Title: MELANOCORTIN AND TRANSFERRIN FUSION PROTEINS

Abstract: The present invention discloses fusion proteins comprising a transferrin moiety and a melanocortin or melanocortin receptor agonist moiety. In one embodiment of the invention, the transferrin moiety is modified to exhibit reduced or no glycosylation. In another embodiment, the transferrin moiety is modified to exhibit reduced or no binding to a transferrin receptor. The fusion protein of the invention can optionally comprise a linker peptide and/or a second therapeutic peptide such as GLP-1 or exendin-4. Also disclosed are methods of treating or preventing a disease or condition in a subject by administering a fusion protein of the invention.
— with sequence listing part of description published separately in electronic form and available upon request from
the International Bureau
MELANOCORTIN AND TRANSFERRIN FUSION PROTEINS

RELATED APPLICATIONS

This application is related to International Application PCT/US03/26818, filed August 28, 2003, which claims the benefit of U.S. Application 10/378,094, filed March 4, 2003, and U.S. Application 10/231,494, filed August 30, 2002, which claims the benefit of U.S. Provisional Application 60/315,745, filed August 30, 2001 and U.S. Provisional Application 60/334,059, filed November 30, 2001, all of which are herein incorporated by reference in their entirety. This application is related to U.S. Application 11/367,692, filed March 6, 2006 which claims the benefit of U.S. Provisional Application 60/658,140, filed March 4, 2005 and is related to U.S. Provisional Application 60/663,757, filed March 4, 2005; U.S. Provisional Application 60/832,582 filed July 24, 2006; and U.S. Provisional Application 60/837,323, filed August 14, 2006, all of which are herein incorporated by reference in their entirety.

FIELD OF THE INVENTION

The present invention relates to a melanocortin and transferrin fusion protein and use thereof for the treatment of diseases treatable with melanocortin receptor agonists. In one embodiment, the fusion protein of the present invention can be used to treat diseases associated with inflammation.

BACKGROUND OF THE INVENTION

Therapeutic proteins or peptides in their native state or when recombinantly produced are typically labile molecules exhibiting short periods of serum stability or short in vivo circulatory half-lives. In addition, these molecules are often extremely labile when formulated, particularly when formulated in aqueous solutions for diagnostic and therapeutic purposes.

Few practical solutions exist to extend or promote the stability in vivo or in vitro of proteinaceous therapeutic molecules. Polyethylene glycol (PEG) is a substance that can be attached to a protein, resulting in longer-acting, sustained activity of the protein. If the activity of a protein is prolonged by the attachment to PEG, the frequency that the protein needs to be administered may be decreased. PEG attachment, however, often decreases or destroys the protein's therapeutic activity. While in some instance PEG attachment can reduce immunogenicity of the protein, in other instances it may increase immunogenicity.

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Therapeutic proteins or peptides have also been stabilized by fusion to a protein capable of extending the in vivo circulatory half-life of the therapeutic protein. For instance, therapeutic proteins fused to albumin or to antibody fragments may exhibit extended in vivo circulatory half-life when compared to the therapeutic protein in the unfused state. See U.S. Patents 5,876,969 and 5,766,883.

Another serum protein, glycosylated human transferrin (Tf) has also been used to make fusions with therapeutic proteins to target delivery to the interior of cells or to carry agents across the blood-brain barrier. These fusion proteins comprising glycosylated human Tf have been used to target nerve growth factor (NGF) or ciliary neurotrophic factor (CNTF) across the blood-brain barrier by fusing full-length Tf to the agent. See U.S. Patents 5,672,683 and 5,977,307. In these fusion proteins, the Tf portion of the molecule is glycosylated and binds to two atoms of iron, which is required for Tf binding to its receptor on a cell and, according to the inventors of these patents, to target delivery of the NGF or CNTF moiety across the blood-brain barrier. Transferrin fusion proteins have also been produced by inserting an HIV-1 protease target sequence into surface exposed loops of glycosylated transferrin to investigate the ability to produce another form of fusion protein for targeted delivery to the inside of a cell via the Tf receptor (Ali et al., 1999, J. Biol. Chem. 274(34):24066-24073).


The structure of Tf has been well characterized and the mechanisms of receptor binding, iron binding and release and carbonate ion binding have been elucidated (U.S. Patents 5,026,651, 5,986,067 and MacGillivray et al., 1983, J. Biol. Chem. 258(6):3543-3546).

Transferrin and antibodies that bind the transferrin receptor have also been used to deliver or carry toxic agents to tumor cells as cancer therapy (Baselga and Mendelsohn,
and transferrin has been used as a non-viral gene therapy vector to deliver DNA to cells (Frank et al., 1994; Wagner et al., 1992). The ability to deliver proteins to the central nervous system (CNS) using the transferrin receptor as the entry point has been demonstrated with several proteins and peptides including CD4 (Walus et al., 1996), brain derived neurotrophic factor (Pardridge et al., 1994), glial derived neurotrophic factor (Albeck et al.), a vasointestinal peptide analogue (Bickel et al., 1993), a beta-amyloid peptide (Saito et al., 1995), and an antisense oligonucleotide (Pardridge et al., 1995).

Melanocortins (α-MSH, β-MSH, γ-MSH and ACTH) are involved in diverse physiological functions, including, but not limited to, pigmentation, inflammation, temperature control, exocrine secretion, control of food intake, sexual function, cardiovascular regulation and neuromuscular regeneration. For instance, α-MSH ameliorates the effects of UV radiation on skin, exhibits anti-inflammatory properties and is believed to have antimicrobial effects (Catania et al., 2004, Pharmacol. Rev. 56: 1-29). β-MSH variants have been shown to exhibit anti-obesity effects (Hsiung et al., 2005, Endocrinology. 146(12): 5257-5266). On the other hand, a γ-MSH variant has been shown to stimulate appetite via the melanocortin 3 receptor (Marks et al., 2006, Peptides. 27(2): 259-64). γ-MSH has also been shown to be involved in the regulation of adrenal steroidogenesis and has been shown to have effects on the cardiovascular and renal systems (Harmer and Bicknell, 2005, Peptides. 26(10): 1944-1951).

