat. No. 4,658,639 (Nicoletti et al.).

[0123] Paramagnetic metal ions, suitable for use as imaging agents in MRI include the lanthanide elements of atomic number 57-70, or the transition metals of atomic numbers 21-29, 42 or 44. U.S. Pat. No. 4,647,447 (Gries et al.) teaches MRI imaging via chelated paramagnetic metal ions.

[0124] Examples of therapeutic radionuclides are the $\beta$- emitters. Suitable $\beta$- emitters include $^{67}Cu$, $^{186}Rh$, $^{188}Rh$, $^{189}Rh$, $^{153}Sm$, $^{90}Y$, and $^{111}In$.

[0125] Antisense oligonucleotides have a potential use in the treatment of any disease caused by overexpression of a normal gene, or expression of an aberrant gene. Antisense oligonucleotides can be used to reduce or stop expression of that gene. Examples of oncogenes which can be treated with antisense technology and references which teach specific antisense molecules which can be used include: c-Jun and cFos (U.S. Patent No. 5,985,558); HER-2 (U.S.
Proteins which may be used as therapeutic agents include apoptosis inducing agents such as pRB and p53 which induce apoptosis when present in a cell (Xu et al. U.S. Patent No. 5,912,236), and proteins which are deleted or underexpressed in disease such as erythropoietin (Sytkowski, et al. U.S. Patent No. 6,048,971).

It can be envisioned that the therapeutic moiety can be any chemotherapeutic agent for neoplastic diseases such as alkylating agents (nitrogen mustards, ethylenimines, alkyl sulfonates, nitrosoureas, and triazenes), antimetabolites (folic acid analogs such as methotrexate, pyrimidine analogs, and purine analogs), natural products and their derivatives (antibiotics, alkaloids, enzymes), hormones and antagonists (adrenocorticosteroids, progestins, estrogens), and the like. Alternatively, the therapeutic moiety can be an antisense oligonucleotide which acts as an anti-neoplastic agent, or a protein which activates apoptosis in a neoplastic cell.

The therapeutic moiety can be any type of neuroeffector, for example, neurotransmitters or neurotransmitter antagonists may be targeted to an area where they are needed without the wide variety of side effects commonly experienced with their use.

The therapeutic moiety can be an anesthetic such as an opioid, which can be targeted specifically to the area of pain. Side effects, such as nausea, are commonly experienced by patients using opioid pain relievers. The method of the present invention would allow the very specific localization of the drug to the area where it is needed, such as a surgical wound or joints in the case of arthritis, which may reduce the side effects.

The therapeutic moiety can be an anti-inflammatory agent such as histamine, H1-receptor antagonists, and bradykinin. Alternatively, the anti-inflammatory agent can be a non-steroidal anti-inflammatory such as salicylic acid derivatives, indole and indene acetic acids, and alkanones. Alternatively, the anti-inflammatory agent can be one for the treatment of asthma such as corticosteroids, cromolyn sodium, and nedocromil. The anti-inflammatory agent can be administered with or without the bronchodilators such as B2-selective androgenic drugs and theophylline.

The therapeutic moiety can be a diuretic, a vasopressin agonist or antagonist, angiotensin, or renin which specifically effect a patient's blood pressure.

The therapeutic moiety can be any pharmaceutical used for the treatment of heart disease. Such pharmaceuticals include, but are not limited to, organic nitrites (amyl nitrites, nitroglycerin, isosorbide dinitrate), calcium channel blockers, antiplatelet and antithrombotic agents, vasodilators, vasoinhibitors, anti - digitalis antibodies, and nodal blockers.
[0133] The therapeutic moiety can be any pharmaceutical used for the treatment of protozoan infections such as tetracycline, clindamycin, quinines, chloroquine, mefloquine, trimethoprim-sulfanethoxazole, metronidazole, and oramin. The ability to target pharmaceuticals or other therapeutics to the area of the protozoal infection is of particular value due to the very common and severe side effects experienced with these antibiotic pharmaceuticals.

[0134] The therapeutic moiety can be any anti-bacterial such as sulfonamides, quinolones, penicillins, cephalosporins, aminoglycosides, tetracyclines, chloramphenicol, erythromycin, isoniazids and rifampin.

[0135] The therapeutic moiety can be any pharmaceutical agent used for the treatment of fungal infections such as amphotericins, flucytosine, miconazole, and fluconazole.

[0136] The therapeutic moiety can be any pharmaceutical agent used for the treatment of viral infections such as acyclovir, vidarabine, interferons, ribavirin, zidovudine, zalcitabine, reverse transcriptase inhibitors, and protease inhibitors. It can also be envisioned that virally infected cells can be targeted and killed using other therapeutic moieties, such as toxins, radioactive atoms, and apoptosis-inducing agents.

[0137] The therapeutic moiety can be chosen from a variety of anticoagulant, anti-thrombolyic, and anti-platelet pharmaceuticals.

[0138] It can be envisioned that diseases resulting from an over- or under-production of hormones can be treated using such therapeutic moieties as hormones (growth hormone, androgens, estrogens, gonadotropin-releasing hormone, thyroid hormones, adrenocortical steroids, insulin, and glucagon). Alternatively, if the hormone is over-produced, antagonists or antibodies to the hormones may be used as the therapeutic moiety.

[0139] Various other possible therapeutic moieties include vitamins, enzymes, and other under-produced cellular components and toxins such as diphtheria toxin or botulism toxin.

[0140] Alternatively, the therapeutic moiety may be one that is typically used in \textit{in vitro} diagnostics. Thus, the ligand and linker are labeled by conventional methods to form all or part of a signal generating system. The ligand and linker can be covalently bound to radioisotopes such as tritium, carbon 14, phosphorous 32, iodine 125 and iodine 131 by methods well known in the art. For example, 125I can be introduced by procedures such as the chloramine-T procedure, enzymatically by the lactoperoxidase procedure or by the prelabeled Bolton-Hunter technique. These techniques plus others are discussed in H. Van Vunakis and J.J. Langone, Editors, Methods in Enzymology, Vol. 70, Part A, 1980. See also U.S. Patent No. 3,646,346, issued February 29, 1972, and Edwards \textit{et al.}, U.S. Patent No. 4,062,733, issued December 13, 1977, respectively, for further examples of radioactive labels.
Therapeutic moieties also include chromogenic labels, which are those compounds that absorb light in the visible ultraviolet wavelengths. Such compounds are usually dyestuffs and include quinoline dyes, triarylmethane dyes, phthaleins, insect dyes, azo dyes, anthraquimoid dyes, cyanine dyes, and phenazoxonium dyes.

Fluorogenic compounds can also be therapeutic moieties and include those which emit light in the ultraviolet or visible wavelength subsequent to irradiation by light. The fluorogens can be employed by themselves or with quencher molecules. The primary fluorogens are those of the rhodamine, fluorescein and umbelliferone families. The method of conjugation and use for these and other fluorogens can be found in the art. See, for example, J.J. Langone, H. Van Vunakis et al., Methods in Enzymology, Vol. 74, Part C, 1981, especially at page 3 through 105. For a representative listing of other suitable fluorogens, see Tom et al., U.S. Patent No. 4,366,241, issued December 28, 1982, especially at column 28 and 29. For further examples, see also U.S. Patent No. 3,996,345.

These non-enzymatic signal systems are adequate therapeutic moieties for the present invention. However, those skilled in the art will recognize that an enzyme-catalyzed signal system is in general more sensitive than a non-enzymatic system. Thus, for the instant invention, catalytic labels are the more sensitive non-radioactive labels.

Catalytic labels include those known in the art and include single and dual (“channeled”) enzymes such as alkaline phosphatase, horseradish peroxidase, luciferase, β-galactosidase, glucose oxidase (lysozyme, malate dehydrogenase, glucose-6-phosphate dehydrogenase) and the like. Examples of dual (“channeled”) catalytic systems include alkaline phosphatase and glucose oxidase using glucose-6-phosphate as the initial substrate. A second example of such a dual catalytic system is illustrated by the oxidation of glucose to hydrogen peroxide by glucose oxidase, which hydrogen peroxide would react with a leuco dye to produce a signal generator. (A further discussion of catalytic systems can be found in Tom et al., U.S. Patent No. 4,366,241, issued December 28, 1982 (see especially columns 27 through 40). Also, see Weng et al., U.S. Patent No. 4,740,468, issued April 26, 1988, especially at columns 2 and columns 6, 7 and 8.

The procedures for incorporating enzymes into the instant therapeutic complexes are well known in the art. Reagents used for this procedure include glutaraldehyde, p-toluene diisocyanate, various carbodiimide reagents, p-benzoquinone m-periodate, N,N1-o-phenylenedimaleimide and the like (see, for example, J.H. Kennedy et al., Clin. Chim Acta 70, 1 (1976)). As another aspect of the invention, any of the above devices and formats may be provided in a kit in packaged combination with predetermined amounts of reagents for use in assaying for a tissue-specific endothelial protein.
Chemiluminescent labels are also applicable as therapeutic moieties. See, for example, the labels listed in C.L. Maier, U.S. Patent No. 4,104,029, issued August 1, 1978.

The substrates for the catalytic systems discussed above include simple chromogens and fluorogens such as para-nitrophenyl phosphate (PNPP), β-D-glucose (plus possibly a suitable redox dye), homovanillic acid, α-dianisidine, bromoresol purple powder, 4-alkyl-umbelliferone, luminol, para-dimethylaminolophine, paramethoxypholine, AMPDD, and the like.

Depending on the nature of the label and catalytic signal producing system, one would observe the signal by irradiating with light and observing the level of fluorescence; providing for a catalyst system to produce a dye, fluorescence, or chemiluminescence, where the dye could be observed visually or in a spectrophotometer and the fluorescence could be observed visually or in a fluorometer; or in the case of chemiluminescence or a radioactive label, by employing a radiation counter. Where the appropriate equipment is not available, it will normally be desirable to have a chromophore produced which results in a visible color. Where sophisticated equipment is involved, any of the techniques are applicable.

Alternatively, the therapeutic moiety can be a prodrug or a promolecule which is converted into the corresponding pharmaceutical agent by a change in the chemical environment or by the action of a discrete molecular agent, such as an enzyme. Preferably, the therapeutic moiety is administered with the specific molecule needed for conversion of the promolecule. Alternatively, the promolecule can be cleaved by a natural molecule found in the microenvironment of the target tissue. Alternatively, the prodrug is pH sensitive and converted upon change in environment from the blood to the cell or vesicle (Greco et al., J. Cell. Physiol. 187:22-36, 2001).

USES OF THE THERAPEUTIC COMPLEXES

The therapeutic complex may be used to treat or diagnose any disease for which a tissue- or organ-specific treatment would be efficacious. Examples of such tissues and diseases follow:

In one embodiment, the therapeutic complex may be used to treat or alleviate the symptoms of diseases which affect the brain. Examples of such diseases include but are not limited to: bacterial infections, viral infections, fungal and parasitic infections, epilepsy, schizophrenia, bipolar disorder, neurosis, depression, brain cancer, Parkinson's disease, Alzheimer's disease and other forms of dementia, prion-related diseases, stroke, migraine, ataxia, multiple sclerosis, meningitis, brain abscess, and Wernicke's disease or other metabolic disorders.

In a further embodiment, the therapeutic complex may be used to treat diseases which affect the lungs. Examples of such diseases include but are not limited to: bacterial
infections (i.e. *S. pneumoniae, M. tuberculosis*), viral infections (i.e. Hantavirus), fungal and parasitic infections (i.e. *Pneumocystis carinii*), asthma, lung cancer, emphysema, lung transplant rejection, cystic fibrosis, pulmonary hypertension, pulmonary thromboembolism, and pulmonary edema.

[0153] In a further embodiment, the therapeutic complex may be used to treat or alleviate the symptoms of diseases which affect the pancreas. Examples of such diseases include but are not limited to: parasitic infections, pancreatic cancer, chronic pancreatitis, and pancreatic insufficiency, endocrine tumors, and diabetes.

[0154] In one embodiment, the therapeutic complex may be used to treat or alleviate the symptoms of diseases which affect the heart. Examples of such diseases include but are not limited to: bacterial infections, viral infections, fungal and parasitic infections, infarction, cyanotic heart disease, coronary heart disease, congestive heart disease, cardiac arrhythmias and heart valve disease.

[0155] In a further embodiment, the therapeutic complex may be used as a diagnostic of disease or tissue type or to quantify or identify the tissue-specific luminally expressed protein.

[0156] The cells bearing target proteins interact with the therapeutic complex in two general ways, by transcytosis or passive diffusion. These interactions allow the therapeutic complex to interact directly with the vascular endothelial cell bearing the target protein, become enmeshed in the endothelial matrix containing said endothelial cell, or cross through the endothelial matrix into the encapsulated tissue or organ.

[0157] Transcytosis occurs when, after attachment of the complex with the target protein on the endothelial cell, the therapeutic complex is transcytosed across the vasculature into the endothelial matrix tissue or endothelial cell of choice. Preferably, the binding of the ligand to the target protein will stimulate the transport of the therapeutic complex across the endothelium within a transcytotic vesicle. During transcytosis, the conditions within the microenvironment of the vesicle are more highly acidic and can be used to selectively cleave the therapeutic moiety. For this to happen, preferably, the linker should be pH sensitive, so as to be cleaved due to the change in pH upon going from the blood stream (pH 7.5) to transcytotic vesicles or the interior of the cell (pH 6.0) such as the acid sensitive linkers disclosed. Alternatively, a separate linker may not be necessary when the bond between the ligand and the therapeutic moiety is itself acid sensitive.

[0158] In passive diffusion, the ligand in the complex may attach to the exterior cell membrane, following which there is release of the therapeutic moiety which crosses into the endothelial cell or tissue by passive means, but there is no entry of the entire therapeutic complex into the cell. Preferably, the therapeutic agent is released in high concentrations in microproximity to the endothelium within the specific target tissue. These higher concentrations are expected to
result in relatively greater concentrations of the drug reaching the target tissue versus systemic tissues.

[0159] The therapeutic complexes may be taken up by the cell and stay within the cell or cellular matrix or may cross into the organs and become diffuse within the organ.

[0160] The therapeutic complexes of the present invention advantageously bind to a target protein on a specific tissue, organ or cell and can be used for a number of desired outcomes. In one embodiment, the therapeutic complexes are used to keep toxic substances in a specific environment, allowing for a more specific targeting of a therapeutic moiety to that environment and preventing systemic effects of the therapeutic moiety. In addition, a lower concentration of the substance would be needed for the same effect.

[0161] In a further embodiment, the therapeutic complex is used to keep substances from getting into tissues. The therapeutic moiety might be used to block receptors, that if activated, would cause further harm to the surrounding tissue.

[0162] In a further embodiment the therapeutic complex is used to replace a substance, such as a surfactant protein, or a hormone which is in some way dysfunctional or absent from a specific tissue.

**PRODRUGS**

[0163] The concept of prodrugs are well known in the art and are used herein in a similar manner. The instant prodrugs possess different pharmaceutical characteristics before and after their conversion from prodrug to the corresponding pharmaceutical agent. The therapeutic complexes of the present invention may advantageously incorporate the use of a prodrug in two ways. The therapeutic complexes may have a prodrug attached as a therapeutic moiety which can be converted either by the subsequent injection of a non-indigenous enzyme, or by an enzyme found in the tissue of choice. Alternatively, the therapeutic moiety may be the enzyme which is needed to convert the prodrug. For example, the enzyme β-lactamase may be a part of the therapeutic complex and the prodrug (i.e., doxocillin) is subsequently added and, because the β-lactamase is only found in the targeted tissue, the doxocillin is only unmasked in that area. Unfortunately, neoplastic tissues usually share the enzyme repertoire of normal tissues, making the use of an indigenous enzyme less desirable. However, it can be envisioned that diseased tissues, particularly those diseased by pathogens, may be producing an enzyme specific to the pathogen which is infecting the tissue and this could be used to design an effective prodrug treatment which would be very specific to the infected tissue. For example, a prodrug which is converted by a viral enzyme (i.e., HBV) could be used with a liver-specific antiviral therapeutic complex to get very specific antiviral effect because the prodrug would only be converted in the microenvironment containing the virus.
Therefore, in one embodiment, a "ligand-enzyme" therapeutic complex is used in combination with the unattached prodrug. The prodrug is cleaved by an enzyme and enters the cell. Preferably, the prodrug is hydrophilic, blocking its access into endothelial cells, while the (cleaved) drug is lipophilic, enhancing its ability to enter cells. Alternatively, a "ligand-prodrug" is used as the therapeutic complex in combination with the administration of an unattached non-indigenous enzyme or an indigenous enzyme. The prodrug is cleaved by the enzyme, thus, separated from the therapeutic wherein its lipophilic qualities allow it to enter the cell.

Two of the advantages of the prodrug approach include bystander killing and amplification. One problem with the previous use of antibodies or immunoconjugates in the treatment of cancer was that they were inefficiently taken up by the cells and poorly localized. However, when using a prodrug treatment, because a single molecule of enzyme can convert more than one prodrug molecule the chance of uptake is increased or amplified considerably. In addition, as the active drug diffuses throughout the tumor, it provides a bystander effect, killing or otherwise effecting the therapeutic action on antigen-negative, abnormal cells. Although this bystander effect may also effect normal cells, they will only be those in the direct vicinity of the tumor or diseased organ.

A number of prodrugs have been widely used for cancer therapy and are presented below as examples of prodrugs which can be used in the present invention (Greco et al., J. Cell. Phys. 187:22-36, 2001; and Konstantinos et al., Anticancer Research 19:605-614, 1999). However, it is to be understood that these are some of many examples of this embodiment of the invention.

The most well-studied enzyme/prodrug combination is Herpes simplex virus thymidine kinase (HSV TK) with the nucleotide analog GCV. GCV and related agents are poor substrates for the mammalian nucleoside monophosphate kinase, but can be converted (1000 fold more) efficiently to the monophosphate by TK from HSV 1. Subsequent reactions catalyzed by cellular enzymes lead to a number of toxic metabolites, the most active ones being the triphosphates. GCV-triphosphate competes with deoxyguanosine triphosphate for incorporation into elongating DNA during cell division, causing inhibition of the DNA polymerase and single strand breaks.

The system consisting of cytosine deaminase and 5-fluorocytosine (CD and 5-FC respectively) is similarly based on the production of a toxic nucleotide analog. The enzyme CD, found in certain bacteria and fungi but not in mammalian cells, catalyses the hydrolytic deamination of cytosine to uracil. It can therefore convert the non-toxic prodrug 5-FC to 5-fluorouracil (5-FU), which is then transformed by cellular enzymes to potent pyrimidine antimetabolites (5-FdUMP, 5-FdUTP, and 5-FUTP). Three pathways are involved in the induced
cell death: thymidylate synthase inhibition, formation of (5-FU) RNA and of (5-FU) DNA complexes.

[0169] The mustard prodrug CB1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide] is a weak monofunctional alkylator, but it can be efficiently activated by the rodent enzyme DT diaphorase into a potent DNA cross-linking agent. However, the human enzyme DT diaphorase shows a low reactivity with the prodrug, causing side effects. This problem was overcome when the E. coli enzyme nitroreductase (NTR) was found to reduce the CB1954 prodrug 90 times faster then the rodent DT diaphorase. The prodrug was converted to an alkylating agent which forms poorly repairable DNA crosslinks.

[0170] The oxazaphosphorine prodrug cyclophosphamide (CP) is activated by liver cytochrome P450 metabolism via a 4-hydroxylation reaction. The 4-hydroxy intermediate breaks down to form the bifunctional alkylating toxin phosphoramidine mustard, which leads to DNA cross-links, G2-M arrest and apoptosis in a cycle-independent fashion.

[0171] In the enzyme/prodrug systems described so far the prodrug is converted to an intermediate metabolite, which requires further catalysis by cellular enzymes to form the active drug. The decreased expression of or total lack of these enzymes in the target cells would lead to tumor resistance. The bacterial enzyme carboxypeptidase G2 (CPG2), which has no human analog, is able to cleave the glutamic acid moiety from the prodrug 4-[2-chloroethyl](2-mesyloxyethyl)amino]benzoic acid without further catalytic requirements.