Although the therapeutic potential of melanocortins has been recognized, the extremely short half-life of melanocortins has discouraged their use as therapeutics (Catania et al., 2004, Pharmacol. Rev. 56: 1-29; Rudman et al., 1983, Am. J. Physiol. Endocrinol. Metab. 245: E47-E54; and Wright and Wilson, 1983, Peptides. 4: 5-9). Melanocortin variants and melanocortin receptor agonists have been developed with increased half-life. The majority of prolonged acting variants such as NDP-alpha-MSH, MT-II and SHU-9119, however, have suffered from drastically reduced receptor selectivity (Cai et al., 2005, Peptides. 26(8): 1481-1485; Hol et al., 1994, J. Neurosci. Res. 39: 178-185; and Adan et al., 1994, Eur. J. Pharmacol. 269: 331). Further, although variants have been created which exhibit prolonged half-life, the half-life of these molecules still tends to be fairly short.

Accordingly, a need remains for a long-lasting melanocortin molecule. The present invention fulfills this need by providing transferrin and melanocortin fusion proteins which extend the in vivo circulatory half-life of the melanocortin protein while maintaining or increasing bioactivity and/or receptor selectivity.
SUMMARY OF THE INVENTION

As described in more detail below, the present invention includes a fusion protein comprising a melanocortin peptide or melanocortin variant and a transferrin protein. For instance, the present invention includes fusion proteins comprising α-MSH, β-MSH, γ-MSH or ACTH fused to a transferrin protein. The present invention also includes a fusion protein comprising a melanocortin receptor agonist, melanocortin receptor antagonist or melanocortin receptor binding protein and a transferrin protein. For instance, the present invention includes fusion proteins comprising an MC1R, MC2R, MC3R, MC4R or MC5R agonist or antagonist fused to a transferrin protein.

The melanocortin fusion proteins of the present invention exhibit prolonged serum half-life compared to unfused melanocortin peptides. The melanocortin fusion proteins of the invention are also capable of exhibiting increased bioactivity.

In one embodiment of the invention, the transferrin moiety of the fusion protein is a modified transferrin protein. The present invention includes a fusion protein comprising a modified transferrin moiety that exhibits reduced or no glycosylation as compared to a native transferrin protein. For instance, the invention includes a fusion protein containing a fusion protein comprising a modified Tf protein which exhibits no N-linked glycosylation as a result of a mutation within or adjacent to the N-linked glycosylation site H-X-SfT.

In one embodiment of the invention, the transferrin moiety of the fusion protein is modified to exhibit reduced or no binding to iron. The invention also includes a fusion protein comprising a transferrin moiety which exhibits reduced or no affinity for a Tf receptor.

The fusion protein of the invention can take on multiple configurations. For instance, the melanocortin peptide or melanocortin receptor agonist can be fused at either the N-terminal end or C-terminal end of Tf or at both ends of Tf. The melanocortin peptide or melanocortin receptor agonist can also be inserted within the Tf moiety, for instance, within surface exposed loops on one or both lobes of Tf.

Further, the fusion protein of the invention may contain additional peptide moieties. For instance, the present invention includes a melanocortin fusion protein comprising a peptide linker such as a substantially non-helical peptide linker. In another embodiment, the fusion protein can contain one or more additional therapeutic peptides. For instance, the fusion protein can contain a GLP-1 receptor agonist such as GLP-1 or an exendin peptide (e.g., exendin-4) or an interferon such as interferon β (IFNβ) or interferon α (IFNα). The invention includes methods of treating diseases or disorders that can be treated or ameliorated by the administration of a GLP-1 agonist or an interferon. For instance, a fusion
protein comprising IFNβ, a melanocortin peptide and Tf can be used for the treatment of multiple sclerosis.

The present invention also includes a pharmaceutical composition comprising the fusion protein of the invention and a carrier. The fusion protein of the invention can be administered to treat or prevent a disease in a subject. For instance, the fusion protein of the invention can be administered to a patient in need thereof for prophylactic treatment of a disease. The fusion protein of the invention can be used to treat any disease or condition known to be affected by melanocortins. For instance, the fusion protein of the invention can be administered to a subject to treat or prevent allergic inflammation, arthritis (rheumatoid arthritis, gouty arthritis, acute arthritis, juvenile chronic arthritis), autoimmune uveoretinitis, inflammatory bowel disease, brain injury, ischemic brain damage, experimental brain inflammation, neurodegenerative disorder (multiple sclerosis, Alzheimer’s disease), septic shock, hepatic inflammation, vasculitis, acute respiratory distress syndrome, hemorrhagic shock, reperfusion injury, myocardial ischemia, myocardial arrhythmias, myocardial infarction, organ transplant (heart), renal injury, renal ischemia, mesenteric ischemia, infection, fever, skin cancer, obesity and erectile dysfunction.

The present invention also includes the administration of the fusion protein of the invention for non-therapeutic purposes. For instance, the fusion protein of the invention can be administered to a subject to induce tanning and/or weight loss.

The fusion protein of the present invention can also be administered to a patient suffering from a disease or condition associated with activation of NF-κB or expression of TNF-α.

In another embodiment of the invention, the melanocortin peptide moiety of the fusion protein targets the fusion protein to a particular cell type, for instance, a cancer cell. In this embodiment, the fusion protein may optionally comprise a second therapeutic protein. The Tf moiety may optionally be bound to a metal. The invention includes methods of using the fusion protein to treat diseases such as cancer including, but not limited to, melanoma. For instance, a fusion protein comprising IFNβ, a melanocortin peptide and Tf can be administered to a patient for the treatment of melanoma. In another embodiment of the invention, labeled melanocortin peptide fusion proteins can be used for imaging cells that express one or more melanocortin receptors. For example, the invention provides methods of imaging melanoma cells.
BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the location of a number of Tf surface exposed insertion sites for therapeutic proteins, polypeptides or peptides.