[0172] The reaction between the plant enzyme horseradish peroxidase (HRP) and the non-toxic plant hormone indole-3-acetic acid (IAA) has been analyzed in depth, but not yet completely elucidated. At neutral pH, IAA is oxidized by HRP-compound I to a radical cation, which undergoes scission of the exocyclic carbon-carbon bond to yield the carbon-centered skatolyl radical. In the presence of oxygen, the skatolyl radical rapidly forms a peroxyl radical, which then decays to a number of products, the major ones being indole-3-carbinol, oxindole-3-carbinol and 3-methylene-2-oxindole. In anoxic solution, decarboxylation of the radical cation can still take place and the carbon-centered radical preferentially reacts with hydrogen donors.

[0173] As can readily be seen, the prodrug/enzyme systems advantageously use an enzyme which is not produced by human cells to provide specificity. However, it can readily be seen by one of skill in the art that a human enzyme which is specifically produced in a particular organ or cell type could also be used to achieve this specificity, with the advantage that it would not be immunogenic.

[0174] Finally, heterogeneity could be circumvented by the application of a "cocktail" of conjugates constructed with the same enzyme and a variety of antibodies directed against different organ-associated antigens or different antigenic determinants of the same antigen.
ADMINISTRATION OF THE THERAPEUTIC COMPLEXES

[0175] The therapeutic complexes of the present invention are said to be "substantially free of natural contaminants" if preparations which contain them are substantially free of materials with which these products are normally and naturally found.

[0176] The therapeutic complexes include antibodies, and biologically active fragments thereof, (whether polyclonal or monoclonal) which are capable of binding to tissue-specific luminally-expressed molecules. Antibodies may be produced either by an animal, or by tissue culture, or recombinant DNA means.

[0177] In providing a patient with the therapeutic complex, or when providing the therapeutic complex to a recipient patient, the dosage of administered agent will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition, previous medical history, and the like. In addition, the dosage will vary depending on the therapeutic moiety and the desired effect of the therapeutic complex. As discussed below, the therapeutically effective dose can be lowered if the therapeutic complex is administered in combination with a second therapy or additional therapeutic complexes. As used herein, one compound is said to be additionally administered with a second compound when the administration of the two compounds is in such proximity of time that both compounds can be detected at the same time in the patient's serum.

[0178] The therapeutic complex may be injected via arteries, arterioles, capillaries, sinuses, lymphatic ducts, epithelial cell perfusible spaces or the like. When administering the therapeutic complex by injection, the administration may be by continuous infusion, or by single or multiple boluses.

[0179] The therapeutic complex may be administered either alone or in combination with one or more additional immunosuppressive agents (especially to a recipient of an organ or tissue transplant), antibiotic agents, chemotherapeutic agents, or other pharmaceutical agents, depending on the therapeutic result which is desired. The administration of such compound(s) may be for either a "prophylactic" or a "therapeutic" purpose.

[0180] A composition is said to be "pharmacologically acceptable" if its administration can be tolerated by a recipient patient. Such an agent is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. A typical range is 0.1 µg to 500 mg/kg of therapeutic complex per the amount of the patients weight. One or multiple doses of the therapeutic complex may be given over a period of hours, days, weeks, or months as the conditions suggest. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient patient. The term "pharmacologically effective amount" refers to an amount effective in treating or ameliorating an IL-1 mediated disease
in a patient. The term "pharmaceutically acceptable carrier, adjuvant, or excipient" refers to a non-toxic carrier, adjuvant, or excipient that may be administered to a patient, together with a compound of the preferred embodiment, and which does not destroy the pharmacological activity thereof. The term "pharmaceutically acceptable derivative" means any pharmaceutically acceptable salt, ester, or salt of such ester, of a compound of the preferred embodiments or any other compound which, upon administration to a recipient, is capable of providing (directly or indirectly) a compound of the preferred embodiment. Pharmaceutical compositions of this invention comprise any of the compounds of the present invention, and pharmaceutically acceptable salts thereof, with any acceptable carrier, adjuvant, excipient, or vehicle.

[0181] The therapeutic complex of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby these materials, or their functional derivatives, are combined in admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation, inclusive of other human proteins, e.g., human serum albumin, are described, for example, in Remington's Pharmaceutical Sciences (18th ed., Gennaro, Ed., Mack, Easton Pa. (1990)). In order to form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the therapeutic complex, together with a suitable amount of carrier vehicle.

[0182] Additional pharmaceutical methods may be employed to control the duration of action. Controlled release preparations may be achieved through the use of polymers to complex or absorb the therapeutic complex. Alternatively, it is possible to entrap the therapeutic complex in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxyethylcellulose or gelatine-microcapsules and poly(methylmethacrylate) microcapsules, respectively, or in colloidal drug delivery systems, for example, liposomes, albumin microspheres, microemulsions, nanoparticles, and nanocapsules or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences (1990).

LUMINALLY-EXPOSED TARGET PROTEINS

[0183] Methods for the isolation of tissue-specific luminally-exposed target of the above therapeutic complexes, including cell-surface polypeptides expressed in a tissue-specific manner, are described herein and in United States Patent Application Number 09/528,742, filed March 20, 2000. These luminally-exposed molecules can be present on the surface of cells that line any perfusable space in a tissue, organ or whole intact organism. These perfusable spaces include, e.g., vascular, ductal, CSF space, peritoneum, eye, fascial spaces, and other perfusable tissue spaces.

[0184] Briefly, the methods described herein, which can be used to generate a substantially pure preparation of luminally-exposed molecules, employ cell membrane
impermeable reagents comprising multiple domains. For example, the cell membrane impermeable reagents comprise a first domain comprising a chemical moiety capable of covalently and non-specifically binding to a molecule exposed on the luminal surface of a cell lining a perfusible space in situ or in vivo, a second domain comprising a binding domain (e.g., biotin), and a third domain situated between the first and second domains linking the first domain to the second domain by a cleavable chemical moiety (e.g., a disulfide group), wherein the cleavable chemical moiety will not cleave under in vivo conditions.

[0185] The first domain of the cell membrane impermeable reagent comprises a chemical moiety capable of covalently and non-specifically binding to a molecule expressed on the luminal surface of a cell lining a perfusible space in situ or in vivo. The moiety can be reactive to, e.g., amine, carboxyl, carbohydrate or sulfhydryl groups on the luminally-expressed molecule. The chemistry and reagents for such reactions are well known in the art; see, e.g., catalog of Pierce Chemicals (Rockville, IL); http://www.piercenet.com/Products/.

[0186] Chemical moieties capable of covalently and non-specifically binding luminaire-exposed molecules include amine reactive moieties, e.g., sulfo-NHS ester groups. These moieties react to form a stable covalent bond with amine groups at a pH of about 7 to 9. Such exemplary membrane impermeable cross-linking reagents (which are cleavable) include: thiobis-(sulfosuccinimidyl) propionate groups or sulfosuccinimidyl suberate (see, e.g., Conrad (1985) Int. Arch. Allergy Appl. Immunol. 77:228-231); sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate, e.g., SulfoBiotin-X-NHS™, catalog 21331T, Pierce Chemicals. These compounds can be designed to be cleavable under mild reducing conditions, using, e.g., dithiothreitol (DTT); mild conditions being, e.g., 10 mg/ml, pH 9, RT). See also, e.g., Shimkus (1985) Proc. Natl. Acad. Sci. 82:2593-2597; Duhamel (1985) J. Histochem. Cytochem. 33:711-714; Gottardi (1995) Am. J. Physiol. 268: F285-F295; Soukup (1995) Bioconjugate Chemistry 6: 135-138.

[0187] Other useful chemical moieties capable of covalently and non-specifically binding luminaire-exposed molecules are consumable catalysts, e.g., crosslinking agents such as carbodiimide or carbamoylronium (see, e.g., U.S. Patent Nos. 4,421,847; 4,877,724). With these crosslinking agents, one of the reactants must have a carboxyl group and the other an amine or sulfhydryl group. The crosslinking agent first reacts selectively with the carboxyl group, preferably a carboxyl group on a protein, then is split out during reaction of the "activated" carboxyl group with an amine on the cross-linking reagent, to form an amide linkage between the protein and crosslinking agent, thus covalently bonding the two moieties. See, e.g., U.S. Patent No. 5,817,774.

[0188] Alternatively, sulfhydryl reactive moieties can be used, e.g., maleimide reactive groups such as N-(4-carboxycyclohexylmethyl)maleimide groups can acylate in aqueous
or organic media within 2 minutes at room temperature. Maleimide reacts with -SH groups at pH 6.5 to 7.5, forming stable thioether linkages. See, e.g., U.S. Patent Nos. 5,063,109; 5,053,520.

[0189] Carbohydrate-binding moieties can also be used, e.g., an oxidized carbohydrate specific hydrazide, such as 4-(4-N-Maleimidophenyl) butyric acid hydrazide hydrochloride and its homologues having 2 to 8 carbon atoms in the aliphatic chain connecting the carbonyl and phenyl groups of the spacer. See, e.g., U.S. Patent Nos. 6,015,556; 5,889,155.

[0190] The second domain of the cell membrane impermeable reagent comprises a binding domain. The binding domain can be any chemical moiety having a known ligand that can be manipulated to isolate the luminal-exposed molecule. A binding domain that is preferred for use in the isolation and purification of luminal-exposed molecules is a moiety that has substantially little affinity for most naturally occurring molecules. Alternatively, if the binding domain moiety has significant affinity for naturally occurring molecules, they are present in relatively lesser amounts, or, have less affinity for the binding domain than the ligand chosen to be used in the purification process.

[0191] Such purification-facilitating domains include, but are not limited to, polypeptides and peptides, e.g., metal chelating peptides such as polyhistidine tracts and histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp, Seattle WA).

[0192] Certain cell membrane impermeable reagents comprise a binding domain that is biotin. In such cases, a purification scheme can be developed wherein avidin or streptavidin comprise an immobilized ligand to which the biotin domain can bind. While mammalian cells have significant amounts of naturally biotinylated polypeptides, the use of cleavable (described below) membrane impermeable reagents permits the purification of the reagent bound luminal-exposed molecules from the naturally biotinylated polypeptides.

[0193] The third domain of the cell membrane impermeable reagent comprises a cleavable chemical moiety that will not cleave under in vivo conditions. This domain is a “linking domain” which is situated between the first and second domains. This linking domain can comprise any cleavable chemical moiety that will not cleave under in vivo conditions and that can be cleaved without disrupting the binding of the binding domain to a binding domain ligand. Such cleavable chemical moieties are well known in the art. For example, disulfide groups can be used; with exemplary conditions for cleavage including, e.g., at 37°C with about 10 to 50 mm dithiothreitol (DTT) at pH 8.5 within 30 minutes disulfides are quantitatively cleaved (the disulfides reduced, in this example); or, disulfides also cleaved with, e.g., about 1% to about 5% β-mercaptoethanol (2-ME), or equivalents.
Alternatively, peptide or oligonucleotide linking domains can be cleaved by addition of enzymes that recognize specific sequences (e.g., restriction enzymes for specific nucleic acid sequences and proteases for specific peptides). For example, the cleavable linking domain can include linker sequences cleavable by endopeptidases, such as, e.g., Factor Xa, enterokinase (Invitrogen, San Diego CA) plasmin, enterokinase, kallikrein, urokinase, tissue plasminogen activator, clostripain, chymosin, collagenase, Russell's Viper Venom Protease, post-proline cleavage enzyme, V8 protease, thrombin.

Cell membrane impermeable reagents may also comprise a labeled domain that facilitates detection of luminally-exposed molecules that are bound to these reagents. The cell membrane impermeable reagent can be constructed so that the labeled domain remains with the portion of the cell membrane impermeable reagent that is bound to the luminally-exposed molecule after cleavage of the cleavable chemical moiety.

A cell membrane impermeable reagent can be introduced into an animal, organ or tissue by perfusion or infusion thus allowing the reagent to come into contact with molecules exposed on the luminal surface of cells which line the perfusible space. Methods are well known in the art for perfusing arteries, arterioles, capillaries, sinuses (e.g., as in the liver, placenta) and the like. For example, aortic arch flush, as in, e.g., Woods (1999) J. Trauma 47:1028-1036; arterial cannula in the suprarenal aorta, as in e.g., Mishima (1999) Ann. Thorac. Surg. 67:874-875; coaxial catheter systems permitting movement in three dimensions, as in , e.g., Lauer (1999) J. Am. Coll. Cardiol. 34:1663-1670; cardiac catheterization by a transhepatic approach as in , e.g., McLeod (1999) Heart 82:694-696; central venous catheterization as in , e.g., Albuquerque (1998) Curr. Opin. Clin. Nutr. Metab. Care 1:297-304; placement of central venous catheters as in , e.g., Cavatorta (1999) Clin. Nephrol. 52:191-193, or Ball (1999) Anaesthesia 54:819, and the like.


Additionally, the cell membrane impermeable reagent can be perfused or infused into epithelial lined perfusible spaces, such as, e.g., exocrine and endocrine ducts and pores, respiratory epithelium (nasal epithelium, bronchi, lungs, sinuses), cerebral spinal fluid space (CSF) by, e.g., intrathecal administration, digestive tract epithelium (mouth, pharynx, esophagus, stomach, intestines, colon), bladder, and the like.

During the isolation procedure, the luminally-exposed molecule complex is reacted with a binding domain ligand (e.g., avidin, where the binding domain is biotin), which can be immobilized. A tissue preparation (e.g., tissue extract or homogenate, or the like) comprising
the ligand-bound (tagged) luminally-exposed molecule is first bound to an immobilized binding agent ligand, followed by washing away of non-bound molecules (e.g., substantially all non-bound molecules). Because the cleavable chemical moiety can be cleaved under conditions wherein no or an insignificant amount of binding domain is separated from its ligand, the methods of the invention can generate preparations comprising no or insignificant amounts of contaminating “non-luminally-exposed” molecules (including, e.g., naturally biotinylated molecules).

[0200] Example 1 demonstrates the methods which can be used to isolate a tissue-specific luminally-exposed polypeptide. The methods used in this example are capable of distinguishing proteins exposed on the luminal surface of cells lining a perfusible space, such as the vasculature, from all other proteins present in the tissue. While the data in Example 1 was obtained using rat and swine animal models, the methods used therein are applicable to any animal or human system.

[0201] Luminally-exposed polypeptides can be isolated from a variety of intact organs and/or specific tissues so as to permit identification of polypeptides that are expressed differentially with respect to organ or tissue type. To facilitate such identification, preparations of polypeptides that are isolated from an intact organ or a tissue using the methods described herein are analyzed so as to create a polypeptide profile that is representative of the polypeptides that are expressed in that intact organ or tissue. Such polypeptide profiles can be generated using methods well known in art such as gel electrophoresis, protein chromatography or any other methods used for the separation of proteins. After a polypeptide profile is generated for each intact organ or tissue of interest, these profiles are compared to identify polypeptides that are present only in one or a few organ or tissue types. Polypeptides that are identified as organ or tissue-specific can be further separated or purified from other luminally-exposed molecules in the preparation by methods well known in the art. Such methods include, but are not limited to, ammonium sulfate precipitation, PEG precipitation, immunoprecipitation, standard chromatography, immunochromatography, size exclusion chromatography, ion exchange chromatography, hydrophobic interaction chromatography, affinity chromatography, HPLC two-dimensional electrophoresis and preparative electrophoresis. These and other well known methods of protein purification may be found in Guide to Protein Purification (M. V. Deutcher, ed.), Methods Enzymol. vol. 182, Academic Press, San Diego, Calif. (1990). The purity of the protein product obtained can be assessed using techniques such as SDS PAGE.

[0202] Purified and partially purified polypeptides that have been identified as organ or tissue-specific can be sequenced using methods well known in the art. If the final step of the purification protocol is electrophoresis, the purified or partially purified band (or spot for a two-dimensional electrophoresis) corresponding to the polypeptide of interest can be excised from the gel.
The polypeptide is then recovered from the polyacrylamide gel using known techniques such as, electroelution into membrane traps, diffusion out of homogenized gel slices, or homogenization then processing using a Microcon® filter (Millipore). N-terminal amino acid sequence can be obtained by subjecting the purified polypeptide to Edman degradation. In addition, the internal amino acid sequence can be obtained by digesting the polypeptide of interest with proteases or cyanogen bromide. For example, the polypeptide of interest can be trypsinized or subject to digestion with V8 protease. The peptide fragments are then separated by HPLC. The sequence of purified peptide fragments are determined by standard amino acid sequencing methods, such as Edman degradation, digestion with carboxypeptidase Y followed by Matrix Assisted Laser Desorption Ionization-Time Of Flight (MALDI-TOF) mass spectrometry or Quadrupole-Time Of Flight (Q-TOF) tandem mass spectrometry.

[0203] A tissue-specific luminally-exposed polypeptide or a portion thereof that was identified and purified using the methods described herein can be sequenced using the above techniques. Specifically, a the sequencing of a tissue-specific luminally-exposed polypeptide, or portion thereof, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs.: 3-37 is contemplated. The sequencing of a polypeptide homologous to a tissue-specific luminally-exposed polypeptide, or portion thereof, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs.: 3-37 is also contemplated.

[0204] It will be appreciated that methods other than those described above may be used to determine the full or partial amino acid sequence of tissue-specific luminally-exposed polypeptides.

IDENTIFICATION OF TARGET PROTEINS

[0205] The amino acid sequences or partial amino acid sequences obtained for the tissue-specific luminally-exposed polypeptides can be used as a query sequence for database searching methods using software such as Basic Local Alignment Search Tool (BLAST). BLAST is a family of programs for database similarity searching. The BLAST family of programs includes: BLASTN, a nucleotide sequence database searching program, BLASTX, a protein database searching program where the input is a nucleic acid sequence; and BLASTP, a protein database searching program. BLAST programs embody a fast algorithm for sequence matching, rigorous statistical methods for judging the significance of matches, and various options for tailoring the program for special situations. For example, the N-terminal or internal polypeptide sequence obtained for a tissue-specific luminally-exposed polypeptide, or portion thereof, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs.: 3-37 can be used to query a nonredundant protein database (National Center for Biotechnology Information). The identity or similarity of the polypeptide sequence to database sequences can be identified using BLASTP with

Alternatively, the peptide sequences that are identified as described herein can be analyzed against protein database sequences using the MS PATTERN ver 4.0.0 software available from the University of California San Francisco, Protein Prospector internet site (prospector.ucsf.edu). For example, each sequenced fragment can be used as a query sequence against various publicly available protein sequence databases, such as the NCBI non redundant (nr) database, SwissProt and Owl. For each fragment, the database set is restricted to proteins having a molecular mass within about +/- 25 kDa of the molecular mass of the protein from which the query fragment is obtained. Further specificity can be obtained by requiring the N-terminal query sequences align near the N-terminus of a matching database sequence. If the N-terminal query sequence matches within 60 amino acids of the N-terminus of a database sequence, the N-terminal portion of the database sequence is further analyzed by using the program SIGNALP to determine the location of any N-terminal signal sequences and cleavage sites.

For each of the sequenced fragments, the first query of the analysis requires that the amino acid sequence of the fragment exactly match a database sequence. If no match is obtained from the first query, successive iterations are performed until a sequence match is obtained for each of the fragments analyzed. A match is considered significant only if the aligned portions of the polypeptides display at least 60% sequence identity. If tryptic sequence fragments are used as query sequences, both sequence fragments are required to match the same database protein at level of at least 60% identity. Those sequence fragments that have less than 60% sequence identity to a polypeptide in the database are considered to be unmatched.

Database searching also provides a method for identifying the polynucleotide sequences that encode the polypeptides identified using BLAST or other equivalent search algorithm. These polynucleotide sequences as well as polynucleotide sequences encoding homologous polypeptides from other species can then be used to design oligonucleotide primers which can be used to obtain a full-length cDNA or a cDNA fragment which encodes the polypeptide of interest or a portion thereof. For peptide sequences which have no database match, a degenerate primer can be designed using the sequenced peptide fragment. Using RACE PCR, the entire full-length cDNA or a portion thereof can be obtained. (See Bertling, W. M., et al. (1993) PCR Methods Appl. 3: 95–99; Frohman, M. A. (1991) Methods Enzymol. 218: 340–362; PCR Protocols: A Guide to Methods and Applications, (M. A. Innis, ed.), Academic Press, San Diego, Calif. (1990)). These polynucleotides can then be sequenced using methods well known in the art.

The polynucleotide sequences obtained using the above methods can be used in further database searching to identify homologous polynucleotide sequences and corresponding
homologous polypeptide sequences from other organisms. Homologous polypeptides can also be used for tissue-specific targeting using therapeutic complexes.

[0210] The homologous polypeptides described herein are those that have both a similar amino acid sequence or the same or substantially similar biological activity as a tissue-specific luminally-exposed polypeptide identified as described herein. Homologous polypeptides can be from the same or different species. Homologous polypeptides can contain amino acid substitutions, additions or deletions provided that the molecules remain biologically equivalent to the polypeptides that are obtained by the methods described herein.

[0211] Homologous polypeptides are proteins which are encoded by polynucleotides that are capable of hybridizing with an oligonucleotide probe that hybridizes with a cDNA sequence that encodes a tissue-specific luminally-exposed polypeptide. For example, such cDNAs include polynucleotides that encode luminally-exposed polypeptides comprising an amino acid sequence selected from the group consisting of SEQ ID NOs.: 3-37. Complementary strands, allelic forms and fragments of the polynucleotide sequences encoding homologous polypeptides are also contemplated.

[0212] The oligonucleotide probes which bind the above-described polynucleotides can be considerably shorter than the entire sequence, but should be at least 25, preferably at least 40, more preferably at least 100, even more preferably at least 200, and still more preferably at least 400 nucleotides in length. Longer probes can also be used. Both DNA and RNA probes can be used. The probes are labeled for detecting the corresponding gene (for example, with 32P, 33P, biotin, or avidin).

[0213] The full-length cDNAs encoding the homologous tissue-specific luminally-exposed polypeptides identified as described herein can be obtained by nucleic acid hybridizations under moderate stringency conditions. Such methods are well known in the art. (J. Sambrook, E. F. Fritsch, and T. Maniatis, Molecular Cloning, A Laboratory Manual, 2d edition, Cold Spring Harbor, N.Y., (1989)). An example of a hybridization performed at moderate stringency conditions is the hybridization of an oligonucleotide probe to carrier-bound polynucleotides in 6x sodium chloride/sodium citrate (SSC) at about 45°C followed by one or more washes in 0.2xSSC containing 0.1% SDS at about 42-65°C.

[0214] The amino acid sequences of the homologous polypeptides can differ from the amino acid sequence of tissue-specific luminally-exposed polypeptides which comprise an amino acid sequence selected from the group consisting of SEQ ID NOs.: 3-37 by an insertion or deletion of one or more amino acid residues and/or the substitution of one or more amino acid residues by different amino acid residues. Preferably, amino acid changes are of a minor nature, that is conservative amino acid substitutions that do not significantly affect the folding and/or activity of
the protein; small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small connector peptide of up to about 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

[0215] Nucleic acid expression vectors, containing a polynucleotide that encodes a tissue-specific luminally-exposed polypeptide or a portion thereof can be constructed. Specifically, expression vectors containing a polynucleotide that encodes a tissue-specific luminally-exposed polypeptide, or portion thereof, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs.: 3-37 is contemplated. Expression vectors containing a polynucleotide that encodes a polypeptide homologous to a tissue-specific luminally-exposed polypeptide, or portion thereof, comprising an amino acid sequence selected from the group consisting of SEQ ID NOs.: 3-37 are also contemplated.

[0216] A variety of nucleic acid expression vectors suitable for the expression of tissue-specific luminally-exposed polypeptides are well known in the art. Many of these expression vectors include one or more regulatory sequences that are selected on the basis of the host cells to be used for expression. These regulatory sequences are operably linked to the polynucleotide of interest that is to be expressed. Several of these regulatory sequences, which include promoters, enhancers and other expression control elements, are described in Gene Expression Technology (Goeddel, D.V., ed.), Methods Enzymol. vol. 185, Academic Press, San Diego, Calif. (1990).

[0217] It will be appreciated by those of ordinary skill in the art that the design of an expression vector depends on a variety of factors. Some of these factors include, but are not limited to, the choice of the host cell to be transformed, the level of expression of protein desired, regulatability of protein expression, localization of the expressed protein, and ease of purification of the expressed protein. Recombinant expression vectors that are useful in the expression of the polypeptides described herein can be introduced into a host cell then induced to produce proteins or peptides, including fusion proteins or peptides, that are encoded by the polynucleotides obtained by the methods described herein.

[0218] Recombinant expression vectors can be designed for expression of tissue-specific luminally-exposed polypeptides in prokaryotic or eukaryotic cells. For example, a polypeptide of interest can be expressed in bacterial cells such as E. coli, insect cells (using baculovirus expression vectors), yeast cells or mammalian cells. Suitable host cells are discussed further in Gene Expression Technology (Goeddel, D.V., ed.), Methods Enzymol. vol. 185, Academic Press, San Diego, Calif. (1990). Alternatively, the recombinant expression vector can be transcribed and translated in vitro, for example using T7 promoter regulatory sequences and T7 polymerase.
Expression of proteins in prokaryotes is most often carried out in E. coli with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification (affinity handle). Fusion expression vectors often contain a proteolytic cleavage site that is introduced at the junction of the fusion moiety and the recombinant protein. This cleavage site enables separation of the recombinant protein from the fusion moiety during or subsequent to the purification of the fusion protein. Enzymes useful in facilitating the cleavage of fusion proteins at their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith, D. B. and Johnson, K. S. (1988) Gene 67:31-40), pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia, Piscataway, N.J.) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Examples of suitable inducible non-fusion E. coli expression vectors include pTrc (Amann et al., (1988) Gene 69:301-315) and pET 11d (Studier et al., Gene Expression Technology (Goeddel, D.V., ed.), Methods Enzymol. vol. 185, Academic Press, San Diego, Calif., pp. 60-89, (1990)). Target gene expression from the pTrc vector relies on host RNA polymerase transcription from a hybrid trp-lac fusion promoter. Target gene expression from the pET 11d vector relies on transcription from a T7 gn10-lac fusion promoter mediated by a coexpressed viral RNA polymerase (T7 gn1). This viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident prophage harboring a T7 gn1 gene under the transcriptional control of the lacUV 5 promoter.

One strategy that can be used to maximize recombinant protein expression in E. coli is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, S., Gene Expression Technology (Goeddel, D.V., ed.), Methods Enzymol. vol. 185, Academic Press, San Diego, Calif., pp. 119-128, (1990)). Another strategy is to alter the nucleic acid sequence of the polynucleotide to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in E. coli (Wada et al., (1992) Nucleic Acids Res. 20:2111-2118). Such alteration of nucleic acid sequences of the invention can be carried out by standard techniques known in the art.

Vectors that are used for the expression of recombinant proteins in yeast are also useful in the expression of a tissue-specific luminal-exposed polypeptide. Examples of vectors useful for expression in the yeast Saccharomyces cerevisiae include pYepSec1 (Baldari, et
(Schultz et al., (1987) Gene 54:113-123), pYES2 (Invitrogen Corporation, San Diego, Calif.), and
picZ (Invitrogen Corp, San Diego, Calif.).

[0223] Alternatively, the tissue-specific luminally-exposed polypeptide can be
expressed in insect cells using baculovirus expression vectors. Examples of baculovirus vectors
available for expression of proteins in cultured insect cells (e.g., Sf 9 cells) include the pAc series
(Smith et al. (1983) Mol. Cell Biol. 3:2156-2165) and the pVL series (Lucklow and Summers

[0224] In cases where expression in mammalian cells is desired, the tissue-specific
luminally-exposed polypeptide can be expressed using a mammalian expression vector. Examples
of mammalian expression vectors include pCDM8 (Seed, B. (1987) Nature 329:840) and pMT2PC
(Kaufman et al. (1987) EMBO J. 6:187-195). When used in mammalian cells, the expression
vector's control functions are often provided by viral regulatory elements. For example, commonly
used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40.
For other suitable expression systems for both prokaryotic and eukaryotic cells see chapters 16 and
17 of Sambrook, J., Fritsh, E. F., and Maniatis, T. Molecular Cloning: A Laboratory Manual. 2nd,
ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor,

[0225] The host cell into which the expression vector is introduced can be any
prokaryotic or eukaryotic cell. The expression vector can be introduced into these cells via
conventional transformation or transfection techniques, including but not limited to calcium
phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection,
or electroporation. Suitable methods for transforming or transfecting host cells can be found in
Sambrook, et al. (Molecular Cloning: A Laboratory Manual 2nd, ed, Cold Spring Harbor
Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989, as well as
other laboratory manuals.

[0226] For stable transfection of mammalian cells, it is known that, depending upon
the expression vector and transfection technique used, only a small fraction of cells may integrate
the foreign DNA into their genome. In order to identify and select these integrants, a gene that
encodes a selectable marker (e.g., resistance to antibiotics) is generally introduced into the host
cells along with the gene of interest. Preferred selectable markers include those which confer
resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a
selectable marker can be introduced into a host cell on the same vector as that encoding the tissue-
specific luminally-exposed polypeptide of interest or can be introduced on a separate vector. Cells
stably transfected with the introduced nucleic acid can be identified by drug selection (e.g., cells that have incorporated the selectable marker gene will survive, while the other cells die).

[0227] Among other things, the purified fusion proteins can be utilized to generate antibodies directed to the tissue-specific luminaly-exposed polypeptide of interest.

[0228] A number of embodiments have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. For example, a variety of cleavable chemical moieties, surface molecules, and therapeutic moieties can be used in the instant methods. Accordingly, other embodiments are within the scope of the invention.

[0229] Having now generally described the invention, the following examples are offered to illustrate, but not to limit the claimed invention.

EXAMPLES

[0230] The following examples are offered to illustrate, but not to limit, the claimed invention.

EXAMPLE 1

Isolation of Molecules Exposed on Luminal Surfaces

[0231] The following example describes the methods used to selectively isolate molecules expressed on the luminal surface of vascular endothelial cells. Such methods have been described in detail in United States Patent Application Number 09/528,742, filed on March 20, 2000. In particular, this example demonstrates the selective isolation of polypeptides present on the cell surface of vascular endothelium from various tissues of rats and pigs. Such organs include tissues of the brain, lung, heart and pancreas.

[0232] In some experiments, male Fisher rats were used. Each rat was anesthetized by injection with 1.6 ml of ketamine:xyazine mixture (7.5 mg/ml ketamine: 5 mg/ml xylazine). A tracheotomy was then performed by inserting a catheter into the trachea of the rat and attaching this to a bulb to provide ventilation. The thorax of the animal was then opened and pericardium removed. 0.5 ml heparin (2000 units/ml) was injected into each of the left and right ventricles. A 14-guage catheter was then attached to a perfusion line and inserted into the left ventricle and an incision was made to the right atrium to permit flow of the perfusion buffer. Although the amount of pressure was not critical, a range of between about 10 mm Hg and 80 mm Hg was typically used. In most experiments, perfusion was at 20 mm Hg.

[0233] To clear the vasculature of blood, a buffer of 60 ml Ringers at pH 7.5 with nitroprusside at 0.1 mg/ml was perfused. Second, the vasculature was prepared for reaction with the cell membrane impermeable reagent by perfusion with 60 ml of borate-buffered saline at pH
9.0. Third, about 20 ml of this same buffer containing the DTT cleavable reagent sulfo- \textit{succinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate} (purchased as Sulfobiotin-\textsuperscript{X-NHS} from Pierce Chemicals) was injected in the tissue and allowed to react for about one to two minutes. It will be appreciated that the time of reaction is not critical and may be varied significantly from the reaction time just described.

[0234] One of ordinary skill in the art will recognize that the amount of buffer that is used to deliver the cell membrane impermeable reagent is not critical provided that a sufficient amount is used to permit contact of the reagent with the vasculature of the tissues that will be examined. Additionally, the pH of the buffer is not critical. A range of between about 7.5 and about 9.5 can be used with this particular reagent. A skilled artisan will also recognize that the pH may be adjusted for use with other cell membrane impermeable reagents. It will also be appreciated that the concentration of the cell membrane impermeable reagent that is used may be varied. Concentrations of reagent from about 2 to about 50 mg/ml can also be successfully used to label luminally-exposed molecules.

[0235] After the reaction with reagent, 60 ml Ringers at pH 7.5 with 1.8 mg/ml glycine was perfused to remove excess biotin and to quench any remaining activated biotin. The pH of this quench buffer is not critical. A pH range of between about 7.5 and about 9.5 can be used. After this wash, 60 ml of 25 mM HEPES at pH 7.5 with 0.25 M sucrose and 10 mg/ml of various protease inhibitors, including leupeptin, pepstatin, E64 and PMSF, was introduced to prevent proteolysis. Organs and tissues were then separately removed and stored at -80°C until ready for use.

[0236] It will be appreciated that the exact choice of protease inhibitors and their concentrations is not critical; however, a mixture which includes serine, cysteine, acid, metallo protease inhibitors is desirable.

[0237] Organ and tissue homogenization was carried out by mincing a known weight of tissue with a razor blade. The minced tissue was placed in ten volumes (v/w) PBS at pH 7.4, 1.0 mM EDTA, 1.8 mg/ml glycine with a cocktail of protease inhibitors, including AEBSF, leupeptin, pepstatin A, bestatin, aprotinin (Sigma Cat. # P8340), E64 and PMSF. The tissue suspension was homogenized in a dounce homogenizer with about ten to twelve up and down strokes at approximately 1500 rpm. The homogenate was then centrifuged in about 20 ml aliquots at 500 x g for ten minutes in order to remove cell debris and nuclei. The supernatant was removed and placed in a fresh tube. Each pellet was washed with about ten ml homogenization buffer and the centrifugation was repeated. Supernatants were pooled and spun at 40,000 x g for about two hours to pellet the membrane fractions. Each of these pellets was resuspended in about ten ml homogenization buffer and re-homogenized as before. SDS and Triton X-100 detergents were then
added to a final concentration of about 1% each to solubilize the cell membranes and release proteins.

[0238] These solubilized membrane protein fractions were aliquoted into ten ml aliquots. Thirty ml of a 50% suspension of strepavidin beads (Pierce Chemicals) at 4 mg/ml binding capacity were added to each tube and this was inverted overnight at room temperature (RT). The beads were then allowed to settle into a pellet and the supernatant discarded. The pellet was washed five times with one ml homogenization buffer, 1% SDS, 1% Triton X-100 in order to remove non-specifically bound protein. Molecules modified with the biotin tag (i.e., the luminally-exposed vascular endothelial polypeptides bound to the membrane impermeable reagent) were specifically eluted from the beads by washing twice in mild conditions (i.e. 50 ml homogenization buffer with 50 mM DTT, 1% SDS, 1% Triton-X 100) Under these conditions, the DTT cleaved the internal disulfide domain of the membrane impermeable reagent, releasing the luminally-exposed vascular endothelial polypeptides and leaving the biotin bound to the immobilized streptavidin.

[0239] The eluted luminally-exposed vascular endothelial proteins were then precipitated with four volumes methanol, one volume chloroform and three volumes water, with mixing after each addition. The solution was centrifuged at 14,000 rpm for five minutes in a standard laboratory microcentrifuge to separate the phases. The upper phase was removed and three volumes of methanol were added. The solution was centrifuged again to repellet the protein.

[0240] It will be appreciated that the general isolation procedures described herein for rats can be adapted for use with any animal. For example, the above method was used to isolate luminally-exposed polypeptides from pigs by increasing the volume of the buffers used for perfusion.

EXAMPLE 2

Identification of Luminally-exposed Molecules Expressed in a Tissue-Specific Manner

[0241] The following example describes methods used to determine the profile of luminally-exposed polypeptides that were isolated from tissue samples using the methods described in Example 1. These profiles were then compared to identify those luminally-exposed polypeptides that are expressed in a tissue-specific manner.

[0242] In pig, polypeptides expressed on the luminal surface of vascular endothelial cells from brain, lung, heart and pancreas tissues were isolated using the methods described in Example 1. In preparation for polyacrylamide electrophoresis (PAGE), pellets of the isolated polypeptides were resuspended in sample buffer, which comprised 83 mM Tris HCl, pH 6.8, 1% 2-mercaptoethanol (2-ME), 2% SDS, 10% glycerol, then boiled for five minutes. After boiling, the samples were loaded onto a 4 to 20% gradient acrylamide gel (Novex) and subjected to
electrophoresis for 1.5 hours at 150 volts. The resulting gels were stained with Gelcode Blue™
stain (Pierce Chemical) in order to visualize the polypeptide profile for each of the different tissues
that were analyzed.

[0243] In some cases, samples of the isolated luminally-exposed polypeptides
obtained as described herein were subjected to two-dimensional electrophoresis to facilitate further
isolation from similar sized polypeptides. Methods for performing two-dimensional gel

[0244] Pig brain was studied to identify any luminally-exposed polypeptides
expressed solely or predominantly in cerebral tissues. FIG. 2 shows an approximately 40 kDa
polypeptide that was found to be present in the sample of pig brain but was not found in the other
tissues analyzed, such as heart or lung. Similarly, an approximately 85 kDa and an approximately
35 kDa polypeptide were found to be present in brain tissue but were not found in the other tissue
types that were analyzed (see FIGs. 3 and 4, respectively).

[0245] In subsequent studies, polypeptide profiles obtained from pig heart (cardiac
tissue) were compared to the profiles of other tissues, such as brain and lung. In these
comparisons, six proteins were found to be specific for heart tissue. FIG. 5 shows an
approximately 80 kDa protein that appeared to be associated with the heart tissue but not brain or
lung. FIG. 6 shows two approximately 47 kDa bands that are also specific for heart tissues. FIGs.
7A-C show the presence of an approximately 55 kDa polypeptide that is not associated with either
the lung or the brain. Additionally, an approximately 17 kDa and an approximately 125 kDa were
found to be present in the heart but in none the other tissues examined (see FIGs. 8 and 9,
respectively).

[0246] Lungs were also studied to identify any potential tissue-specific cell surface
polypeptides associated with pulmonary tissues. FIG. 10 shows an approximately 100 kDa protein
that is present in association with lung and heart tissue. FIG. 11 shows a polypeptide at about 25
kDa that was found to be present only in lung tissue. FIGs. 12A-D show the presence of a 48 kDa
polypeptide that was similarly found only in lung tissue. A 125 kDa polypeptide that was present
only in lung tissue is shown in FIGs. 13A-D.

[0247] In other studies, pancreas tissue was examined to identify any luminally-
exposed polypeptides associated therewith. An approximately 45 kDa luminally-exposed
polypeptide having an isoelectric point between pH 5 and 6 was found to be localized only to
pig pancreatic tissue (see FIGs. 14A-D).

[0248] As demonstrated by these stained gels, the expression of isolated luminally-
exposed polypeptides in a variety of perfusible tissue types can be directly compared. More
specifically, luminally-exposed proteins specific for a given tissue or a limited number of tissues can be readily isolated and identified by using the methods of Examples 1 and 2.

EXAMPLE 3

Determination of the Amino Acid Sequence of Tissue-Specific Polypeptides

[0249] The following example describes the methods used to determine either N-terminal amino acid sequence or internal peptide fragment sequence for each of the tissue-specific proteins isolated as described in Examples 1 and 2.

[0250] After electrophoresis, proteins were transferred from the gel to a polyvinylidene difluoride (PVDF) membrane then stained with Coomassie Brilliant Blue. Polypeptide bands (or spots in the case of two-dimensional gel electrophoresis) that were present in only one or a few of the analyzed tissue types were excised from the membrane for protein sequence determination. For most of the excised polypeptides, N-terminal protein sequence was obtained using Edman degradation. Proteins having a blocked N-terminus were digested by incubating the excised membrane containing the polypeptide of interest with approximately 150 ng of trypsin in the presence of 1% zwittergent 3-16 for approximately 20 hours. The tryptic fragments were separated using microbore HPLC. Selected fragments were then subjected to Edman degradation.