Figure 2 is a diagram of an mTf αMSH 279 PCR product.

Figure 3 is a diagram of an mTf γ-MSH279 PCR product.

Figure 4 is a graph showing activity of mTf (PEAPTD)2 α-MSH in a melanin biosynthesis assay.

Figure 5 is a graph showing activity of mTf (PEAPTD)2 α-MSH in a cAMP assay.

DETAILED DESCRIPTION

General Description

Melanocortin therapeutic proteins can be stabilized to extend their serum half-life and/or activity in vivo by genetically fusing a melanocortin peptide, melanocortin receptor agonist or antagonist or fragment thereof to transferrin, modified transferrin, or a portion of transferrin or modified transferrin sufficient to extend the half-life of the therapeutic protein in serum. In one embodiment, the fusion proteins include a transferrin protein or domain covalently linked to a melanocortin peptide, melanocortin receptor agonist, melanocortin receptor antagonist or melanocortin receptor binding peptide wherein the transferrin portion is modified to contain one or more amino acid substitutions, insertions or deletions compared to a wild-type transferrin sequence. In one embodiment, the fusion proteins are engineered to reduce or prevent glycosylation within the Tf or a Tf domain. In other embodiments, the Tf protein or Tf domain(s) is modified to exhibit reduced or no binding to iron or carbonate ion, or to have a reduced affinity or not bind to a Tf receptor (TfR).

The present invention also includes inserting a linker between a therapeutic peptide and a transferrin molecule to increase the stability and availability of a therapeutic molecule for binding to its receptor. Substantially non-helical linkers, including but not limited to PEAPTD (SEQ ID NO.: 13), (PEAPTD)2 (SEQ ID NO.: 10), PEAPTD in combination with an IgG hinge linker (SEQ ID NOS.: 118-123 and 126-129), and (PEAPTD)2 in combination with an IgG hinge linker (SEQ ID NOS.: 205-214), can substantially increase serum half-life, productivity of expression of the fusion protein and/or the activity of a therapeutic molecule such as a melanocortin peptide or melanocortin receptor agonist.

The present invention therefore includes fusion proteins, therapeutic compositions comprising the fusion proteins, and methods of treating, preventing, or ameliorating diseases or disorders by administering melanocortin peptide or melanocortin receptor agonist and transferrin fusion proteins. The melanocortin peptide, once part of the fusion protein, may be
referred to as a melanocortin "portion," "region" or "moiety" of the fusion protein. The melanocortin receptor agonist, once part of the fusion protein, may be referred to as a melanocortin receptor agonist "portion," "region" or "moiety" of the fusion protein. Tranferrin, once part of the fusion protein, may be referred to as a Tf "portion," "region" or "moiety" of the fusion protein. Likewise, a linker peptide, once part of the transferrin fusion protein, may be referred to as a "linker" or linker "portion," "region" or "moiety" of the transferrin fusion protein.

In one embodiment, the invention provides a fusion protein comprising, or alternatively consisting of, a melanocortin peptide and a transferrin protein. In other embodiments, the invention provides a fusion protein comprising, or alternatively consisting of, a biologically active and/or therapeutically active fragment of a melanocortin peptide and a transferrin protein. For instance, the invention includes, but is not limited to, a fusion protein comprising the peptide KPV (α-MSH 11-13). Although the biologically active and/or therapeutically active fragment of a melanocortin peptide may contain or comprise the core melanocortin active sequence HFRW (for example, residues 6-9 of α-MSH), as illustrated by the KPV peptide, other melanocortin peptide fragments are capable of possessing biological and/or therapeutic activity. In other embodiments, the invention provides a fusion protein comprising, or alternatively consisting of, a biologically active and/or therapeutically active variant of a melanocortin peptide and a transferrin protein. For instance, a variant of melanocortin peptide includes, but is not limited to, a melanocortin hybrid peptide such as an α-MSH and γ-MSH hybrid peptide. The fusion proteins of the invention can contain an unmodified or modified transferrin protein. Further, the fusion proteins of invention may optionally contain a linker, for instance, a substantially non-helical linker.

In another embodiment, the invention provides a fusion protein comprising, or alternatively consisting of, a melanocortin receptor agonist, melanocortin receptor antagonist or melanocortin receptor binding peptide and a transferrin protein. In other embodiments, the invention provides a fusion protein comprising, or alternatively consisting of, a biologically active and/or therapeutically active fragment of a melanocortin receptor agonist, melanocortin receptor antagonist or melanocortin receptor binding peptide and a transferrin protein. The fusion proteins of the invention can contain an unmodified or modified transferrin protein. Further, the fusion proteins of invention may optionally contain a linker, for instance, a substantially non-helical linker.

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

Definitions

As used herein, an "amino acid corresponding to" or an "equivalent amino acid" in a peptide or protein sequence is identified by alignment to maximize the identity or similarity between a first peptide or protein sequence and at least a second peptide or protein sequence. The number used to identify an equivalent amino acid in a second sequence is based on the number used to identify the corresponding amino acid in the first sequence. In certain cases, these phrases may be used to describe the amino acid residues in human transferrin compared to certain residues in rabbit serum transferrin.

As used herein, the term "biological activity" or "activity" refers to a function or set of activities performed by a melanocortin, a melanocortin variant or melanocortin receptor agonist or antagonist, or melanocortin receptor binding peptide, or fragment thereof in a biological context (i.e., in an organism or an in vitro facsimile thereof). Biological activities may include, but are not limited, to activities related to α-melanocortin stimulating hormone (α-MSH), β-melanocortin stimulating hormone (β-MSH), γ-melanocortin stimulating hormone (γ-MSH), adrenocorticotropin (ACTH). For instance, biological activities include, but are not limited to, modulation of inflammation, reduction of damage caused by UV radiation to the skin, reduction of damage caused by reperfusion injury, anti-obesity effects, appetite regulation and amelioration of sexual dysfunction. In one embodiment of the invention, the biological activity is binding to a melanocortin receptor. A fusion protein or peptide of the invention is considered to be biologically active if it exhibits one or more biological activities of its therapeutic protein's native counterpart, i.e., unfused counterpart.

A fusion protein, with or without a substantially non-helical linker, substantially exhibits prolonged biological activity if it exhibits one or more biological activities by at least about 2 fold, at least about 5 fold, at least about 10 fold, at least about 20 fold, at least about 30 fold, at least about 40 fold, at least about 50 fold, at least about 60 fold, at least about 70 fold, at least about 80 fold, at least about 90 fold, at least about 100 fold, at least about 200 fold, or at least about 300 fold or more, longer in duration, i.e., period of time, than the same one or more biological activities of its unfused melanocortin counterpart (e.g., unfused α-MSH, Y-MSH, β-MSH or ACTH), either in vivo or in vitro. In another embodiment, a fusion protein with substantially non-helical linker substantially exhibits prolonged activity if it exhibits one or more biological activities by at least about 2 fold, at least about 5 fold, at least about 10 fold, at least about 20 fold, at least about 30 fold, at least about 40 fold, at least
about 50 fold, at least about 60 fold, at least about 70 fold, at least about 80 fold, at least about 90 fold, at least about 100 fold, at least about 200 fold, or at least about 300 fold or more, longer in duration, *i.e.*, period of time, than the same one or more biological activities of a melanocortin and Tf fusion protein lacking a linker sequence or a melanocortin and Tf fusion protein with a flexible linker, either *in vivo* or *in vitro*.

As used herein, "binders" are agents used to impart cohesive qualities to the powdered material. Binders, or "granulators" as they are sometimes known, impart cohesiveness to the tablet formulation, which insures the tablet remaining intact after compression, as well as improving the free-flowing qualities by the formulation of granules of desired hardness and size. Materials commonly used as binders include starch; gelatin; sugars, such as sucrose, glucose, dextrose, molasses, and lactose; natural and synthetic gums, such as acacia, sodium alginate, extract of Irish moss, panwar gum, ghatti gum, mucilage of isapol husks, carboxymethylcellulose, methylcellulose, polyvinylpyrrolidone, Veegum, microcrystalline cellulose, microcrystalline dextrose, amylose, and larch arabogalactan and the like.

As used herein, the term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which a composition is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like.

As used herein, "coloring agents" are agents that give tablets a more pleasing appearance, and in addition help the manufacturer to control the product during its preparation and help the user to identify the product. Any of the approved certified water-soluble FD&C dyes, mixtures thereof, or their corresponding lakes may be used to color tablets. A color lake is the combination by adsorption of a water-soluble dye to a hydrous oxide of a heavy metal, resulting in an insoluble form of the dye.

As used herein, "diluents" are inert substances added to increase the bulk of the formulation to make the tablet a practical size for compression. Commonly used diluents include calcium phosphate, calcium sulfate, lactose, kaolin, mannitol, sodium chloride, dry starch, powdered sugar, silica, and the like.

As used herein, "disintegrators" or "disintegrants" are substances that facilitate the breakup or disintegration of tablets after administration. Materials serving as disintegrants have been chemically classified as starches, clays, cellulosics, algins or gums. Other disintegrators include Veegum HV, methyl cellulose, agar, bentonite, cellulose and wood products, natural sponge, cation-exchange resins, alginic acid, guar gum, citrus pulp, cross-linked polyvinylpyrrolidone, carboxymethylcellulose and the like.
The term "dispersibility" or "dispersible" means a dry powder having a moisture content of less than about 10% by weight (%w) water, usually below about 5%w and preferably less than about 3%w; a particle size of about 1.0-5.0 µm mass median diameter (MMD), usually 1.0-4.0 µm MMD, and preferably 1.0-3.0 µm MMD; a delivered dose of about >30%, usually >40%, preferably >50%, and most preferred >60%; and an aerosol particle size distribution of 1.0-5.0 µm mass median aerodynamic diameter (MMAD), usually 1.5-4.5 µm MMAD, and preferably 1.5-4.0 µm MMAD.

The term "dry" means that the composition has a moisture content such that the particles are readily dispersible in an inhalation device to form an aerosol. This moisture content is generally below about 10% by weight (%w) water, usually below about 5%w and preferably less than about 3%w.

As used herein, "effective amount" means an amount of a melanocortin or pharmacologically active agent that is sufficient to provide the desired local or systemic effect and performance at a reasonable benefit/risk ratio attending any medical treatment.

As used herein, "flavoring agents" vary considerably in their chemical structure, ranging from simple esters, alcohols, and aldehydes to carbohydrates and complex volatile oils. Synthetic flavors of almost any desired type are now available.

As used herein, the terms "fragment of a Tf protein" or "Tf protein," or "portion of a Tf protein" refer to an amino acid sequence comprising at least about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99% or 100% of a naturally occurring Tf protein or mutant thereof.

As used herein, the term "gene" refers to any segment of DNA associated with a biological function. Thus, genes include, but are not limited to, coding sequences and/or the regulatory sequences required for their expression. Genes can also include non-expressed DNA segments that, for example, form recognition sequences for other proteins. Genes can be obtained from a variety of sources, including cloning from a source of interest or synthesizing from known or predicted sequence information, and may include sequences designed to have desired parameters.

As used herein, a "heterologous polynucleotide" or a "heterologous nucleic acid" or a "heterologous gene" or a "heterologous sequence" or an "exogenous DNA segment" refers to a polynucleotide, nucleic acid or DNA segment that originates from a source foreign to the particular host cell, or, if from the same source, is modified from its original form. A heterologous gene in a host cell includes a gene that is endogenous to the particular host cell, but has been modified. Thus, the terms refer to a DNA segment which is foreign or heterologous to the cell, or homologous to the cell but in a position within the host cell.
nucleic acid in which the element is not ordinarily found. As an example, a signal sequence native to a yeast cell but attached to a human Tf sequence is heterologous.