[0251] For each of the polypeptides that were isolated and sequenced, Table 1 displays the SEQ ID NO., molecular mass, organism from which the polypeptide was isolated, tissue specificity and type of peptide sequence that was obtained.

<table>
<thead>
<tr>
<th>SEQ ID NO.</th>
<th>Organism</th>
<th>Tissue Specificity</th>
<th>Molecular Mass (kDa)</th>
<th>Sequence Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEQ ID NO.: 3</td>
<td>Pig</td>
<td>Brain</td>
<td>40</td>
<td>N-terminal</td>
</tr>
<tr>
<td>SEQ ID NO.: 4</td>
<td>Pig</td>
<td>Brain</td>
<td>85</td>
<td>N-terminal</td>
</tr>
<tr>
<td>SEQ ID NO.: 5</td>
<td>Pig</td>
<td>Brain</td>
<td>35</td>
<td>N-terminal</td>
</tr>
<tr>
<td>SEQ ID NOs.: 6 &amp; 7</td>
<td>Pig</td>
<td>Heart</td>
<td>80</td>
<td>Tryptic fragments</td>
</tr>
<tr>
<td>SEQ ID NO.: 8</td>
<td>Pig</td>
<td>Heart</td>
<td>47</td>
<td>N-terminal</td>
</tr>
<tr>
<td>SEQ ID NO.: 9</td>
<td>Pig</td>
<td>Heart</td>
<td>47</td>
<td>N-terminal</td>
</tr>
<tr>
<td>SEQ ID NO.: 10</td>
<td>Pig</td>
<td>Heart</td>
<td>55</td>
<td>N-terminal</td>
</tr>
<tr>
<td>SEQ ID NO.: 11</td>
<td>Pig</td>
<td>Heart</td>
<td>17</td>
<td>N-terminal</td>
</tr>
<tr>
<td>SEQ ID NOs.: 12 &amp; 13</td>
<td>Pig</td>
<td>Heart</td>
<td>125</td>
<td>Tryptic fragments</td>
</tr>
<tr>
<td>SEQ ID NO.: 14</td>
<td>Pig</td>
<td>Lung</td>
<td>25</td>
<td>N-terminal</td>
</tr>
<tr>
<td>SEQ ID NO.: 15</td>
<td>Pig</td>
<td>Lung</td>
<td>48</td>
<td>N-terminal</td>
</tr>
<tr>
<td>SEQ ID NO.: 16</td>
<td>Pig</td>
<td>Lung</td>
<td>125</td>
<td>N-terminal</td>
</tr>
<tr>
<td>SEQ ID NO.: 17</td>
<td>Pig</td>
<td>Lung</td>
<td>25</td>
<td>N-terminal</td>
</tr>
<tr>
<td>SEQ ID NOs.: 18 &amp; 19</td>
<td>Pig</td>
<td>Lung/Heart</td>
<td>100</td>
<td>Tryptic fragments</td>
</tr>
<tr>
<td>SEQ ID NO.: 20</td>
<td>Pig</td>
<td>Pancreas</td>
<td>45</td>
<td>N-terminal</td>
</tr>
</tbody>
</table>
EXAMPLE 4

Comparison of the Sequences of Isolated Tissue-Specific Polypeptides to Known Protein Sequences

[0252] The following example describes the methods used to determine the functional identity of the tissue-specific luminally-exposed polypeptides that were sequenced using the methods described in Example 3.

[0253] The amino acid sequence obtained for each tissue-specific luminally-exposed polypeptide was compared to amino acid sequences available in public databases. The amino acid sequence of both N-terminal and tryptic peptide fragments identified in the above examples were analyzed using MS PATTERN ver. 4.0.0, which is available at prospector.ucsf.edu. Specifically, each fragment was used as a query sequence against various publicly available protein sequence databases, such as the NCBI non redundant (nr) database, SwissProt and Owl. For each fragment, the database set was restricted to proteins having a molecular mass within about +/- 25 kDa of the molecular mass of the protein from which the query fragment was obtained. Further specificity was obtained by requiring the N-terminal query sequences align near the N-terminus of a matching database sequence. If the N-terminal query sequence matched within 60 amino acids of the N-terminus of a database sequence, the N-terminal portion of the database sequence was further analyzed by using the program SIGNALP to determine the location of any N-terminal signal sequences and cleavage sites.

[0254] For each of the sequenced fragments, the first query of the analysis required that the amino acid sequence of the fragment exactly match a database sequence. If no match was obtained from the first query, successive iterations were performed until a sequence match was obtained for most of the fragments analyzed. A match was considered significant only if the aligned portions of the polypeptides displayed at least 60% sequence identity. When tryptic sequence fragments were used as query sequences, both sequence fragments were required to match the same database protein at level of at least 60% identity. Those sequence fragments that had less than 60% sequence identity to a polypeptide in the database were considered to be unmatched.

[0255] Table 2 displays the results of the database comparisons using each amino acid sequence (SEQ ID NO.) listed in Table 1 as a query sequence.
<table>
<thead>
<tr>
<th>SEQ ID NO.</th>
<th>Homologous Protein Sequence</th>
<th>NCBI Accession No.</th>
<th>Percent Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEQ ID NO.: 3</td>
<td>Folate Binding Protein (Human)</td>
<td>4928859</td>
<td>100</td>
</tr>
<tr>
<td>SEQ ID NO.: 4</td>
<td>Unmatched</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>SEQ ID NO.: 5</td>
<td>Unmatched</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>SEQ ID NO.: 6</td>
<td>CD36 (Human)</td>
<td>I59613</td>
<td>80</td>
</tr>
<tr>
<td>SEQ ID NO.: 7</td>
<td>CD36 (Human)</td>
<td>I59613</td>
<td>100</td>
</tr>
<tr>
<td>SEQ ID NO.: 8</td>
<td>Cell Adhesion Regulator (Rat)</td>
<td>AAD00260</td>
<td>89</td>
</tr>
<tr>
<td>SEQ ID NO.: 9</td>
<td>Sarcoglycan Epsilon (Human)</td>
<td>043556</td>
<td>100</td>
</tr>
<tr>
<td>SEQ ID NO.: 10</td>
<td>NAR3 (Human)</td>
<td>Q13508</td>
<td>80</td>
</tr>
<tr>
<td>SEQ ID NO.: 11</td>
<td>Aquaporin 2 (Dog)</td>
<td>CAA71663</td>
<td>83</td>
</tr>
<tr>
<td>SEQ ID NO.: 12</td>
<td>Cadherin-13 (Human)</td>
<td>NP001248</td>
<td>100</td>
</tr>
<tr>
<td>SEQ ID NO.: 13</td>
<td>Cadherin-13 (Human)</td>
<td>NP001248</td>
<td>100</td>
</tr>
<tr>
<td>SEQ ID NO.: 14</td>
<td>CD9 (Human)</td>
<td>XP_033314</td>
<td>100</td>
</tr>
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<td>SEQ ID NO.: 15</td>
<td>RAGE (Cow)</td>
<td>Q28173</td>
<td>80</td>
</tr>
<tr>
<td>SEQ ID NO.: 16</td>
<td>Integrin Alpha-X (Human)</td>
<td>P20702</td>
<td>86</td>
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<td>SEQ ID NO.: 17</td>
<td>CD81 (Human)</td>
<td>XP_006475</td>
<td>100</td>
</tr>
<tr>
<td>SEQ ID NO.: 18</td>
<td>VAP-1 (Human)</td>
<td>Q16853</td>
<td>100</td>
</tr>
<tr>
<td>SEQ ID NO.: 19</td>
<td>VAP-1 (Human)</td>
<td>Q16853</td>
<td>100</td>
</tr>
<tr>
<td>SEQ ID NO.: 20</td>
<td>MDP-1 (Human)</td>
<td>P16444</td>
<td>100</td>
</tr>
</tbody>
</table>

[0256] Table 3 displays the SEQ ID NOs. for each of the proteins identified from its source organism or a related organism. The SEQ ID NOs. for each of the corresponding polypeptide homologs identified from humans is also provided. Additionally, the SEQ ID NOs. of the polynucleotide sequences which encode each protein from the source or related organism and the corresponding human homolog are indicated. The term “N/A” in Table 3 means that the sequence was not available.
TABLE 3

<table>
<thead>
<tr>
<th>Identified Protein</th>
<th>Protein from Source or Related Organism</th>
<th>Homologous Human Protein</th>
<th>DNA Encoding Protein from Source or Related Organism</th>
<th>DNA Encoding the Homologous Human Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folate Binding Protein</td>
<td>SEQ ID NO.: 21 (Pig)</td>
<td>SEQ ID NO.: 22</td>
<td>SEQ ID NO.: 38 (Pig)</td>
<td>SEQ ID NO.: 39</td>
</tr>
<tr>
<td>CD36</td>
<td>N/A</td>
<td>SEQ ID NO.: 23</td>
<td>N/A</td>
<td>SEQ ID NO.: 40</td>
</tr>
<tr>
<td>Cell Adhesion Regulator</td>
<td>SEQ ID NO.: 24 (Rat)</td>
<td>N/A</td>
<td>SEQ ID NO.: 41 (Rat)</td>
<td>N/A</td>
</tr>
<tr>
<td>Sarcoglycan Epsilon</td>
<td>N/A</td>
<td>SEQ ID NO.: 25</td>
<td>N/A</td>
<td>SEQ ID NO.: 42</td>
</tr>
<tr>
<td>NAR3</td>
<td>N/A</td>
<td>SEQ ID NO.: 26</td>
<td>N/A</td>
<td>SEQ ID NO.: 43</td>
</tr>
<tr>
<td>Aquaporin 2</td>
<td>SEQ ID NO.: 27 (Dog)</td>
<td>SEQ ID NO.: 28</td>
<td>SEQ ID NO.: 44 (Dog)</td>
<td>SEQ ID NO.: 45</td>
</tr>
<tr>
<td>Cadherin-13</td>
<td>N/A</td>
<td>SEQ ID NO.: 29</td>
<td>N/A</td>
<td>SEQ ID NO.: 46</td>
</tr>
<tr>
<td>CD9</td>
<td>N/A</td>
<td>SEQ ID NO.: 30</td>
<td>N/A</td>
<td>SEQ ID NO.: 47</td>
</tr>
<tr>
<td>RAGE</td>
<td>SEQ ID NO.: 31 (Cow)</td>
<td>SEQ ID NO.: 32</td>
<td>SEQ ID NO.: 48 (Cow)</td>
<td>SEQ ID NO.: 49</td>
</tr>
<tr>
<td>Integrin Alpha-X</td>
<td>N/A</td>
<td>SEQ ID NO.: 33</td>
<td>N/A</td>
<td>SEQ ID NO.: 50</td>
</tr>
<tr>
<td>CD81</td>
<td>N/A</td>
<td>SEQ ID NO.: 34</td>
<td>N/A</td>
<td>SEQ ID NO.: 51</td>
</tr>
<tr>
<td>VAP-1</td>
<td>N/A</td>
<td>SEQ ID NO.: 35</td>
<td>N/A</td>
<td>SEQ ID NO.: 52</td>
</tr>
<tr>
<td>MDP-1</td>
<td>SEQ ID NO.: 36 (Pig)</td>
<td>SEQ ID NO.: 37</td>
<td>SEQ ID NO.: 53 (Pig)</td>
<td>SEQ ID NO.: 54</td>
</tr>
</tbody>
</table>

EXAMPLE 5

Identification and Isolation of Polynucleotides that Encode Tissue-Specific Luminally-Exposed Polypeptides

[0257] The following provides exemplary methods that are used to identify and isolate polynucleotides that encode tissue-specific luminally-exposed polypeptides identified by the methods described herein.

[0258] Separate single stranded cDNA libraries (sscDNA) are constructed for each organism of interest. To create tissue-specific sscDNA libraries, portions of a tissue of interest from organisms, such as monkey, pig or rat, are excised and total RNA is isolated using methods commonly known in the art. For example, the commonly known guanidine salts/phenol extraction protocol is one of many methods which can be used to produce total RNA from isolated tissues. Chomczynski & Sacchi, 1987, Anal. Biochem. 162: 156. The total RNA extracts are then used to generate sscDNA using methods well known in the art. For example, an oligo dT primer flanked
by two or more degenerate nucleotides at its 3' end and a specific 15 to 21 base oligonucleotide at its 5' end (RPBT), which is included to facilitate the binding of a reverse primer, can be used to prime first ssDNA synthesis from the preparations of total RNA.

[0259] The tissue-specific ssDNA is used as a template for PCR to obtain double-stranded cDNAs (cDNA) which contain the coding regions of the polypeptides identified using the methods described herein. Different cDNA cloning strategies are used depending on whether the tissue-specific luminal-exposed polypeptide sequence that was obtained using the methods described herein matches a polypeptide sequence contained in publicly available databases. In cases in which a database match is found, the full-length DNA which encoded the polypeptide is often available. Such full-length DNA sequences can be used to design specific PCR primers which correspond to the 5' and 3' ends of the polypeptide coding sequence. These primers are then used to amplify the corresponding full-length cDNA using a high fidelity polymerase (e.g. pfu) and the ssDNA library as template. To facilitate subsequent directional cloning of the full-length DNA into an expression vector, each primer contains an additional short nucleotide sequence at its 5' end. The additional sequences are complementary to the overhanging sequence generated by a different restriction endonuclease.

[0260] All oligonucleotides used in these methods can be synthesized with an Applied Biosystems 394 DNA synthesizer using established phosphoramidite chemistry. Ethanol precipitated primers can be used for PCR without further purification.

[0261] Alternative cloning approaches can be used in those instances in which the sequence of the tissue-specific luminal-exposed polypeptides obtained using the methods described herein do not match a polypeptide sequence contained in publicly available databases. In cases where the N-terminus portion of the polypeptide of interest has been identified, a corresponding degenerate primer can be designed which includes all possible nucleotide sequence variations capable of encoding the identified N-terminal peptide sequence. This degenerate primer and a primer which corresponds to the RPBS (incorporated into the ssDNA during synthesis) are then used to amplify the full-length cDNA using a high fidelity polymerase (e.g. pfu) and the ssDNA library as template. As previously described herein, each of these primers may include additional sequences which facilitate subsequent directional cloning of the full-length cDNA.

[0262] In cases where N-terminal amino acid sequence is not available but one or more internal peptide sequences are present, RACE PCR is used to obtain the 5' and 3' ends of the full-length cDNA which encodes the polypeptide of interest. Methods for performing RACE PCR are well known in the art. (See Bertling, W. M., et al. (1993) PCR Methods Appl. 3: 95–99; Frohman, M. A. (1991) Methods Enzymol. 218: 340–362; PCR Protocols: A Guide to Methods and Applications, (M. A. Innis, ed.), Academic Press, San Diego, Calif. (1990)). Briefly, RACE PCR is
based on the construction of a specialized cDNA library that includes primer binding templates located at each end of the double stranded cDNA. The primer binding template that is ultimately located at the 3' end of the coding strand of the dscDNA (RPBT) is formed as described previously described herein. The primer binding template that is ultimately located at the 5' end of the coding strand of the dscDNA (FPBT) is formed by blunt end ligation of an adapter to the dscDNA after the completion of second strand synthesis. If a small internal portion of the sequence of a specific cDNA that lies between the FPBT and the RPBT is known, the region of DNA between the FPBT and this internal sequence can be amplified. Likewise, the region between the RPBT and the internal sequence can also be amplified.

[0263] To obtain the full-length cDNA of interest by RACE, an internal peptide sequence fragment of a polypeptide of interest is used to design a degenerate oligonucleotide that includes all possible nucleotide sequence variations capable of encoding the identified internal peptide fragment. This degenerate primer and a primer which corresponds to the RPBT are then used to amplify a region of the cDNA between the internal primer and the 3' terminus of the cDNA coding strand (3' end fragment) using a high fidelity polymerase (e.g. pfu) and the RACE cDNA library as template. Subsequent to the amplification, Taq polymerase can be used to add a single adenine nucleotide to the 3' ends of each strand of the double stranded PCR product to facilitate cloning. The 3' end fragment is subjected preparative gel electrophoresis then purified from the gel using a commercially available kit (Qiagen Gel Extraction Kit, Qiagen Corp.) according to the manufacturer's instructions. The gel-purified, 3' end fragment is then inserted into a T-tailed PCR cloning vector and ligated at 15°C overnight using T4 DNA ligase (New England BioLabs, Beverly, MA). A portion of the ligation mixture is then used to transform competent Escherichia coli and 100 µl of the transformation mixture is plated onto Luria broth plates containing 100 µg/ml of ampicillin. Isolated ampicillin-resistant transformants are picked, and streaked to obtain single colony isolates. Plasmid DNA is then obtained from these single colony isolates. The presence of the insert in each construct can be confirmed by amplification of the cloned region using oligonucleotide primers flanking the insert site. Clones having the appropriate size inserts are then sequenced using a cycle sequencing dye-terminator kit with AmpliTaq DNA Polymerase, FS (ABI, Foster City, Calif.). The sequencing reaction products are run on an ABI Prism 377 DNA Sequencer.

[0264] Using the nucleotide sequence of the 3' end fragment, a gene specific primer complementary to the coding strand of the cDNA can be designed. This gene specific primer and a primer that corresponds to the FPBT are then used in conjunction with the RACE cDNA library and a high fidelity polymerase (e.g. pfu) to amplify a fragment that corresponds to a region of the cDNA between the internal primer and the 5' terminus of the cDNA coding strand (5' end
fragment). This 5’ end fragment is processed as previously described for the 3’ end fragment so as to obtain nucleotide sequence.

[0265] Having knowledge of the nucleotide sequence of both the 5’ and 3’ ends of the full-length cDNA, one can design oligonucleotide primers that correspond to each end of the cDNA sequence. These primers can then be used to amplify the full-length cDNA using the RACE cDNA library and a high fidelity polymerase such as pfu polymerase.

EXAMPLE 6

Identification of cDNAs Encoding Homologs of Tissue-Specific Luminally-Exposed Polypeptides

[0266] The following example describes methods that are used to obtain cDNAs which encode the homologs of the tissue-specific luminally-exposed polypeptides described herein, including cDNA which encodes polypeptides homologous to luminally-exposed polypeptides comprising an amino acid sequence selected from the group consisting of SEQ ID NOs.: 3-37.

[0267] Polynucleotides encoding homologous polypeptides may be obtained by screening a cDNA library constructed from an appropriate tissue of an organism other than the organism from which the tissue-specific luminally-exposed polypeptide was originally identified.

[0268] To identify a polynucleotide which encodes a polypeptide homologous to a luminally-exposed polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs.: 3-37, an oligonucleotide probe is constructed using the appropriate full-length cDNA sequence described in Example 5 herein. Methods of oligonucleotide probe construction are well known in the art.

[0269] A cDNA library from an organism other than the organism from which the tissue-specific luminally-exposed polypeptide was originally identified is prepared. This library is then screened for a polynucleotides which hybridize with the probes described above and which encode polypeptide homologous to a luminally-exposed polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs.: 3-37. The cDNA library containing the polynucleotide which encodes the homologous polypeptide from such other organism can be plated using methods known in the art. (J. Sambrook, E. F. Fritsch, and T. Maniatis, Molecular Cloning, A Laboratory Manual, 2d edition, Cold Spring Harbor, N.Y., (1989)). The polynucleotides are then transferred to and immobilized on nitrocellulose or other carrier. In order to identify a polynucleotide that is homologous with luminally-exposed polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs.: 3-37, the carrier containing the library is incubated with the radiolabeled probe sequence for 1 hour at 6xSSC at 45°C. The carrier is then washed three times for 30 minutes each in 0.2xSSC with 0.1% SDS at
42°C. Polynucleotides to which the oligonucleotide probe hybridizes under these conditions are detected using X-ray film.