As used herein, an "isolated" nucleic acid sequence refers to a nucleic acid sequence which is essentially free of other nucleic acid sequences, e.g., at least about 20% pure, preferably at least about 40% pure, more preferably about 60% pure, even more preferably about 80% pure, most preferably about 90% pure, and even most preferably about 95% pure, as determined by methods known in the art, for instance, by agarose gel electrophoresis. For example, an isolated nucleic acid sequence can be obtained by standard cloning procedures used in genetic engineering to relocate the nucleic acid sequence from its natural location to a different site where it will be reproduced. The cloning procedures may involve excision and isolation of a desired nucleic acid fragment comprising the nucleic acid sequence encoding the polypeptide, insertion of the fragment into a vector molecule, and incorporation of the recombinant vector into a host cell where multiple copies or clones of the nucleic acid sequence will be replicated. The nucleic acid sequence may be of genomic, cDNA, RNA, semi-synthetic, synthetic origin or any combinations thereof.

As used herein, two or more DNA coding sequences are said to be "joined" or "fused" when, as a result of in-frame fusions between the DNA coding sequences, the DNA coding sequences are translated into a fusion polypeptide. The term "fusion" in reference to the fusion proteins of the invention includes, but is not limited to, attachment of at least one melanocortin peptide, melanocortin variant or melanocortin receptor agonist or fragment thereof to the N-terminal end of Tf, attachment to the C-terminal end of Tf, and/or insertion between any two amino acids within Tf.

As used herein, "lubricants" are materials that perform a number of functions in tablet manufacture, such as improving the rate of flow of the tablet granulation, preventing adhesion of the tablet material to the surface of the dies and punches, reducing interparticle friction, and facilitating the ejection of the tablets from the die cavity. Commonly used lubricants include talc, magnesium stearate, calcium stearate, stearic acid, and hydrogenated vegetable oils. Typical amounts of lubricants range from about 0.1% by weight to about 5% by weight.

As used herein, "modified transferrin" as used herein refers to a transferrin molecule that exhibits at least one modification of its amino acid sequence, compared to wild-type transferrin.

As used herein, "modified transferrin fusion protein" as used herein refers to a protein formed by the fusion of at least one molecule of modified transferrin (or a fragment or variant
to at least one molecule of a melanocortin peptide or melanocortin receptor agonist (or fragment or variant thereof).

As used herein, the terms "nucleic acid" or "polynucleotide" refer to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. Unless specifically limited, the terms encompass nucleic acids containing analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al., 1991, Nucleic Acid Res. 19:5081; Ohtsuka et al., 1985, J. Biol. Chem. 260:2605-2608; Cassol et al., 1992; and Rossolini et al., 1994, Mol. Cell. Probes 8:91-98). The term nucleic acid is used interchangeably with gene, cDNA, and mRNA encoded by a gene.

As used herein, a DNA segment is referred to as "operably linked" when it is placed into a functional relationship with another DNA segment. For example, DNA for a signal sequence is operably linked to DNA encoding a fusion protein of the invention if it is expressed as a preprotein that participates in the secretion of the fusion protein; a promoter or enhancer is operably linked to a coding sequence if it stimulates the transcription of the sequence. Generally, DNA sequences that are operably linked are contiguous, and in the case of a signal sequence or fusion protein both contiguous and in reading phase. However, enhancers need not be contiguous with the coding sequences whose transcription they control. Linking, in this context, is accomplished by ligation at convenient restriction sites or at adapters or linkers inserted in lieu thereof.

As used herein, "pharmaceutically acceptable" refers to materials and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. Typically, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans.
As used herein, "physiologically effective amount" is that amount delivered to a subject to give the desired palliative or curative effect. This amount is specific for each drug and its ultimate approved dosage level.

As used herein, "potency" refers to the ability of melanocortin or a melanocortin receptor agonist or other peptide moiety of the invention to activate one or more melanocortin receptors, for instance, one or more of melanocortin 1 receptor (MC1R), melanocortin 2 receptor (MC2R), melanocortin 3 receptor (MC3R), melanocortin 4 receptor (MC4R) and melanocortin 5 receptor (MC5R). For instance, a melanocortin and Tf fusion protein of the invention can exhibit a potency of at least about 1 fold, at least about 2 fold, at least about 3 fold, at least about 4 fold, at least about 5 fold, at least about 6 fold, at least about 7 fold, at least about 8 fold, at least about 9 fold, at least about 10 fold, at least about 20 fold, or at least about 50 fold or more compared to the corresponding unfused melanocortin peptide or melanocortin receptor agonist. In one embodiment of the invention, a fusion protein comprising a melanocortin peptide or melanocortin receptor agonist, a substantially non-helical linker peptide and transferrin can exhibit a potency of at least about 1 fold, at least about 2 fold, at least about 3 fold, at least about 4 fold, at least about 5 fold, at least about 6 fold, at least about 7 fold, at least about 8 fold, at least about 9 fold, at least about 10 fold, at least about 20 fold, or at least about 50 fold or more compared to the corresponding fusion protein without a substantially non-helical linker or compared to a fusion protein with a flexible linker.

As used herein, the term "powder" means a composition that consists of finely dispersed solid particles that are free flowing and capable of being readily dispersed in an inhalation device and subsequently inhaled by a subject so that the particles reach the lungs to permit penetration into the alveoli. Thus, the powder is said to be "respirable." Preferably the average particle size is less than about 10 microns (µm) in diameter with a relatively uniform spheroidal shape distribution. More preferably the diameter is less than about 7.5 µm and most preferably less than about 5.0 µm. Usually the particle size distribution is between about 0.1 µm and about 5 µm in diameter, particularly about 0.3 µm to about 5 µm.