[0270] The hybridizing polynucleotides can then be isolated, cloned and sequenced using methods commonly known in the art. Once the sequence of the hybridizing polynucleotide is determined, this sequence can be used to obtain the full-length polynucleotide homolog using the methods previously described in Example 5. The full-length homolog is then compared to the polynucleotide from which the probe was constructed to determine the percent nucleotide identity. Using commonly available computer programs, such as the Wisconsin Package developed and distributed by the Genetics Computer Group, the amino acid sequence of the homologous polypeptide can be determined. The homologous polypeptide is then compared to the polypeptide encoded by the polynucleotide from which the probe was constructed to determine the percent similarity of the two polypeptide sequences.

[0271] Database searching can also be used to identify a polypeptide homologous to a luminally-exposed polypeptide which comprises an amino acid sequence selected from the group consisting of SEQ ID NOs.: 3-37. The polynucleotide which encodes polypeptide which comprises an amino acid sequence selected from the group consisting of SEQ ID NOs.: 3-37 is obtained using the method described in Example 5. This sequence or fragment thereof is then used as a query sequence against the polynucleotide sequences in the NCBI nonredundant sequence database. The database search and sequence comparison is performed by using the NCBI BLASTN 2.0.9 computer algorithm with the BLOSUM62 matrix and the default parameters except that filtering is turned off.

[0272] A polynucleotide which encodes a polypeptide homologous to a luminally-exposed polypeptide which comprises an amino acid sequence selected from the group consisting of SEQ ID NOs.: 3-37 can be expressed, purified and used to generate antibodies thereto using the methods described herein.

EXAMPLE 7

Expression and Purification of Recombinant Tissue
Specific Luminally-Exposed Polypeptides and Fragments Thereof

[0273] The following example provides an exemplary method for the expression of tissue-specific luminally-exposed polypeptides (and fragments thereof) that are encoded by cDNA sequences identified by the methods described herein. This method is based on an E. coli expression system; however, one of ordinary skill in the art will recognize that a variety of host organisms and expression systems exist that can be used to express these tissue-specific luminally-exposed polypeptides.
Several vector systems for protein expression in *E. coli* are well known and available to someone knowledgeable in the art. A full-length cDNA, which encodes a polypeptide of interest and which contains restriction endonuclease sequences appropriate for directional insertion of the coding sequences into the vector, can be inserted into any of these vectors and placed under the control of the promoter such that the coding sequences can be expressed from the vector’s promoter. Alternatively, the full-length cDNA can be selectively digested or used as a template for the amplification of select fragments which can be placed under the control of a promoter in an expression vector. Vectors such as the pGEX and pET3 series vectors can be for such expression. (see, Gene Expression Technology (D. V. Goeddel, ed.), *Methods Enzymol.* vol. 185, Academic Press, San Diego, Calif. (1990)).

The expression vector is then transformed into DH5α or some other *E. coli* strain suitable for the over expression of proteins. Transformation can be facilitated using the calcium chloride method, electroporation protocols, or any other method for introducing nucleic acids into *E. coli* that is known in the art. Positive transformants are selected after growing the transformed cells on plates containing an antibiotic to which the vector confers resistance.

In one embodiment of the invention, the protein is expressed and maintained in the cytoplasm as the native sequence. In another embodiment, the expression vector can include a targeting sequence which allows for differential cellular targeting, such as to the periplasmic space or to the exterior medium. In yet other embodiments, a protein tag is included that facilitates purification of the protein from either fractionated cells or from the culture medium by affinity chromatography. A skilled artisan will recognize that embodiments represented by translational fusions require that the cDNA coding sequence be linked to the fusion partner in the appropriate reading frame so that translation of the desired fusion protein results.

Expressed proteins, whether in the culture medium or liberated from the periplasmic space or the cytoplasm, are then purified or enriched from the supernatant using conventional techniques such as ammonium sulfate precipitation, PEG precipitation, immunoprecipitation, standard chromatography, immunochromatography, size exclusion chromatography, ion exchange chromatography, hydrophobic interaction chromatography, affinity chromatography, HPLC two-dimensional electrophoresis and preparative electrophoresis. (see, Guide to Protein Purification (M. V. Deutscher, ed.), *Methods Enzymol.* vol. 182, Academic Press, San Diego, Calif. (1990)). Alternatively, if the polypeptide is secreted from the host cell into the surrounding medium in a state that is sufficiently enriched, the polypeptide or fragment thereof may be used for its intended purpose without further purification. The purity of the protein product obtained can be assessed using techniques such as SDS PAGE.
Antibodies capable of specifically recognizing the protein of interest can be generated using synthetic peptides using methods well known in the art. See, Antibodies: A Laboratory Manual, (Harlow and Lane, Eds.) Cold Spring Harbor Laboratory (1988). For example, synthetic peptides can be injected into mice to generate antibodies which recognize the full-length polypeptide. Antibodies prepared using these peptide fragments can be used to purify the full-length polypeptide by using standard immunochromatography techniques.

In an alternative protein purification scheme, a polynucleotide encoding the tissue-specific luminally-exposed polypeptide of interest or portion thereof can be incorporated as a translational fusion into expression vectors designed for use in affinity-based purification schemes. In such strategies the coding sequence of the polynucleotide of interest or portion thereof is inserted in-frame with the gene encoding the other portion of the fusion polypeptide (the affinity handle). In some embodiments, the affinity handle is polyhistidine.

In other embodiments the affinity handle is maltose binding protein (MBP). A chromatography matrix having nickel (if polyhistidine affinity handles are used) or an antibody to MBP (if MBP affinity handles are used) attached thereto is then used to purify polypeptide fusion. Protease cleavage sites can be engineered between the polyhistidine gene or the MBP gene and the polynucleotide of interest, or portion thereof. Thus, during of subsequent to the final purification step, the polypeptide of interest can be separated from the affinity handle by proteolysis.

Expression and purification of a tissue-specific luminally-exposed polypeptide in E. coli

In this example, a tissue-specific luminally-exposed polypeptide is expressed as a recombinant glutathione-S-transferase (GST) fusion polypeptide in E. coli and the fusion polypeptide is isolated and characterized. Specifically, the polypeptide of interest, such as a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs.: 3-37, is fused to GST and this fusion polypeptide is expressed in E. coli, e.g., strain PEB199. Expression of the GST-tissue-specific luminally-exposed polypeptide fusion protein in PEB199 is induced with IPTG. The crude bacterial lysates of the induced PEB199 strain, which contains the recombinant fusion polypeptide, is then passed over a column of glutathione beads. Elution of the bound tissue-specific luminally-exposed polypeptide is accomplished by using thrombin to cleave the peptide linker which separates the glutathione-S-transferase affinity handle from the polypeptide of interest. The purity of this recombinant tissue-specific luminally-exposed polypeptide is determined by subjecting a sample of the eluate to PAGE and silver staining the resulting gel.
EXAMPLE 8

Preparation of Polyclonal Antibodies to Tissue

Specific Luminally-Exposed Polypeptides or Fragments Thereof

[0282] The following example illustrates the preparation of polyclonal antibodies directed to a full-length tissue-specific luminally-exposed polypeptide or a fragment thereof identified using the methods described herein.

[0283] Polyclonal antibodies directed to a tissue-specific luminally-exposed polypeptide identified using the methods described herein are prepared by inoculating a host animal with the polypeptide of interest. The polypeptide comprising the inoculum is substantially pure, preferably comprising less than about 1% contaminant. To increase the immune response of the host animal, the polypeptide of interest is combined with an adjuvant. Suitable adjuvants include alum, dextran, sulfate, large polymeric anions, oil & water emulsions, e.g. Freund's adjuvant, Freund's complete adjuvant, and the like. The polypeptide of interest may also be conjugated to synthetic carrier proteins or synthetic antigens.

[0284] A variety of hosts can be immunized to produce the polyclonal antibodies. Such hosts include rabbits, guinea pigs, rodents, e.g. mice, rats, sheep, goats, and the like. The polypeptide of interest is administered to the host, usually intradermally, with an initial dosage followed by one or more, usually at least two, additional booster dosages. Following immunization, the blood from the host is collected, followed by separation of the serum from the blood cells. The immunoglobulin present in the resultant antiserum may be further fractionated using known methods, such as ammonium salt fractionation, DEAE chromatography, and the like.

Preparation of polyclonal antibodies to a fragment of a tissue-specific luminally-exposed polypeptide

[0285] New Zealand white female rabbits are used for the production of polyclonal antibodies to one or more fragments of a tissue-specific luminally-exposed polypeptide identified using the methods described herein. Specifically, peptides comprising an amino acid sequence selected from the group consisting of SEQ ID NOs.: 3-37 are used. A synthetic peptide corresponding to a 28 amino acid residue fragment of a polypeptide identified using the methods described herein is linked to Kehole Limpet Hemocyanin (KLH) for use as an antigen. Subdermal injection is carried out using 1 mg of KLH-linked peptide that has been emulsified in Freund's complete adjuvant. After 3 weeks the animals are bled and tested for reactivity. The animals are injected again after 3 weeks using 1 mg of KLH-linked peptide in Freund's incomplete adjuvant. Two weeks later the serum is tested. The serum that is obtained is then tested to determine it reactivity to the full-length polypeptide antigen.
EXAMPLE 9

Localization of Tissue-Specific Luminally-Exposed Polypeptides Using Polyclonal Antibodies

[0286] The antibody localization methods described in the following example can be used to verify the tissue specificity of luminally-exposed target molecules, including the tissue-specific luminally-exposed polypeptides identified using the methods described herein. In some cases, where the polypeptide of interest has been previously isolated, commercial antibodies may be available. In other cases, where the polypeptide of interest has not been previously characterized antibodies may be prepared using the methods described in Examples 5-8.

[0287] Experiments which demonstrate the tissue-specificity of a polypeptide can be performed both in vitro and in vivo. For example, Western blot is an in vitro method that can be used to confirm the tissue specificity of polypeptides separated by PAGE as described previously in Example 2. In vivo localization can be achieved by injecting the appropriate labeled antibody into a host animal. After a sufficient incubation time, tissues can be removed and examined to determine the localization of the label.

In vitro tissue-specific localization of rat transferrin receptor

[0288] The transferrin receptor (CD71) is a luminally-exposed transcytotic receptor present on the surface of endothelial cells that line the capillaries of the brain. Friden, P. M., et al. (1991). PNAS 88:4771-5. Using the methods previously described herein, CD71 was shown to be expressed in a brain-specific manner. Cell-surface polypeptides isolated from brain, heart, kidney and lung tissues were separated by gel electrophoresis as described in Example 2. The separated polypeptides were then transferred to nitrocellulose by blotting at 25 milliamp overnight. The filter blots were then blocked with 2% BSA in TBS, 0.1% Tween-20 buffer for about one hour at RT. The blocking solution was removed and the OX-26 monoclonal antibody (Accurate), which is specific for CD71 (see, e.g., U.S. Patent No. 6,004,814), contained in 0.2% BSA buffer was incubated with the blot for about one hour at RT. The filters were washed three times for about ten minutes in TBS-TWEEN then incubated with the “secondary” horse radish peroxidase (HRP)-labeled antibody. After washing three times, the blots were developed with ECL-Plus™ (Amersham/Pharmacia) and photographed over UV light.

[0289] In polypeptide preparations from isolated brain tissue, a band at about 90 kDa corresponding to the monomeric form of CD71 was present. No bands were detected in the polypeptide preparations obtained from isolated rat heart, kidney or lung tissues. Such results show that CD71 is expressed specifically in the brain tissues.
In vivo tissue-specific localization of rat transferrin receptor

[0290]  In vivo localization studies with OX-26 antibody demonstrated that CD71 is only expressed in brain capillaries thus confirming the ability of the methods described herein to identify tissue-specific luminaly-exposed polypeptides. For these localization studies, OX-26 and a control antibody of the same isotype but a different specificity (specific for albumin) were labeled with biotin. About 0.5 ml of a 1 mg/ml solution of each antibody was injected into the tail vein of separate rats under light anesthesia. The antibody was allowed to circulate for about thirty minutes after which time the animal was sacrificed and its organs/tissues were removed individually. Sections of each were made of each tissue by placing a small cube in embedding medium (HistoPrep™, Fisher), in a small plastic cube. This preparation was then immersed for about twenty seconds in 2-methylpentane which had been prechilled in liquid nitrogen. The frozen cubes were kept on dry ice until they were sectioned. The tissues were sectioned at five mm slices on a cryostat, air dried overnight and fixed in acetone for two min. The slides were then stained with streptavidin-HRP.

[0291]  FIGs. 15 A-D show the immunohistochemistry of tissue sections from a rat which was injected with either OX-26 or a control antibody. FIG. 15A is brain from a rat injected with OX-26, FIG. 15B is brain from a rat injected with the anti-albumin control antibody, FIG. 15C is lung from a rat injected with OX-26, FIG. 15D is lung from a rat injected with the anti-albumin control antibody. These results demonstrate that the antibody localized to the capillaries of the brain, and to no other tissue. Such specificity is particularly advantageous in that it is often difficult to find therapeutics which can cross the blood-brain barrier.

In vivo localization of CD81

[0292]  In another experiment, 50 µg of biotinylated antibody specific for rat CD81 (clone eaa2 from Research Diagnostics, Inc.) was administered by to adult rats by tail vein injection. Thirty minutes after the administration of the antibody, the rats were sacrificed and organs were prepared for immunohistochemistry as described above.

[0293]  Tissue sections of heart and liver and other organs were analyzed. The biotinylated antibody was only seen associated with the endothelium of the lung.

[0294]  The polypeptide sequence of human CD81 is provided as SEQ ID NO.: 34. The corresponding nucleotide sequence is SEQ ID NOs.: 51.

In vivo localization of folate binding protein

[0295]  Using a biotinylated antibody directed to rat folate binding protein (clone LK26 from Signet Pathology Systems) in conjunction with the in vivo administration and
immunohistochemistry techniques described above, folate binding protein (FBP) was shown to be tissue specific.

[0296] FIGs. 16 A-E show the localization of the biotinylated antibody specific for FBP to the cells of the choroid plexus of the brain. Binding of the FBP specific antibody is not observed in any other tissues that were tested including heart, kidney, liver, and pancreas.

[0297] Although exemplary methods have been described for confirming the tissue specificity of polypeptides identified using the methods described herein, it will be appreciated that variations of the above-described methods can be utilized to confirm the tissue specificity of the polypeptides described herein.

EXAMPLE 10

Tissue-Specific Delivery of a Therapeutic Moiety Linked to a Ligand

[0298] The following example describes the construction of a therapeutic moiety linked to a tissue-specific ligand and localization of the therapeutic moiety in a tissue-specific manner.

Localization of toxin to the brain using OX-26 antibody

[0299] In a follow-up experiment to the in vivo localization of CD71, OX-26 was used to deliver ricin A chain (Sigma, Catalog number L9514) to the choroid plexus of the brain. First, the ricin (therapeutic moiety) was mixed with the OX-26 antibody (ligand) and a disulfide-containing biotin (Pierce, catalog number 21331). The ricin and OX-26 were then linked by the addition of Nuetravidin (Pierce, catalog number 31000) which bound both biotins, thus forming a complex of ricin and the antibody. This therapeutic complex was then administered to rats through tail vein injection and brain and lung tissues were processed as described above.

[0300] It was found that the antibody not only facilitated the localization of the toxin to the vasculature of the brain, but presumably also its entry into the tissue via transcytosis. Once in the tissue, the toxin elicited an inflammatory response in the brain, a reaction typically seen for any toxin introduced into the brain. No inflammatory response was seen in any other sectioned tissue.

Localization of gentamicin to the choroid plexus using folate

[0301] Folate, which is a ligand for the transcytotic receptor folate binding protein, was selected as a ligand to illustrate the role of transcytosis in the delivery of therapeutic molecules to specific tissues. A therapeutic complex comprising folate linked to gentamicin (therapeutic
moiety) was constructed. This therapeutic complex was then administered to rats through tail vein injection and colon, heart, kidney, liver, lung and brain tissues were processed as described above.

[0302] FIGs. 17 A-F show that the therapeutic complex containing gentamicin localized only to the choroid plexus of the brain. No staining was observed for the other tissues examined. These results indicated that the ligand for folate binding protein FBP is useful as a tissue-specific ligand for therapeutic moieties and that the therapeutic moieties can be linked to folate without affecting its recognition of or specificity for its cell-surface target molecule. Furthermore, these results show that therapeutic moieties can be delivered across endothelial cell sheet that lines the vasculature thus permitting concentration of the therapeutic moiety in the underlying tissues.

Localization of liposome encapsulated molecules to the brain using an antibody specific for the polypeptide comprising SEQ ID NO.: 4 or a homolog thereto

[0303] The full-length cDNA which encodes the polypeptide comprising an amino acid sequence having SEQ ID NO.: 4 is used as a brain-specific target for the delivery of a liposome-encapsulated drug. The full-length cDNA which encodes the polypeptide comprising an amino acid sequence having SEQ ID NO.: 4 can be obtained using the methods described in Example 5. This cDNA is expressed, purified then used to generate polyclonal antibodies using the methods described herein. These polyclonal antibodies, which are specific for the cell-surface luminally-exposed polypeptide comprising an amino acid sequence having SEQ ID NO.: 4, are used as a ligand for the targeting of a therapeutic moiety to the brain in a tissue-specific manner.

[0304] The therapeutic moiety comprises gentamicin which is linked to the ligand via a liposomal linker. The liposomes are linked to the polyclonal antibody ligands through polyethylene glycol (PEG) molecules that are attached to phospholipids present at the surface of the liposome. To facilitate PEG-mediated antibody attachment, distearoylphosphatidylethanolamine (DSPE) is first derivatized with PEG having a molecule weight between 1000 and 5000 kDa then the free end of the attached PEG group is converted to a reactive maleimide using methods well known in the art, such as those described in United States Patent Number 5,527,528. This reactive pegylated DSPE is incorporated into liposomes in about 0 to 10 mole percent. Other components of the liposome include unreactive pegylated DSPE in the range of about 0 to 10 mole percent, distearoylphosphatidylcholine (DSPC) or egg phosphatidylcholine in the range of 50 to 100 mole percent, and cholesterol in the range of about 0 to 50 mole percent.

[0305] Liposomes are formed by the reverse phase evaporation method described in United States Patent Number 4,235,871. Gentamicin is entrapped in the liposomes by adding this compound in the aqueous phase during liposome formation.
[0306] It will be appreciated that liposomes can be produced by a variety of methods known in the art. For example, liposomes can be formed using the methods described in Storm et al., PSTT 1:19-31 (1998) and United States Patent Numbers 4,522,803 and 4,885,172. It will also be appreciated that a variety of methods for encapsulating compounds within liposomes are known in the art. Such examples include the methods described in Mayer et al., Cancer Res. 49:5922-5930 and United States Patent Number 4,885,172.

[0307] Gentamicin containing liposomes are linked to the polyclonal antibody specific to a polypeptide comprising an amino acid sequence having SEQ ID NO.: 4 by adding the antibody to the liposomes in a solution of phosphate buffered saline at pH 8.0 and incubating the suspension for 16 hours with gentle shaking under reducing conditions.

[0308] The liposome-linked antibodies are then intravenously administered to swine. After about 30 minutes, the animals are sacrificed and the brain, heart, and lung tissues are prepared as previously described. Gentamicin is expected to be found to accumulate only in the brain.

EXAMPLE 11

Use of anti-VAP-1/Doxorubicin Therapeutic Complex with an Acid Sensitive Linker for the Treatment of Lung Cancer

[0309] The following example describes a the construction of an acid cleavable therapeutic complex that is formed between the anticancer agent doxorubicin and Fab2 fragments specific for VAP-1. Also described is a method of using this complex in the tissue-specific treatment of lung cancer.

[0310] Anti-VAP-1/doxorubicin therapeutic complexes can be constructed using the methods described in Example 10. Initially, a therapeutic level of a human anti-VAP-1/doxorubicin complex is administered to a patient intravenously. An effective amount of the complex is delivered to the patient, preferably 1 μg to 100 mg/Kg of patient weight in saline or an intravenously acceptable delivery vehicle.

[0311] The anti-VAP-1 F(ab')2, which is used as the ligand, is specific for the lung tissue. As the therapeutic complex is taken up into the lung tissue, the acid sensitive linker is cleaved and the doxorubicin is free to intercalate into the DNA. Because the doxorubicin is incorporated into the DNA of cycling cells, the effect on the cancer cells which are in the process of cycling will be marked and the effect on the normal lung cancer cells much reduced. Therefore, the treatment results in a reduction of the number of cancer cells in the lung, with a minimum of side effects. Because doxorubicin generally targets dividing cells and, because of the tissue specificity,
it will only affect the dividing cells of the lung, and therefore, it is envisioned that the number of cells killed due to side effects of the treatment will be minimal.

EXAMPLE 12

Use of anti-VAP-1/doxocillin Therapeutic

Complex for the Treatment of Lung Cancer Using a Prodrug


[0313] The therapeutic complex is an anti-VAP-1/β-lactamase conjugate which includes an F(ab')2 specific for VAP-1 that is linked to β-lactamase via a polypeptide linker, or a covalent bond. An example of an appropriate polypeptide linker is SMCC. The therapeutic agent doxocillin does not cross the endothelium due to a number of negative charges in the structure, which makes it nontoxic for all cells and ineffective as an anticancer drug. However, doxocillin can be thought of as a pro-drug which becomes active upon cleavage of the β-lactam ring to produce doxorubicin. Doxorubicin does cross the endothelium and intercalates into the DNA of cycling cells, making it an effective chemotherapeutic agent.

[0314] Initially, a therapeutic amount of a anti-VAP-1/β-lactamase complex is administered to the patient intravenously. A therapeutic level of the therapeutic complex is administered to the patient at between about 1 μg to 100 mg/Kg of patient weight. The anti-VAP-1 F(ab')2 ligand, which is targeted to the lung tissue, is linked to the β-lactamase prodrug in the therapeutic complex using a linker which is not cleavable. After administration and localization of the therapeutic complex, a therapeutic level of doxocillin is administered to the patient at between about 1 μg to 100 mg/Kg of patient weight, preferably between 10 μg to 10 mg/Kg of patient weight. The doxocillin is taken up systemically, but only in the microenvironment of the lung, the doxocillin is cleaved by the β-lactamase to produce doxorubicin. Therefore, the eukaryotic cytotoxic activity of the prodrug is unmasked only at the location of the β-lactamase, that is, the lungs. The doxorubicin is taken up by the lung tissue and intercalates into the DNA. However, because the doxorubicin is incorporated into the DNA of cycling cells, the effect on the cancer cells which are in the process of cycling will be marked and the effect on the normal lung cancer cells much reduced. The treatment results in a reduction in the number of cancer cells in the lung.
EXAMPLE 13

Use of anti-VAP-1 Therapeutic Complex for the Treatment of Lung Infections

[0315] The following example describes the construction of a therapeutic complex comprising anti-VAP-1 linked to liposomes containing cephalixin and a method of treating pneumonia using such a complex.

[0316] The most common bacterial pneumonia is pneumococcal pneumonia caused by Streptococcus pneumoniae. Other bacterial pneumonias may be caused by Haemophilus influenzae, and various strains of mycoplasma. Pneumococcal pneumonia is generally treated with penicillin. However, penicillin-resistant strains are becoming more common.

[0317] The present invention is used for the treatment of pneumococcal pneumonia in humans (or other mammals) as follows. A therapeutic complex is constructed by linking liposomes containing cephalixin to the F(αβ)2 fragments of human antibodies directed to VAP-1. Polyethylene glycol (PEG) is used to join phosphotidylethanolamine (PE) in the outer lamella of the liposomes to the VAP-1 specific F(αβ)2 fragments. The cephalixin is carried within the liposome. Such liposomes can be produced by using pegylated PE in the construction of the liposome using for example, the thin film hydration technique followed by a few freeze-thaw cycles. The cephalixin is captured within the interior of the liposome during liposome formation. The PEG on the exterior of the liposome is then activated as described above and anti-VAP-1 F(αβ)2 fragments are linked thereto. Similar liposomal suspensions can also be prepared according to methods known to those skilled in the art.

[0318] A dispersion of the therapeutic complex is then prepared and 0.1 to 10 nmol is injected intravenously. The liposomes carrying the cephalixin are targeted to the lung by the VAP-1 specific F(αβ)2 fragments. Upon binding to the endothelium, the liposomes are taken up and the cephalixin is taken into the lung tissue. The cephalixin can then act on the cell walls of the dividing S. pneumoniae organisms. One advantage of the targeting of antibiotics to a specific region is that less antibiotic is needed for the same result, there is less likelihood of side effects, and the likelihood of contributing to the drug resistance of the microorganism is considerably reduced.

EXAMPLE 14

Use of anti-VAP-1 Therapeutic Complex for the Treatment of Tuberculosis

[0319] In the following example, a method is set out for the construction and use of a VAP-1/rifampin prodrug therapeutic complex to treat tuberculosis.

[0320] It can readily be envisioned that diseases such as tuberculosis, caused by the bacterium M. tuberculosis, which is often treated using rifampin or isoniazid for a very long period of time, would be more effectively treated using the therapeutic agent of the present invention.
Much of the reason for the high incidence of disease and drug resistance in this microbe is the noncompliance with the extremely long course of treatment. It can be envisioned that using a method that directly targets the lungs with a high concentration of antibiotic would reduce the need for an unworkably long treatment and thus reduce the incidence of noncompliance and drug resistance.

[0321] The preferred embodiment is used for the treatment of tuberculosis in humans (or other mammals) as follows. A therapeutic complex is constructed by linking liposomes containing rifampin to the F(ab′)₂ fragments of human antibodies directed to VAP-1. PEG is used to join phosphatidylethanolamine (PE) in the outer lamella of the liposome to the VAP-1 specific F(ab′)₂ fragments. The rifampin is carried within the liposome. Such liposomes can be produced by using pegylated PE in the construction of the liposome using for example, the thin film hydration technique followed by a few freeze-thaw cycles. The cephalixin is captured within the interior of the liposome during liposome formation. The PEG on the exterior of the liposome is then activated as described above and anti-VAP-1 F(ab′)₂ fragments are linked thereto. Similar liposomal suspensions can also be prepared according to methods known to those skilled in the art.

[0322] A dispersion of the therapeutic complex is then prepared and 0.1 to 10 nmol is injected intravenously. The liposomes carrying the rifampin are targeted to the lung by the VAP-1 specific F(ab′)₂ fragments. Upon binding to the endothelium, the liposomes are taken up and the rifampin is taken into the lung tissue. The rifampin can then act on the M. tuberculosis organisms.

EXAMPLE 15

Use of anti-VAP-1 Therapeutic Complex for the Treatment of Surfactant Deficiencies

[0323] The following example describes, a method for the synthesis and use of an anti-VAP-1/surfactant protein therapeutic complex to treat lung diseases resulting from underproduction of surfactant proteins.

[0324] A number of lung diseases, including emphysema, include, as part of the cause or effect of the disease, deficiencies of surfactant proteins. The present invention is used for the treatment of surfactant deficiencies as follows. A therapeutic complex is constructed by linking a surfactant protein, such as surfactant protein A (SP-A), to F(ab′)₂ fragments of antibodies directed to VAP-1. The bonding linking this therapeutic moiety with the ligand is a pH sensitive bond.

[0325] The therapeutic complex is then injected intravenously into a patient. The complex is targeted to the lung by the VAP-1 specific F(ab′)₂ fragments. After binding to the target, the therapeutic complex is taken up by the lung tissue and the change in pH cleaves the bond, thus releasing the surfactant protein.
EXAMPLE 16

Use of anti-VAP-1 Therapeutic Complex for the Treatment of Lung Transplantation Rejection

[0326] In the following example, a method is set out for the synthesis and use of a VAP-1/corticosteroid therapeutic complex to treat rejection of transplanted lung tissue.

[0327] The present invention is used for the treatment of lung transplantation rejection as follows. A therapeutic complex is constructed by linking an immunosuppressant, such as a corticosteroid or cyclosporin, to F(ab')\textsubscript{2} fragments of VAP-1 specific antibodies using a pH sensitive linker.

[0328] This therapeutic complex is then injected intravenously into a patient and is targeted to the lung by the VAP-1 specific F(ab')\textsubscript{2} fragments. After binding to the target, the therapeutic complex is taken up by the lung tissue and the change in pH cleaves the bond, thus releasing the immunosuppressant only in the area of the lungs. It can readily be seen that the advantage of such a treatment is that the patient is not immunosuppressed and still has a healthy active immune system during recovery from the surgery. The lung (or other transplanted organ) is the only organ which is immunosuppressed and is carefully monitored.

[0329] It will be appreciated that any method which permits the therapeutic complex to contact tissue-specific luminal-exposed target molecules present on the desired tissues can be used to administer the therapeutic complex. Such methods, include but are not limited to, intravenous injection, intraperitoneal injection, ingestion, inhalation, and rectal administration.

[0330] These results indicate that therapeutic moieties can be linked to ligands such as antibodies and/or ligands specific for a cell-surface polypeptide. Such a cell-surface polypeptide can include a luminal-exposed polypeptide which comprises an amino acid sequence selected from the group consisting of SEQ ID NOs.: 3-37 and homologs thereof. Accordingly, therapeutic moieties can then be successfully delivered to such target polypeptides in a tissue-specific manner.

[0331] Other embodiments of the invention can be envisioned within the scope of the following claims.
WHAT IS CLAIMED IS:

1. A brain-specific therapeutic complex comprising:
   a ligand which binds in a tissue-specific manner to a polypeptide exposed on the
   luminal surface of cells which comprise cerebral tissue;
   a linker; and
   a therapeutic moiety wherein said linker links the ligand to the therapeutic moiety.

2. The therapeutic complex of Claim 1, wherein said luminally-exposed polypeptide
   comprises an amino acid sequence selected from the group consisting of SEQ ID NOs.: 3-5, 21, 22
   and homologs thereof.

3. The therapeutic complex of Claim 1, wherein said luminally-exposed polypeptide
   comprises SEQ ID NO.: 21, 22 or a homolog thereof.

4. The therapeutic complex of Claim 1, wherein said luminally-exposed polypeptide
   comprises SEQ ID NO.: 4 or a homolog thereof.

5. The therapeutic complex of Claim 1, wherein said luminally-exposed polypeptide
   comprises SEQ ID NO.: 5 or a homolog thereof.

6. The therapeutic complex of Claim 2, wherein said ligand is an antibody.

7. The therapeutic complex of Claim 6, wherein said antibody is a polyclonal
   antibody.

8. The therapeutic complex of Claim 6, wherein said antibody is a monoclonal
   antibody.

9. The therapeutic complex of Claim 6, wherein said antibody is a humanized
   antibody.

10. The therapeutic complex of Claim 2, wherein said ligand is an antibody fragment
    selected from the group consisting of Fab, Fab’ and Fab’2.

11. The therapeutic complex of Claim 2, wherein said ligand is a single chain Fv.

12. The therapeutic complex of Claim 2, wherein said ligand binds to a cell-surface
    molecule.

13. The therapeutic complex of Claim 2, wherein said ligand binds to a cell-surface
    receptor.

14. The therapeutic complex of Claim 13, wherein said ligand does not activate said
    cell-surface receptor.

15. The therapeutic complex of Claim 2, wherein said therapeutic moiety is selected
    from the group consisting of enzymes, antibiotics, immunomodulators, chemotherapeutic agents,
    antiviral agents, antifungal agents, contrast agents, prodrugs, hormones and a mixture thereof.
16. The therapeutic complex of Claim 15, wherein said enzymes specifically cleave prodrugs to produce the corresponding pharmaceutical agent.

17. The therapeutic complex of Claim 2, wherein said therapeutic moiety is gentamicin.

18. The therapeutic complex of Claim 2, wherein said linker is pH sensitive.

19. The therapeutic complex of Claim 2, wherein said linker comprises a bond that is cleaved under acidic conditions.

20. The therapeutic complex of Claim 2, wherein said linker is sensitive to reducing conditions.

21. The therapeutic complex of Claim 2, wherein said linker is cleavable by an enzyme.

22. The therapeutic complex of Claim 2, wherein said linker is a liposome.

23. The therapeutic complex of Claim 22, wherein said ligand is on the outside of said liposome and said therapeutic moiety is on the inside of said liposome.

24. A pharmaceutical composition comprising the brain-specific therapeutic complex of Claim 2 and one or more pharmaceutically acceptable carriers.

25. A method of determining the presence or concentration of folate binding protein (FBP) in a tissue or cell, comprising administering the therapeutic complex of Claim 3 to said tissue or cell in vitro or in vivo, and identifying or quantitating the amount of the therapeutic complex which bound.

26. A heart-specific therapeutic complex comprising:
   a ligand which binds in a tissue-specific manner to a polypeptide exposed on the luminal surface of cells which comprise cardiac tissue;
   a linker; and
   a therapeutic moiety wherein said linker links the ligand to the therapeutic moiety.

27. The therapeutic complex of Claim 26, wherein said luminally-exposed polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NOs.: 6-13, 23-29 and homologs thereof.

28. The therapeutic complex of Claim 26, wherein said luminally-exposed polypeptide comprises SEQ ID NO.: 23 or a homolog thereof.

29. The therapeutic complex of Claim 26, wherein said luminally-exposed polypeptide comprises SEQ ID NO.: 24 or a homolog thereof.

30. The therapeutic complex of Claim 26, wherein said luminally-exposed polypeptide comprises SEQ ID NO.: 25 or a homolog thereof.
31. The therapeutic complex of Claim 26, wherein said luminaly-exposed polypeptide comprises SEQ ID NO.: 26 or a homolog thereof.

32. The therapeutic complex of Claim 26, wherein said luminaly-exposed polypeptide comprises SEQ ID NO.: 27, 28 or a homolog thereof.

33. The therapeutic complex of Claim 26, wherein said luminaly-exposed polypeptide comprises SEQ ID NO.: 29 or a homolog thereof.

34. The therapeutic complex of Claim 27, wherein said ligand is an antibody.

35. The therapeutic complex of Claim 34, wherein said antibody is a polyclonal antibody.

36. The therapeutic complex of Claim 34, wherein said antibody is a monoclonal antibody.

37. The therapeutic complex of Claim 34, wherein said antibody is a humanized antibody.

38. The therapeutic complex of Claim 27, wherein said ligand is an antibody fragment selected from the group consisting of Fab, Fab', and Fab'2.

39. The therapeutic complex of Claim 27, wherein said ligand is a single chain Fv.

40. The therapeutic complex of Claim 27, wherein said ligand binds to a cell-surface molecule.

41. The therapeutic complex of Claim 27, wherein said ligand binds to a cell-surface receptor.

42. The therapeutic complex of Claim 41, wherein said ligand does not activate said cell-surface receptor.

43. The therapeutic complex of Claim 27, wherein said therapeutic moiety is selected from the group consisting of enzymes, antibiotics, immunomodulators, chemotherapeutic agents, antiviral agents, antifungal agents, contrast agents, prodrugs, hormones and a mixture thereof.

44. The therapeutic complex of Claim 43, wherein said enzymes specifically cleave prodrugs to produce the corresponding pharmaceutical agent.

45. The therapeutic complex of Claim 27, wherein said therapeutic moiety is gentamicin.

46. The therapeutic complex of Claim 27, wherein said linker is pH sensitive.

47. The therapeutic complex of Claim 27, wherein said linker comprises a bond that is cleaved under acidic conditions.

48. The therapeutic complex of Claim 27, wherein said linker is sensitive to reducing conditions.
49. The therapeutic complex of Claim 27, wherein said linker is cleavable by an enzyme.

50. The therapeutic complex of Claim 27, wherein said linker is a liposome.

51. The therapeutic complex of Claim 50, wherein said ligand is on the outside of said liposome and said therapeutic moiety is on the inside of said liposome.

52. A pharmaceutical composition comprising the heart-specific therapeutic complex of Claim 27 and one or more pharmaceutically acceptable carriers.

53. A method of determining the presence or concentration of CD36 in a tissue or cell, comprising administering the therapeutic complex of Claim 28 to said tissue or cell in vitro or in vivo, and identifying or quantitating the amount of the therapeutic complex which bound.

54. A method of determining the presence or concentration of cell adhesion regulator in a tissue or cell, comprising administering the therapeutic complex of Claim 29 to said tissue or cell in vitro or in vivo, and identifying or quantitating the amount of the therapeutic complex which bound.

55. A method of determining the presence or concentration of sarcoglycan epsilon in a tissue or cell, comprising administering the therapeutic complex of Claim 30 to said tissue or cell in vitro or in vivo, and identifying or quantitating the amount of the therapeutic complex which bound.

56. A method of determining the presence or concentration of testis ecto-arginine ADP ribosyltransferase (NAR3) in a tissue or cell, comprising administering the therapeutic complex of Claim 31 to said tissue or cell in vitro or in vivo, and identifying or quantitating the amount of the therapeutic complex which bound.

57. A method of determining the presence or concentration of aquaporin 2 in a tissue or cell, comprising administering the therapeutic complex of Claim 32 to said tissue or cell in vitro or in vivo, and identifying or quantitating the amount of the therapeutic complex which bound.

58. A method of determining the presence or concentration of cadherin-13 in a tissue or cell, comprising administering the therapeutic complex of Claim 33 to said tissue or cell in vitro or in vivo, and identifying or quantitating the amount of the therapeutic complex which bound.

59. A lung-specific therapeutic complex comprising:
   a ligand which binds in a tissue-specific manner to a polypeptide exposed on the luminal surface of cells which comprise pulmonary tissue;
   a linker; and
   a therapeutic moiety wherein said linker links the ligand to the therapeutic moiety.

60. The therapeutic complex of Claim 59, wherein said luminally-exposed polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NOs.: 14-17, 30-34 and homologs thereof.
61. The therapeutic complex of Claim 59, wherein said luminaly-exposed polypeptide comprises SEQ ID NO.: 30 or a homolog thereof.

62. The therapeutic complex of Claim 59, wherein said luminaly-exposed polypeptide comprises SEQ ID NO.: 31, 32 or a homolog thereof.

63. The therapeutic complex of Claim 59, wherein said luminaly-exposed polypeptide comprises SEQ ID NO.: 33 or a homolog thereof.

64. The therapeutic complex of Claim 59, wherein said luminaly-exposed polypeptide comprises SEQ ID NO.: 34 or a homolog thereof.

65. The therapeutic complex of Claim 60, wherein said ligand is an antibody.

66. The therapeutic complex of Claim 65, wherein said antibody is a polyclonal antibody.

67. The therapeutic complex of Claim 65, wherein said antibody is a monoclonal antibody.

68. The therapeutic complex of Claim 65, wherein said antibody is a humanized antibody.

69. The therapeutic complex of Claim 60, wherein said ligand is an antibody fragment selected from the group consisting of Fab, Fab' and Fab'2.

70. The therapeutic complex of Claim 60, wherein said ligand is a single chain Fv.

71. The therapeutic complex of Claim 60, wherein said ligand binds to a cell-surface molecule.

72. The therapeutic complex of Claim 60, wherein said ligand binds to a cell-surface receptor.

73. The therapeutic complex of Claim 72, wherein said ligand does not activate said cell-surface receptor.

74. The therapeutic complex of Claim 60, wherein said therapeutic moiety is selected from the group consisting of enzymes, antibiotics, immunomodulators, chemotherapeutic agents, antiviral agents, antifungal agents, contrast agents, prodrugs, hormones and a mixture thereof.

75. The therapeutic complex of Claim 74, wherein said enzymes specifically cleave prodrugs to produce the corresponding pharmaceutical agent.

76. The therapeutic complex of Claim 60, wherein said therapeutic moiety is gentamicin.

77. The therapeutic complex of Claim 60, wherein said linker is pH sensitive.

78. The therapeutic complex of Claim 60, wherein said linker comprises a bond that is cleaved under acidic conditions.
79. The therapeutic complex of Claim 60, wherein said linker is sensitive to reducing conditions.