As used herein, "productivity" of expression of the fusion protein refers to the ability of the protein to be expressed in a host cell system. For instance, the melanocortin and Tf fusion protein with a substantially non-helical linker can be expressed in a yeast cell. As used herein, "substantially increase productivity" means that expression of the fusion protein in the host is increased at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 100%, at least about 200%, or at least about 300% or
more compared to the expression of a similar fusion protein lacking the linker or a similar fusion protein with a flexible polypeptide linker.

As used herein, the term "promoter" refers to a region of DNA involved in binding RNA polymerase to initiate transcription.

As used herein, the term "recombinant" refers to a cell, tissue or organism that has undergone transformation with a new combination of genes or DNA.

As used herein, the term "serum half-life" or "plasma half-life" refers to the time required for the in vivo serum melanocortin or melanocortin receptor agonist concentration to decline by 50%. The shorter the serum half-life of melanocortin or a melanocortin receptor agonist, the shorter will be the period that the protein can exert a therapeutic effect. For instance, a melanocortin and Tf fusion peptide, with or without a substantially non-helical linker, exhibits extended serum half-life if it exhibits a measurable increase in half-life including, at least about 5 fold, at least about 10 fold, at least about 50 fold, at least about 100 fold, at least about 200 fold, at least about 300 fold, at least about 400 fold, at least about 500 fold, at least about 600 fold, at least about 700 fold, at least about 800 fold, at least about 900 fold, at least about 1,000 fold, at least about 5,000 fold, at least about 10,000, at least about 25,000 fold, at least about 50,000 fold, at least about 75,000 fold, or at least about 100,000 fold increase or more in serum half-life compared to the similar melanocortin peptide or melanocortin receptor agonist in its unfused state.

As used herein, the term "subject" can be a human, a mammal, or an animal. The subject being treated is a patient in need of treatment.

As used herein, the term "substantially non-helical linker" or "rigid linker" refers to a linker that physically separates the melanocortin and transferrin moieties of a fusion protein. "Substantially non helical" means that linker peptide exhibits little or no helical or spiral shape or secondary structure. For instance, a substantially non-helical structure can comprise less than about 20% helical or spiral shape or secondary structure. A typical alpha-helical peptide is right-handed (twists in a clockwise direction), comprises the amino acid R groups extending to the outside of the helix, the helix making a complete turn at every 3.6 amino acids and the carbonyl group of each peptide bond extends parallel to the axis of the helix and points directly at the N-H group of the peptide bond 4 amino acids below it in the helix with a hydrogen bond forming between them. The non-helical linkers typically contain at least about 5%, at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, or about 100% amino acids which disrupt any alpha-helix formation, such as amino acids that induce kinks in the polypeptide chain.
Kinks can be introduced into a naturally occurring peptide by modifying amino acid residues. Amino acids which cause kinks in the polypeptide chain include, for instance, proline and glycine amino acid residues. For example, the addition of a proline or a glycine at or about the middle of a straight α helical barrel will cause the protein to bend, i.e., kink. The introduction of a proline residue will generally cause a greater kink than the introduction of a glycine residue. As can be appreciated by a skilled artisan, the introduction of a proline or glycine residue anywhere in a linker peptide can cause a kink. A linker peptide can contain one or more amino acid residues which induce kinks. For instance, a substantially non-helical linker can have at least about 5% proline content, at least about 10% proline content, at least about 20% proline content, at least about 30% proline content, at least about 40% proline content, at least about 50% proline content, at least about 60% proline content, at least about 70% proline content, at least about 80% proline content, at least about 90% proline content, at least about 95% proline content or about 100% proline content.

Substantially non-helical linkers include, but are not limited to, PEAPTD (SEQ ID NO.: 13), PEAPTDPEAPTD (SEQ ID NO.: 10), PEAPTDPEAPTD (SEQ ID NO.: 14), IgG hinge (SEQ ID NO.: 88, 89, and 117), PEAPTD + IgG hinge (SEQ ID NOS.: 118-123 and 126-129), PPPPPPPPPPPP (SEQ ID NO.: 17), GEAPTDPEAPTD (SEQ ID NO.: 18), PEAGTDPEAPTD (SEQ ID NO.: 19), PEAPTDGEAPTD (SEQ ID NO.: 20), PEAPTDPEAGTD (SEQ ID NO.: 21), PQAPTNPQAPT (SEQ ID NO.: 22), and PEAPDPEAPEA (SEQ ID NO.: 23). Typically, substantially non-helical linkers have at least about 5, at least about 6, at least about 7, at least about 8, at least about 9, at least about 10, at least about 11, at least about 12, at least about 13, at least about 14, at least about 15, at least about 16, at least about 17, at least about 18, at least about 19, at least about 20, or at least about 21 or more amino acids. However, there is no upper limit on linker length.

As used herein, "tablets" are solid pharmaceutical dosage forms containing drug substances with or without suitable diluents and prepared either by compression or molding methods well known in the art. Tablets have been in widespread use since the latter part of the 19th century and their popularity continues. Tablets remain popular as a dosage form because of the advantages afforded both to the manufacturer (e.g., simplicity and economy of preparation, stability, and convenience in packaging, shipping, and dispensing) and the patient (e.g., accuracy of dosage, compactness, portability, blandness of taste, and ease of administration). Although tablets are most frequently discoid in shape, they may also be round, oval, oblong, cylindrical or triangular. They may differ greatly in size and weight depending on the amount of drug substance present and the intended method of
administration. They are divided into two general classes: (1) compressed tablets and (2) molded tablets or tablet triturates. In addition to the active or therapeutic ingredient or ingredients, tablets contain a number or inert materials or additives. A first group of such additives includes those materials that help to impart satisfactory compression characteristics to the formulation, including diluents, binders and lubricants. A second group of such additives helps to give additional desirable physical characteristics to the finished tablet, such as disintegrators, colors, flavors and sweetening agents.

As used herein, the term "therapeutically effective amount" refers to that amount of the fusion protein comprising a melanocortin peptide or melanocortin receptor agonist which, when administered to a subject in need thereof, is sufficient to effect treatment. The amount of fusion protein which constitutes a "therapeutically effective amount" will vary depending on the therapeutic protein used, the severity of the condition or disease, and the age and body weight of the subject to be treated, but can be determined routinely by one of ordinary skill in the art having regard to his/her own knowledge and to this disclosure.

As used herein, "therapeutic protein" or "therapeutic molecule" refers to melanocortin, melanocortin fragments or variants or analogs thereof, melanocortin receptor agonists or melanocortin receptor agonist fragments having one or more therapeutic and/or biological activities. The terms peptides, proteins, and polypeptides are used interchangeably herein. As used herein, a polypeptide displaying a "therapeutic activity" or a protein that is "therapeutically active" is a melanocortin, melanocortin fragment or a variant or analog thereof or a melanocortin receptor agonist, melanocortin receptor antagonist or a melanocortin receptor binding peptide or fragment or variant or analog thereof that possesses one or more known biological and/or therapeutic activities associated with melanocortin such as described herein or otherwise known in the art. A "therapeutic protein" is a melanocortin protein, analog, or melanocortin receptor agonist or other peptide moiety described herein that is useful to treat, prevent or ameliorate a disease, condition or disorder. Such a disease, condition or disorder may be in humans or in a non-human animal, i.e., veterinary use.

As used herein, the term "transformation" refers to the transfer of nucleic acid, i.e., a nucleotide polymer, into a cell. As used herein, the term "genetic transformation" refers to the transfer and incorporation of DNA, especially recombinant DNA, into a cell.

As used herein, the term "transformant" refers to a cell, tissue or organism that has undergone transformation.

As used herein, the term "transgene" refers to a nucleic acid that is inserted into an organism, host cell or vector in a manner that ensures its function.
As used herein, the term "transgenic" refers to cells, cell cultures, organisms, bacteria, fungi, animals, plants, and progeny of any of the preceding, which have received a foreign or modified gene and in particular a gene encoding a melanocortin and transferrin fusion protein by one of the various methods of transformation, wherein the foreign or modified gene is from the same or different species than the species of the organism receiving the foreign or modified gene.

"Variants or variant" refers to a polynucleotide or nucleic acid differing from a reference nucleic acid or polypeptide, but retaining essential properties thereof. Generally, variants are overall closely similar, and, in many regions, identical to the reference nucleic acid or polypeptide. As used herein, "melanocortin variant" or "melanocortin analog" refers to a melanocortin peptide, i.e., moiety, of a fusion protein of the invention, differing in sequence from a native therapeutic protein but retaining at least one functional and/or therapeutic property thereof as described elsewhere herein or otherwise known in the art.

As used herein, the term "vector" refers broadly to any plasmid, phagemid or virus encoding an exogenous nucleic acid. The term is also be construed to include non-plasmid, non-phagemid and non-viral compounds which facilitate the transfer of nucleic acid into virions or cells, such as, for instance, polyllysine compounds and the like. The vector may be a viral vector that is suitable as a delivery vehicle for delivery of the nucleic acid, or mutant thereof, to a cell, or the vector may be a non-viral vector which is suitable for the same purpose. Examples of viral and non-viral vectors for delivery of DNA to cells and tissues are well known in the art and are described, for example, in Ma et al., 1997, Proc. Natl. Acad. Sci. U.S.A. 94:12744-12746. Examples of viral vectors include, but are not limited to, a recombinant vaccinia virus, a recombinant adenovirus, a recombinant retrovirus, a recombinant adeno-associated virus, a recombinant avian pox virus, and the like (Cranage et al., 1986, EMBO J. 5:3057-3063; International Patent Application No. WO 94/17810, published August 18, 1994; International Patent Application No. WO 94/23744, published October 27, 1994). Examples of non-viral vectors include, but are not limited to, liposomes, polyamine derivatives of DNA and the like.

As used herein, the term "wild type" refers to a polynucleotide or polypeptide sequence that is naturally occurring.

**Transferrin and Transferrin Modifications**

The present invention provides fusion proteins comprising a melanocortin peptide, melanocortin analog, a melanocortin receptor agonist or fragment thereof and transferrin or modified transferrin. Any transferrin may be used to make the fusion proteins of the
invention. In one embodiment, the fusion protein also includes a linker, for instance, a substantially non-helical linker.

Any transferrin may be used to make the fusion proteins of the invention. As an example, the wild-type human Tf is a 679 amino acid protein of approximately 75kDa (not accounting for glycosylation), with two main lobes or domains, N (about 330 amino acids) and C (about 340 amino acids), which appear to originate from a gene duplication. See GenBank accession numbers NM_001063, XM_002793, M12530, XM_039845, XM_039847 and S95936, all of which are herein incorporated by reference in their entirety, as well as SEQ ID NOs: 1, 2 and 3. The two domains have diverged over time but retain a large degree of identity/similarity.

Each of the N and C domains is further divided into two subdomains, N1 and N2, C1 and C2. The function of Tf is to transport iron to the cells of the body. This process is mediated by the Tf receptor (TfR), which is expressed on all cells, particularly actively growing cells. TfR recognizes the iron bound form of Tf (two molecules of which are bound per receptor), endocytosis then occurs whereby the TfR/Tf complex is transported to the endosome, at which point the localized drop in pH results in release of bound iron and the recycling of the TfR/Tf complex to the cell surface and release of Tf (known as apoTf in its iron-unbound form). Receptor binding is through the C domain of Tf. The two glycosylation sites in the C domain do not appear to be involved in receptor binding as unglycosylated iron bound Tf does bind the receptor.

Each Tf molecule can carry two iron ions (Fe³⁺). These are complexed in the space between the N1 and N2, C1 and C2 sub domains resulting in a conformational change in the molecule. Tf crosses the blood brain barrier (BBB) via the Tf receptor.