80. The therapeutic complex of Claim 60, wherein said linker is cleavable by an enzyme.

81. The therapeutic complex of Claim 60, wherein said linker is a liposome.

82. The therapeutic complex of Claim 81, wherein said ligand is on the outside of said liposome and said therapeutic moiety is on the inside of said liposome.

83. A pharmaceutical composition comprising the lung-specific therapeutic complex of Claim 60 and one or more pharmaceutically acceptable carriers.

84. A method of determining the presence or concentration of CD9 in a tissue or cell, comprising administering the therapeutic complex of Claim 61 to said tissue or cell in vitro or in vivo, and identifying or quantitating the amount of the therapeutic complex which bound.

85. A method of determining the presence or concentration of receptor for advanced glycosylation end products (RAGE) in a tissue or cell, comprising administering the therapeutic complex of Claim 62 to said tissue or cell in vitro or in vivo, and identifying or quantitating the amount of the therapeutic complex which bound.

86. A method of determining the presence or concentration of integrin alpha-X in a tissue or cell, comprising administering the therapeutic complex of Claim 63 to said tissue or cell in vitro or in vivo, and identifying or quantitating the amount of the therapeutic complex which bound.

87. A method of determining the presence or concentration of CD81 in a tissue or cell, comprising administering the therapeutic complex of Claim 64 to said tissue or cell in vitro or in vivo, and identifying or quantitating the amount of the therapeutic complex which bound.

88. A pancreas-specific therapeutic complex comprising:
   a ligand which binds in a tissue-specific manner to a polypeptide exposed on the luminal surface of cells which comprise pancreatic tissue;
   a linker; and
   a therapeutic moiety wherein said linker links the ligand to the therapeutic moiety.

89. The therapeutic complex of Claim 88, wherein said luminally-exposed polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NOs.: 20, 36, 37 and homologs thereof.

90. The therapeutic complex of Claim 88, wherein said luminally-exposed polypeptide comprises SEQ ID NO.: 36, 37 or a homolog thereof.

91. The therapeutic complex of Claim 89, wherein said ligand is an antibody.

92. The therapeutic complex of Claim 91, wherein said antibody is a polyclonal antibody.
93. The therapeutic complex of Claim 91, wherein said antibody is a monoclonal antibody.

94. The therapeutic complex of Claim 91, wherein said antibody is a humanized antibody.

95. The therapeutic complex of Claim 89, wherein said ligand is an antibody fragment selected from the group consisting of Fab, Fab’ and Fab’2.

96. The therapeutic complex of Claim 89, wherein said ligand is a single chain Fv.

97. The therapeutic complex of Claim 89, wherein said ligand binds to a cell-surface molecule.

98. The therapeutic complex of Claim 89, wherein said ligand binds to a cell-surface receptor.

99. The therapeutic complex of Claim 98, wherein said ligand does not activate said cell-surface receptor.

100. The therapeutic complex of Claim 89, wherein said therapeutic moiety is selected from the group consisting of enzymes, antibiotics, immunomodulators, chemotherapeutic agents, antiviral agents, antifungal agents, contrast agents, prodrugs, hormones and a mixture thereof.

101. The therapeutic complex of Claim 100, wherein said enzymes specifically cleave prodrugs to produce the corresponding pharmaceutical agent.

102. The therapeutic complex of Claim 89, wherein said therapeutic moiety is gentamicin.

103. The therapeutic complex of Claim 89, wherein said linker is pH sensitive.

104. The therapeutic complex of Claim 89, wherein said linker comprises a bond that is cleaved under acidic conditions.

105. The therapeutic complex of Claim 89, wherein said linker is sensitive to reducing conditions.

106. The therapeutic complex of Claim 89, wherein said linker is cleavable by an enzyme.

107. The therapeutic complex of Claim 89, wherein said linker is a liposome.

108. The therapeutic complex of Claim 107, wherein said ligand is on the outside of said liposome and said therapeutic moiety is on the inside of said liposome.

109. A pharmaceutical composition comprising the pancreatic-specific therapeutic complex of Claim 89 and one or more pharmaceutically acceptable carriers.

110. A method of determining the presence or concentration of membrane dipeptidase-1 (MDP-1) in a tissue or cell, comprising administering the therapeutic complex of Claim 90 to said
tissue or cell *in vitro* or *in vivo*, and identifying or quantitating the amount of the therapeutic complex which bound.

111. A method of delivering a substance to a specific tissue, comprising:

administering a therapeutically effective amount of a therapeutic complex, said therapeutic complex comprising a therapeutic moiety linked to a ligand that binds to a tissue-specific luminally-exposed polypeptide, wherein said tissue-specific luminally-exposed polypeptide is selected from a group consisting of folate binding protein, CD36, cell adhesion regulator, sarcoglycan epsilon, NAR3, aquaporin 2, cadherin-13, CD9, RAGE, integrin alpha-X, CD81, VAP-1, and MDP-1.

112. A method for brain-specific delivery of a substance *in vivo* or *in vitro*, comprising:

providing a ligand that binds to folate binding protein, and

administering said ligand *in vivo* or *in vitro*, wherein said substance is delivered to a cerebral tissue as a result of the administration of said ligand.

113. The method of Claim 112, wherein said ligand is selected from the group consisting of an antibody, a protein, a peptide, an oligonucleotide, a small molecule, and a polysaccharide.

114. The method of Claim 112, wherein said substance is covalently or non-covalently bound to said ligand.

115. The method of Claim 112, wherein said substance is administered separately from said ligand.

116. The method of Claim 112, wherein said substance is a therapeutic moiety.

117. The method of Claim 112, wherein said substance is said ligand.

118. The method of Claim 112, wherein said *in vivo* administration is by a method selected from the group consisting of injection, oral delivery, aerosolization, an implantable pump, a patch, and a stent.

119. The method of Claim 112, wherein said *in vitro* administration is to a cerebral tissue to be transplanted.

120. A method for heart-specific delivery of a substance *in vivo* or *in vitro*, comprising:

providing a ligand that binds to a polypeptide selected from the group consisting of CD36, cell adhesion regulator, sarcoglycan epsilon, NAR3, aquaporin 2, and cadherin-13; and

administering said ligand *in vivo* or *in vitro*, wherein said substance is delivered to a cardiac tissue as a result of the administration of said ligand.
121. The method of Claim 120, wherein said ligand is selected from the group consisting of an antibody, a protein, a peptide, an oligonucleotide, a small molecule, and a polysaccharide.

122. The method of Claim 120, wherein said substance is covalently or non-covalently bound to said ligand.

123. The method of Claim 120, wherein said substance is administered separately from said ligand.

124. The method of Claim 120, wherein said substance is a therapeutic moiety.

125. The method of Claim 120, wherein said substance is said ligand.

126. The method of Claim 120, wherein said in vivo administration is by a method selected from the group consisting of injection, oral delivery, aerosolization, an implantable pump, a patch, and a stent.

127. The method of Claim 120, wherein said in vitro administration is to a cardiac tissue to be transplanted.

128. A method for lung-specific delivery of a substance in vivo or in vitro, comprising:
   providing a ligand that binds to a polypeptide selected from the group consisting of CD9, RAGE, integrin alpha-X, and CD81; and
   administering said ligand in vivo or in vitro, wherein said substance is delivered to a pulmonary tissue as a result of the administration of said ligand.

129. The method of Claim 128, wherein said ligand is selected from the group consisting of an antibody, a protein, a peptide, an oligonucleotide, a small molecule, and a polysaccharide.

130. The method of Claim 128, wherein said substance is covalently or non-covalently bound to said ligand.

131. The method of Claim 128, wherein said substance is administered separately from said ligand.

132. The method of Claim 128, wherein said substance is a therapeutic moiety.

133. The method of Claim 128, wherein said substance is said ligand.

134. The method of Claim 128, wherein said in vivo administration is by a method selected from the group consisting of injection, oral delivery, aerosolization, an implantable pump, a patch, and a stent.

135. The method of Claim 128, wherein said in vitro administration is to a pulmonary tissue to be transplanted.

136. A method for pancreas-specific delivery of a substance in vivo or in vitro, comprising:
providing a ligand that binds to MDP-1, and
administering said ligand in vivo or in vitro, wherein said substance is delivered to
a pancreatic tissue as a result of the administration of said ligand.

137. The method of Claim 136, wherein said ligand is selected from the group
consisting of an antibody, a protein, a peptide, an oligonucleotide, a small molecule, and a
polysaccharide.

138. The method of Claim 136, wherein said substance is covalently or non-covalently
bound to said ligand.

139. The method of Claim 136, wherein said substance is administered separately from
said ligand.

140. The method of Claim 136, wherein said substance is a therapeutic moiety.

141. The method of Claim 136, wherein said substance is said ligand.

142. The method of Claim 136, wherein said in vivo administration is by a method
selected from the group consisting of injection, oral delivery, aerosolization, an implantable pump,
a patch, and a stent.

143. The method of Claim 136, wherein said in vitro administration is to a pancreatic
tissue to be transplanted.

144. A lung and heart-specific therapeutic complex comprising:
a ligand which binds in a tissue-specific manner to a polypeptide exposed on the
luminal surface of cells which comprise pulmonary tissue;
a linker; and
a therapeutic moiety wherein said linker links the ligand to the therapeutic moiety.

145. The therapeutic complex of Claim 144, wherein said luminally-exposed
polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NOs.:
18, 19, 35 and homologs thereof.

146. The therapeutic complex of Claim 144, wherein said luminally-exposed
polypeptide comprises SEQ ID NO.: 35 or a homolog thereof.

147. The therapeutic complex of Claim 145, wherein said ligand is an antibody.

148. The therapeutic complex of Claim 147, wherein said antibody is a polyclonal
antibody.

149. The therapeutic complex of Claim 147, wherein said antibody is a monoclonal
antibody.

150. The therapeutic complex of Claim 147, wherein said antibody is a humanized
antibody.
151. The therapeutic complex of Claim 145, wherein said ligand is an antibody fragment selected from the group consisting of Fab, Fab' and Fab’2.

152. The therapeutic complex of Claim 145, wherein said ligand is a single chain Fv.

153. The therapeutic complex of Claim 145, wherein said ligand binds to a cell-surface molecule.

154. The therapeutic complex of Claim 145, wherein said ligand binds to a cell-surface receptor.

155. The therapeutic complex of Claim 154, wherein said ligand does not activate said cell-surface receptor.

156. The therapeutic complex of Claim 145, wherein said therapeutic moiety is selected from the group consisting of enzymes, antibiotics, immunomodulators, chemotherapeutic agents, antiviral agents, antifungal agents, contrast agents, prodrugs, hormones and a mixture thereof.

157. The therapeutic complex of Claim 156, wherein said enzymes specifically cleave prodrugs to produce the corresponding pharmaceutical agent.

158. The therapeutic complex of Claim 145, wherein said therapeutic moiety is gentamicin.

159. The therapeutic complex of Claim 145, wherein said linker is pH sensitive.

160. The therapeutic complex of Claim 145, wherein said linker comprises a bond that is cleaved under acidic conditions.

161. The therapeutic complex of Claim 145, wherein said linker is sensitive to reducing conditions.

162. The therapeutic complex of Claim 145, wherein said linker is cleavable by an enzyme.

163. The therapeutic complex of Claim 145, wherein said linker is a liposome.

164. The therapeutic complex of Claim 163, wherein said ligand is on the outside of said liposome and said therapeutic moiety is on the inside of said liposome.

165. A pharmaceutical composition comprising the lung and heart-specific therapeutic complex of Claim 145 and one or more pharmaceutically acceptable carriers.

166. A method of determining the presence or concentration of vascular adhesion protein-1 (VAP-1) in a tissue or cell, comprising administering the therapeutic complex of Claim 145 to said tissue or cell in vitro or in vivo, and identifying or quantitating the amount of the therapeutic complex which bound.

167. A method for lung or heart-specific delivery of a substance in vivo or in vitro, comprising:

   providing a ligand that binds to VAP-1, and
administering said ligand in vivo or in vitro, wherein said substance is delivered to
a lung or heart tissue as a result of the administration of said ligand.

168. The method of Claim 167, wherein said ligand is selected from the group
consisting of an antibody, a protein, a peptide, an oligonucleotide, a small molecule, and a
polysaccharide.

169. The method of Claim 167, wherein said substance is covalently or non-covalently
bound to said ligand.

170. The method of Claim 167, wherein said substance is administered separately from
said ligand.

171. The method of Claim 167, wherein said substance is a therapeutic moiety.

172. The method of Claim 167, wherein said substance is said ligand.

173. The method of Claim 167, wherein said in vivo administration is by a method
selected from the group consisting of injection, oral delivery, aerosolization, an implantable pump,
a patch, and a stent.

174. The method of Claim 167, wherein said in vitro administration is to a lung or heart
tissue to be transplanted.
FIG. 1

- Linker releases active agent after binding with target
- Target is a tissue-specific endothelial cell surface protein
- Small-Molecule Ligand binds to the Target
- Endothelial Cell
47 kDa

Cell Adhesion Regulator
and Sarcoglycan Epsilon

FIG. 6
FIG. 7A  

FIG. 7B  

FIG. 7C  

Arginine ADP-ribosyltransferase (NAR3)
Standard IHC Rat Signet LK-26 1:50 100X
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      ROBEN, Paul, W.
      STEVENS, Anthony, C.

<120> TISSUE-SPECIFIC ENDOTHELIAL MEMBRANE PROTEINS

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Species:

- Canis familiaris
- Homo sapiens
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Gly Leu Gly Ile Gly Thr Leu Val Gin Ala Leu Gly His Ile Ser Arg
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65  70  75  80
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Arg Gly Asp Leu Ala Val Asn Ala Leu Ser Asn Ser Thr Thr Ala Gly
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Ile Phe Ala Ser Thr Asp Glu Arg Arg Gly Glu Asn Pro Gly Thr Pro
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Ala Leu Ser Ile Gly Phe Ser Val Ala Leu Gly His Leu Leu Gly Ile
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His Tyr Thr Gly Cys Ser Met Asn Pro Ala Arg Ser Leu Ala Pro Ala
180 185 190
Val Val Thr Gly Lys Phe Asp Asp His Trp Val Phe Trp Ile Gly Pro
195 200 205
Leu Val Gly Ala Ile Leu Gly Ser Leu Leu Tyr Asn Tyr Val Leu Phe
210 215 220
Pro Pro Ala Lys Ser Leu Ser Glu Arg Leu Ala Val Leu Lys Gly Leu
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Thr Val Glu Asp Lys Asp Pro Thr Thr Gly Ala Trp Arg Ala Ala
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Tyr Thr Ile Ile Asn Gly Asn Pro Gly Gin Ser Phe Glu Ile His Thr
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130 135 140
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145 150 155 160
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165 170 175
Ile Arg Tyr Ser Val Tyr Lys Asp Pro Ala Gly Trp Leu Asn Ile Asn
180 185 190
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195 200 205
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Ser Gly Asn Pro Pro Ala Thr Gly Thr Gly Thr Leu Leu Ile Thr Leu
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Glu Asp Val Asn Asp Ala Pro Phe Ile Tyr Pro Thr Val Ala Glu
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Val Cys Asp Asp Ala Lys Asn Leu Ser Val Val Ile Leu Gly Ala Ser
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Asp Lys Asp Leu His Pro Asn Thr Asp Pro Phe Lys Phe Glu Ile His
275 280 285
Lys Gln Ala Val Pro Asp Lys Val Trp Lys Ile Ser Lys Ile Asn Asn
290 295 300
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Thr Asn Asn Asn Ser Ser Phe Tyr Thr Gly Val Tyr Ile Leu Ile
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Gly Ala Gly Ala Leu Met Met Leu Val Gly Phe Leu Gly Cys Cys Gly
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Ala Val Gln Glu Ser Gln Cys Met Leu Gly Leu Phe Phe Gly Phe Leu
85 90 95
Leu Val Ile Phe Ala Ile Glu Ile Ala Ala Ala Ile Trp Gly Tyr Ser
100 105 110
His Lys Asp Glu Val Ile Lys Glu Val Gln Glu Phe Tyr Lys Asp Thr
115 120 125
Tyr Asn Lys Leu Lys Thr Lys Asp Glu Pro Glu Arg Glu Thr Leu Lys
130 135 140
Ala Ile His Tyr Ala Leu Asn Cys Cys Gly Leu Ala Gly Gly Val Glu
145 150 155 160
Gln Phe Ile Ser Asp Ile Cys Pro Lys Lys Asp Val Leu Glu Thr Phe
165 170 175
Thr Val Lys Ser Cys Pro Asp Ala Ile Lys Glu Val Phe Asp Asn Lys
180 185 190
Phe His Ile Ile Gly Ala Val Gly Ile Gly Ile Ala Val Val Val Met Ile 195 200 205
Phe Gly Gly Met Ile Phe Ser Met Ile Leu Cys Cys Ala Ile Arg Arg Asn 210 215 220
Arg Glu Met Val 225

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| <211> | 416 |
| <212> | PRT |
| <213> | Bos taurus |

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| Pro Leu Val Leu Asn Cys Lys Gly Ala Pro Lys Lys Pro Pro Gln Gln | 35 40 45 |
| Leu Glu Trp Lys Leu Asn Thr Gly Arg Thr Glu Ala Trp Lys Val Leu | 50 55 60 |
| Ser Pro Gln Gly Asp Pro Trp Asp Ser Val Ala Arg Val Leu Pro Asn | 65 70 75 80 |
| Gly Ser Leu Leu Leu Pro Ala Val Gly Ile Gin Asp Glu Gly Thr Phe | 85 90 95 |
| Arg Cys Arg Ala Thr Ser Arg Ser Gly Lys Glu Thr Lys Ser Asn Tyr | 100 105 110 |
| Arg Val Arg Val Tyr Gln Ile Pro Gly Lys Pro Glu Ile Val Asp Pro | 115 120 125 |
| Ala Ser Glu Leu Met Ala Gly Val Pro Asn Lys Val Gly Thr Cys Val | 130 135 140 |
| Ser Glu Gly Tyr Pro Ala Gly Thr Leu Asn Trp Leu Leu Asp Gly | 145 150 155 160 |
| Lys Thr Leu Ile Pro Asp Gly Lys Gly Val Ser Val Lys Glu Glu Thr | 165 170 175 |
| Lys Arg His Pro Lys Thr Gly Leu Phe Thr Leu His Ser Glu Leu Met | 180 185 190 |
| Val Thr Pro Ala Arg Gly Ala Leu His Pro Thr Phe Ser Cys Ser | 195 200 205 |
| Phe Thr Pro Gly Leu Pro Arg Arg Ala Leu His Thr Ala Pro Ile | 210 215 220 |
| Gln Leu Arg Val Trp Ser Glu His Arg Gly Gly Gly Pro Asn Val | 225 230 235 240 |
| Asp Ala Val Pro Leu Lys Glu Val Gin Leu Val Val Glu Pro Glu | 245 250 255 |
| Gly Ala Val Ala Pro Gly Gly Thr Val Thr Leu Thr Cys Glu Ala Pro | 260 265 270 |
| Ala Gln Pro Pro Pro Ile His Thr Ile Lys Asp Gly Arg Pro Leu | 275 280 285 |
| Pro Leu Pro Pro Gly Pro Met Leu Leu Leu Pro Glu Val Gly Pro Glu | 290 295 300 |
| Asp Gln Gly Thr Tyr Ser Cys Val Ala Thr His Pro Ser His Gly Pro | 305 310 315 320 |
| Gln Glu Ser Arg Ala Val Ser Val Thr Ile Ile Glu Thr Gly Glu Glu | 325 330 335 |
| Gly Thr Thr Ala Gly Ser Val Glu Gly Pro Gly Leu Glu Thr Leu Ala | 340 345 350 |
| Leu Thr Leu Gly Ile Leu Gly Gly Leu Gly Thr Val Ala Leu Leu Ile | 355 360 365 |
| Gly Val Ile Val Trp His Arg Arg Arg Gln Arg Lys Gly Gin Glu Arg |</p>
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35  35  40
Leu Glu Trp Lys Leu Asn Thr Gly Arg Thr Glu Ala Trp Lys Val Leu
50  55  60
Ser Pro Gln Gly Gly Gly Pro Trp Ser Val Ala Arg Val Leu Pro
65  70  75  80
Asn Gly Ser Leu Phe Leu Pro Ala Gly Ile Gln Asp Glu Gly Ile
85  90  95
Pro Arg Cys Arg Ala Met Asn Arg Asn Gly Lys Glu Thr Lys Ser Asn
100 105 110
Tyr Arg Val Arg Val Tyr Gln Ile Pro Gly Lys Pro Glu Ile Val Asp
115 120 125
Val Ser Ala Ser Glu Leu Thr Ala Gly Val Pro Asn Lys Val Gly Thr Cys
130 135 140
Val Ser Glu Gly Ser Tyr Pro Ala Gly Thr Leu Ser Trp His Leu Asp
145 150 155 160
Gly Lys Pro Leu Val Pro Asn Glu Lys Gly Val Ser Val Lys Glu Gln
165 170 175
Thr Arg Arg His Pro Glu Thr Gly Leu Phe Thr Leu Gln Ser Glu Leu
180 185 190
Met Val Thr Pro Ala Arg Gly Gly Asp Pro Arg Pro Thr Phe Ser Cys
195 200 205
Ser Phe Ser Pro Gly Leu Pro Arg His Ala Leu Arg Thr Ala Pro
210 215 220
Ile Gln Pro Arg Val Trp Glu Pro Val Leu Glu Glu Val Glu Leu
225 230 235 240
Val Val Glu Pro Glu Gly Gly Ala Val Ala Pro Gly Gly Thr Val Thr
245 250 255
Leu Thr Cys Glu Val Pro Ala Gln Pro Ser Pro Gln Ile His Trp Met
260 265 270
Lys Asp Gly Val Pro Leu Pro Pro Ser Pro Val Leu Ile Leu
275 280 285
Glu Ile Gly Pro Gln Asp Glu Gly Thr Tyr Ser Cys Val Ala Thr
290 295 300
His Ser Ser His Gly Pro Gln Ser Arg Ala Val Ser Ile Ser Ile
305 310 315 320
Ile Glu Pro Gly Glu Gly Pro Thr Ala Gly Ser Val Gly Glu Ser
325 330 335
Gly Leu Gly Thr Leu Ala Ala Leu Gly Ile Leu Gly Gly Leu Gly
340 345 350
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Val Val Val Gly Ala Pro Gln Lys Ile Thr Ala Ala Asn Gln Thr Gly 50 55 60
Gly Leu Tyr Gln Cys Gly Tyr Ser Thr Gly Ala Cys Glu Pro Ile Gly 65 70 75 80
Leu Gln Val Pro Pro Glu Ala Val Asn Met Ser Leu Gly Leu Ser Leu 85 90 95
 Ala Ser Thr Ser Thr Ser Pro Ser Gln Leu Leu Ala Cys Gly Pro Thr Val 100 105 110
His His Glu Cys Gly Arg Asn Met Tyr Leu Thr Gly Leu Cys Phe Leu 115 120 125
Leu Gly Pro Thr Gln Leu Thr Gln Arg Leu Pro Val Ser Arg Gln Glu 130 135 140
Cys Pro Arg Glu Glu Glu Asp Ile Val Phe Leu Ile Asp Gly Ser Gly 145 150 155 160
Ser Ile Ser Ser Arg Asn Phe Ala Thr Met Met Asn Phe Val Arg Ala 165 170 175
Val Ile Ser Gln Phe Gln Arg Pro Ser Thr Gln Phe Ser Leu Met Gln 180 185 190
Phe Ser Asn Lys Phe Gln Thr His Leu Thr Phe Glu Glu Phe Arg Arg 195 200 205
Thr Ser Asn Pro Leu Ser Leu Leu Ala Ser Val His Gln Leu Gln Gly 210 215 220
Phe Thr Tyr Thr Ala Thr Ala Ile Gln Asn Val Val His Arg Leu Phe 225 230 235 240
His Ala Ser Tyr Gly Ala Arg Arg Asp Ala Thr Lys Ile Leu Ile Val 245 250 255
Ile Thr Asp Gly Lys Gly Gly Asp Thr Leu Asp Tyr Lys Asp Val 260 265 270
Ile Pro Met Ala Asp Ala Ala Gly Ile Ile Arg Tyr Ala Ile Gly Val 275 280 285
Gly Leu Ala Phe Asn Arg Asn Ser Trp Lys Glu Leu Asn Asp Ile 290 295 300
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Glu Gly Thr Glu Thr Thr Ser Ser Ser Ser Phe Glu Leu Glu Met Ala 345 350
Gln Glu Gly Phe Ser Ala Val Phe Thr Pro Asp Gly Pro Val Leu Gly 355 360 365
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Glu Lys Glu Ser His Val Ala Met His Arg Tyr Gln Val Asn Asn Leu 945 950 955 960
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Met Arg Ala Gln Thr Thr Thr Val Leu Glu Lys Tyr Lys Val His Asn 1090 1095 1100
Pro Thr Pro Leu Ile Val Gly Ser Ser Ile Gly Gly Leu Leu Leu Leu 1105 1110 1115 1120
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Leu Gly Asp Lys Pro Ala Pro Asn Thr Phe Tyr Val Gly Ile Tyr Ile 50 55 60
Leu Ile Ala Val Gly Ala Val Met Met Phe Val Gly Phe Leu Gly Cys 65 70 75 80
Tyr Gly Ala Ile Gln Glu Ser Gln Cys Leu Leu Gly Thr Phe Phe Thr 85 90 95
Cys Leu Val Ile Leu Phe Ala Cys Glu Val Ala Ala Gly Ile Trp Gly 100 105 110
Phe Val Asn Lys Asp Gln Ile Ala Lys Asp Val Lys Gln Phe Tyr Asp 115 120 125
Gln Ala Leu Gln Gln Ala Val Val Asp Asp Ala Asn Asn Ala Lys
130  135  140
Ala Val Val Lys Thr Phe His Glu Thr Leu Asp Cys Cys Gly Ser Ser
145  150  155  160
Thr Leu Thr Ala Leu Thr Thr Ser Val Leu Lys Asn Asn Leu Cys Pro
165  170  175
Ser Gly Ser Asn Ile Ile Ser Asn Leu Phe Glu Asp Cys His Gln
180  185  190
Lys Ile Asp Asp Leu Phe Ser Gly Lys Leu Tyr Leu Ile Gly Ile Ala
195  200  205
Ala Ile Val Val Ala Val Ile Met Ile Phe Glu Met Ile Leu Ser Met
210  215  220
Val Leu Cys Cys Gly Ile Arg Asn Ser Ser Val Tyr
225  230  235

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Ile Phe Ala Leu Val Cys Val Leu Leu Val Gly Arg Gly Gly Asp Gly
 20  25  30
Gly Glu Pro Ser Gln Leu Pro His Cys Pro Ser Val Ser Pro Ser Ala
 35  40  45
Gln Pro Trp Thr His Pro Gly Gln Ser Gln Leu Phe Ala Asp Leu Ser
  50  55  60
Arg Glu Glu Leu Thr Ala Val Met Arg Phe Leu Thr Gln Arg Leu Gly
  65  70  75  80
Pro Gly Leu Val Asp Ala Ala Gln Ala Arg Pro Ser Asp Asn Cys Val
  85  90  95
Phe Ser Val Glu Leu Gln Leu Pro Pro Lys Ala Ala Ala Leu Ala His
 100 105 110
Leu Asp Arg Gly Ser Pro Pro Ala Arg Glu Ala Leu Ala Ile Val
 115 120 125
Phe Phe Gly Arg Gln Pro Gln Pro Asn Val Ser Gln Leu Val Val Gly
 130 135 140
Pro Leu Pro His Pro Ser Tyr Met Arg Asp Val Thr Val Glu Arg His
 145 150 155 160
Gly Gly Pro Leu Pro Tyr His Arg Arg Pro Val Leu Phe Gln Glu Tyr
 165 170 175
Leu Asp Ile Asp Gln Met Ile Phe Asn Arg Glu Leu Pro Gln Ala Ser
 180 185 190
Gly Leu Leu His His Cys Cys Phe Tyr Lys His Arg Gly Arg Asn Leu
 195 200 205
Val Thr Met Thr Thr Ala Pro Arg Gly Leu Gln Ser Gly Asp Arg Ala
 210 215 220
Thr Trp Phe Gly Leu Tyr Tyr Asn Ile Ser Gly Ala Gly Phe Phe Leu
 225 230 235 240
His His Val Gly Leu Leu Leu Val Asn His Lys Ala Leu Asp Pro
 245 250 255
Ala Arg Trp Thr Ile Gln Lys Val Phe Tyr Gln Gly Arg Tyr Tyr Asp
 260 265 270
Ser Leu Ala Gln Leu Ala Gln Phe Glu Ala Gly Leu Val Asn Val
 275 280 285
Val Leu Ile Pro Asp Asn Gly Thr Gly Gly Ser Trp Ser Leu Lys Ser
 290 295 300
Pro Val Pro Pro Gly Pro Ala Pro Pro Leu Gln Phe Tyr Pro Gln Gly
 305 310 315 320
Pro Arg Phe Ser Val Gln Gly Ser Arg Val Ala Ser Ser Leu Trp Thr
325  330  335
Phe Ser Phe Gly Leu Gly Ala Phe Ser Gly Pro Arg Ile Phe Asp Val
340  345  350
Arg Phe Gln Gly Glu Arg Leu Val Tyr Glu Ile Ser Leu Gln Glu Ala
355  360  365
Leu Ala Ile Tyr Gly Gly Asn Ser Pro Ala Ala Met Thr Thr Thr Arg Tyr
370  375  380
Val Asp Gly Gly Phe Gly Met Gly Lys Tyr Thr Thr Pro Leu Thr Arg
385  390  395  400
Gly Val Asp Cys Pro Tyr Leu Ala Thr Tyr Val Asp Trp His Phe Leu
405  410  415
Leu Glu Ser Gln Ala Pro Lys Thr Ile Arg Asp Ala Phe Cys Val Phe
420  425  430
Glu Gln Asn Gln Gly Leu Pro Leu Arg Arg His His Ser Asp Leu Tyr
435  440  445
Ser His Tyr Phe Gly Gly Leu Ala Glu Thr Val Leu Val Val Arg Ser
450  455  460
Met Ser Thr Leu Leu Asp Tyr Asp Tyr Val Trp Asp Thr Val Phe His
465  470  475  480
Pro Ser Gly Ala Ile Glu Ile Arg Phe Tyr Ala Thr Gly Tyr Ile Ser
485  490  495
Ser Ala Phe Leu Phe Gly Ala Thr Gly Lys Tyr Gly Asn Gln Val Ser
500  505  510
Glu His Thr Leu Gly Thr Val His Thr His Ser Ala His Phe Lys Val
515  520  525
Asp Leu Asp Val Ala Gly Leu Glu Asn Trp Val Trp Ala Glu Asp Met
530  535  540
Val Phe Val Pro Met Ala Val Pro Trp Ser Pro Glu His Gln Leu Gln
545  550  555  560
Arg Leu Gln Val Thr Arg Lys Leu Leu Glu Met Glu Gln Ala Ala
565  570  575
Phe Leu Val Gly Ser Ala Thr Pro Arg Tyr Leu Tyr Leu Ala Ser Asn
580  585  590
His Ser Asn Lys Trp Gly His Pro Arg Gly Tyr Arg Ile Gln Met Leu
595  600  605
Ser Phe Ala Gly Glu Pro Leu Pro Gln Asn Ser Ser Met Ala Arg Gly
610  615  620
Phe Ser Trp Glu Arg Tyr Gln Leu Ala Val Thr Gln Arg Lys Glu Glu
625  630  635  640
Glu Pro Ser Ser Ser Ser Val Phe Asn Gln Asn Pro Trp Ala Pro
645  650  655
Thr Val Asp Phe Ser Asp Phe Ile Asn Glu Thr Ile Ala Gly Lys
660  665  670
Asp Leu Val Ala Trp Val Thr Ala Gly Phe Leu His Ile Pro His Ala
675  680  685
Glu Asp Ile Pro Asn Thr Val Thr Val Gly Asn Gly Val Gly Phe Phe
690  695  700
Leu Arg Pro Tyr Asn Phe Phe Asp Glu Asp Pro Ser Phe Tyr Ser Ala
705  710  715  720
Asp Ser Ile Tyr Phe Arg Gly Asp Gln Asp Ala Gly Ala Cys Glu Val
725  730  735
Asn Pro Leu Ala Cys Leu Pro Gln Ala Ala Ala Cys Ala Pro Asp Leu
740  745  750
Pro Ala Phe Ser His Gly Gly Phe Ser His Asn
755  760

<210> 36
<211> 409
<212> PRT
<213> Sus scrofa

<400> 36
Met Trp Thr Ser Trp Trp Leu Trp Pro Leu Val Ala Val Cys Ala Ala Ala
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Asp Gln Phe Arg Asp Leu Ala Val Arg Ile Met Gln Asp Thr Pro Val
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Ile Asp Gly His Asn Asp Leu Pro Trp Gln Leu Leu Asn Leu Phe Asn
35 40 45
Asn Gln Leu Gln Asp Pro Gly Ala Asn Leu Ser Ser Leu Ala His Thr
50 55 60
His Thr Asn Ile Pro Lys Leu Lys Ala Gly Phe Val Gly Gly Gln Phe
65 70 75 80
Trp Ser Ala Tyr Val Pro Cys Asp Thr Gln Asn Arg Asp Ala Val Lys
85 90 95
Arg Thr Leu Gln Gln Ile Asp Val Ile Gln Arg Met Cys Gln Ala Tyr
100 105 110
Pro Glu Thr Phe Ala Cys Val Thr Ser Ser Thr Gly Ile Arg Gln Ala
115 120 125
Phe Arg Glu Gly Lys Val Ala Ser Leu Val Gly Val Gly Val Gly Gly His
130 135 140
Ser Ile Asp Ser Ser Leu Gly Val Leu Arg Ala Leu Tyr His Leu Gly
145 150 155 160
Met Arg Tyr Met Thr Leu Thr His Ser Cys Asn Thr Pro Trp Ala Asp
165 170 175
Asn Trp Leu Val Asp Thr Gly Asp Asp Lys Ala Gln Ser Gln Gly Leu
180 185 190
Ser His Phe Gly Gln Ser Val Val Lys Glu Met Asn Arg Leu Gly Val
195 200 205
Met Ile Asp Leu Ala His Val Ser Val Ala Thr Met Arg Ala Ala Leu
210 215 220
Lys Leu Ser Gln Ala Pro Val Ile Phe Ser His Ser Ser Ala Tyr Ser
225 230 235 240
Leu Cys Pro His Arg Arg Asn Val Pro Asp Asp Val Leu Gln Leu Val
245 250 255
Lys Glu Thr Gly Ser Leu Val Met Val Asn Phe Tyr Asn Asp Tyr Val
260 265 270
Ser Cys Ser Ala Lys Ala Asn Leu Ser Gln Val Ala Asp His Leu Asp
275 280 285
His Ile Lys Lys Val Ala Gly Ala Ala Val Gly Phe Gly Gly Asp
290 295 300
Tyr Asp Gly Val Ser Arg Val Pro Ser Gly Leu Glu Asp Val Ser Lys
305 310 315 320
Tyr Pro Asp Leu Val Ala Glu Leu Arg Arg Gln Trp Thr Glu Ala
325 330 335
Glu Val Arg Gly Ala Leu Ala Asp Leu Leu Arg Val Phe Glu Ala
340 345 350
Val Glu Gln Ala Ser Asn His Ala Glu Pro Gly Glu Glu Pro Ile
355 360 365
Pro Leu Gly Gln Leu Glu Ala Ser Cys Arg Thr Asn Tyr Gly Tyr Ser
370 375 380
Ala Ala Pro Ser Leu His Leu Pro Pro Gly Leu Ala Ser Leu
385 390 395 400
Val Pro Leu Leu Leu Leu Ser Leu Pro
405

<210> 37
<211> 405
<212> PRT
<213> Homo sapiens
<400> 37
Met Thr Lys Ser Arg Asp Glu Thr His Glu Gly Cys Cys Gly Ser
 1   5        10       15
Leu Ala Asn Tyr Thr Ser Ala Lys Phe Leu Leu Tyr Leu Gly His
 20  25        30       35
Ser Leu Ser Thr Trp Gly Asp Arg Met Trp His Phe Ala Val Ser Val
 35  40        45       50
Phe Leu Val Glu Leu Tyr Gly Asn Ser Leu Leu Leu Thr Ala Val Tyr
 50  55        60       65
Gly Leu Val Ala Gly Ser Val Leu Val Leu Gly Ala Ile Ile Gly
 65  70        75       80
Asp Trp Val Asp Lys Ala Arg Leu Lys Val Ala Glu Thr Ser Leu
 85  90        95
Val Val Glu Asn Val Ser Val Ile Leu Cys Gly Ile Ile Leu Met Met
100 105       110      115
Val Phe Leu His Lys Asn Glu Leu Leu Asn Met Tyr His Gly Trp Val
120 125
Leu Thr Val Cys Tyr Ile Leu Ile Ile Thr Ile Ala Asn Ile Ala Asn
130 135       140      145
Leu Ala Ser Thr Ala Thr Ala Ile Thr Ile Gln Arg Asp Trp Ile Val
150 155       160      165
Val Val Ala Gly Glu Asn Arg Ser Arg Leu Ala Asp Met Asn Ala Thr
170 175      180      185
Ile Arg Arg Ile Asp Glu Leu Thr Asn Ile Leu Ala Pro Met Ala Val
190 195
Gly Gln Ile Met Thr Phe Gly Ser Pro Val Ile Gly Cys Gly Phe Ile
195 200       205      210
Ser Gly Trp Asn Leu Val Ser Met Cys Val Glu Tyr Phe Leu Leu Trp
215 220
Lys Val Tyr Glu Lys Thr Pro Ala Leu Ala Val Lys Ala Ala Leu Lys
225 230       235      240
Val Glu Glu Ser Glu Leu Lys Gln Leu Thr Ser Pro Lys Asp Thr Glu
245 250       255      260
Pro Lys Pro Leu Glu Gly Thr His Leu Met Gly Gly Lys Asp Ser Asn
265 270
Ile Arg Glu Leu Glu Cys Glu Glu Asn Pro Thr Cys Ala Ser Glu Ile
270 275       280      285
Ala Glu Pro Phe Arg Thr Phe Arg Asp Gly Trp Val Ser Tyr Tyr Asn
290 295       300      305
Gln Pro Val Phe Leu Gly Trp His Gly Pro Gly Phe Pro Leu Tyr Asp
310 315       320      325
Ser Pro Gly Leu Arg Leu Tyr Arg His Ser Ile Cys Leu His Ser Gly
330 335
Thr Glu Trp Phe His Pro Glu Cys Phe Asp Gly Ser Ile Ser Asn Asn
340 345       350      355
Trp Asn Asn Glu Cys Gly Leu His Leu Ala Thr Ser Lys Met Trp
360 365
Pro Cys Ser Asp Trp Ser Val Leu Arg Thr Gly Ser Ala Phe Leu Phe
370 375       380      385
Asp Leu Val Cys Asp Leu Arg Val His Ala Thr Phe Leu Gly Pro
390 395
Val Cys Phe Ser Ile
400

<210> 38
<211> 959
<212> DNA
<213> Sus scrofa
<400> 38
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<210> 39
<211> DNA
<212> DNA
<213> Homo sapiens

<400> 39
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tccctgctcc tctagtgtgg gttggctgta gtagggggga ctcagcaacag gattgctattg 180
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<210> 40
<211> DNA
<212> DNA
<213> Homo sapiens

<220>
<221> DNA
<222> DNA
<223> DNA

<400> 40
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