In human transferrin, the iron binding sites comprise at least amino acids Asp 63 (Asp 82 of SEQ ID NO: 2 which includes the native Tf signal sequence), Asp 392 (Asp 411 of SEQ ID NO: 2), Tyr 95 (Tyr 114 of SEQ ID NO: 2), Tyr 426 (Tyr 445 of SEQ ID NO: 2), Tyr 188 (Tyr 207 of SEQ ID NO: 2), Tyr 514 or 517 (Tyr 533 or 536 SEQ ID NO: 2), His 249 (His 268 of SEQ ID NO: 2), and His 585 (His 604 of SEQ ID NO: 2) of SEQ ID NO: 3. The hinge regions comprise at least N domain amino acid residues 94-96, 245-247 and/or 316-318 as well as C domain amino acid residues 425-427, 581-582 and/or 652-658 of SEQ ID NO: 3. The carbonate binding sites comprise at least amino acids Thr 120 (Thr 139 of SEQ ID NO: 2), Thr 452 (Thr 471 of SEQ ID NO: 2), Arg 124 (Arg 143 of SEQ ID NO: 2), Arg 456 (Arg 475 of SEQ ID NO: 2), Ala 126 (Ala 145 of SEQ ID NO: 2), Ala 458 (Ala 477 of SEQ ID NO: 2), Gly 127 (Gly 146 of SEQ ID NO: 2), and Gly 459 (Gly 478 of SEQ ID NO: 2) of SEQ ID NO: 3.
Any animal Tf molecule may be used to produce the fusion proteins of the invention, including human Tf variants, cow, pig, sheep, dog, rabbit, rat, mouse, hamster, echinida, platypus, chicken, frog, hornworm, monkey, as well as other bovine, canine and avian species. All of these Tf sequences are readily available in GenBank and other public databases. In one embodiment of the invention, the fusion protein includes a modified human transferrin.

The human Tf nucleotide sequence is available (see SEQ ID NOS 1, 2 and 3 and the accession numbers described above) and can be used to make genetic fusions between Tf or a domain of Tf and the therapeutic molecule of choice. Fusions may also be made from related molecules such as lacto transferrin (lactoferrin; GenBank Ace: NM_002343) or melanotransferrin (GenBank Ace. NM_013900; murine melanotransferrin).

Melanotransferrin is a glycosylated protein found at high levels in malignant melanoma cells and was originally named human melanoma antigen p97 (Brown et al., 1982, Nature, 296: 171-173). It possesses high sequence homology with human serum transferrin, human lactoferrin and chicken transferrin (Brown et al., 1982, Nature, 296: 171-173; Rose et al., Proc. Natl. Acad. Sci. USA, 1986, 83: 1261-1265). However, unlike these receptors, no cellular receptor has been identified for melanotransferrin. Melanotransferrin reversibly binds iron and it exists in two forms, one of which is bound to cell membranes by a glycosyl phosphatidylinositol anchor while the other form is both soluble and actively secreted (Baker et al., 1992, FEBS Lett, 298: 215-218; Alemany et al., 1993, J. Cell Sci., 104: 1155-1162; Food et al., 1994, J. Biol. Chem. 274: 701 1-7017).

Lactoferrin (Lf), a natural defense iron-binding protein, has been found to possess antibacterial, antiviral, antineoplastic and anti-inflammatory activity. The protein is present in exocrine secretions that are commonly exposed to normal flora: milk, tears, nasal exudate, saliva, bronchial mucus, gastrointestinal fluids, cervico-vaginal mucus and seminal fluid. Additionally, Lf is a major constituent of the secondary specific granules of circulating polymorphonuclear neutrophils (PMNs). The apoprotein is released on degranulation of the PMNs in septic areas. A principal function of Lf is that of scavenging free iron in fluids and inflamed areas so as to suppress free radical-mediated damage and decrease the availability of the metal to invading microbial and neoplastic cells. In a study that examined the turnover rate of $^{125}$I Lf in adults, it was shown that Lf is rapidly taken up by the liver and spleen, and the radioactivity persisted for several weeks in the liver and spleen (Bennett et al., 1979, Clin. Sci. (Lond.) 57: 453-460).

In one embodiment, the transferrin portion of the transferrin fusion protein of the invention includes a transferrin splice variant. In one example, a transferrin splice variant
be a splice variant of human transferrin. In one specific embodiment, the human transferrin splice variant can be that of Genbank Accession AAA61 140.

In another embodiment, the transferrin portion of the transferrin fusion protein of the invention includes a lactoferrin splice variant. In one example, a human serum lactoferrin splice variant can be a novel splice variant of a neutrophil lactoferrin. In one specific embodiment, the neutrophil lactoferrin splice variant can be that of Genbank Accession AAA59479. In another specific embodiment, the neutrophil lactoferrin splice variant can comprise the following amino acid sequence EDCIALKGEADA (SEQ ID NO: 5), which includes the novel region of splice-variance.

In another embodiment, the transferrin portion of the transferrin fusion protein of the invention includes a melanotransferrin peptide or variant or melanocortin receptor agonist or antagonist or fragment thereof.

Fusion proteins of the invention may be made with any Tf protein, fragment, domain or engineered domain. For instance, fusion proteins may be produced using the full-length Tf sequence, with or without the native Tf signal sequence. The fusion proteins may also be made using a single Tf domain, such as an individual N or C domain or a modified form of Tf comprising 2N or 2C domains (see U.S. Provisional Application 60/406,977, filed August 30, 2002, which is herein incorporated by reference in its entirety). In some embodiments, fusions of melanocortin or a melanocortin receptor agonist to a single C domain may be produced, wherein the C domain is altered to reduce, inhibit or prevent glycosylation. In other embodiments, the use of a single N domain is advantageous as the Tf glycosylation sites reside in the C domain and the N domain, on its own. In yet another embodiment, the fusion protein contains a single N domain which is expressed at a high level.

As used herein, a C terminal domain or lobe modified to function as an N-like domain is modified to exhibit glycosylation patterns or iron binding properties substantially like that of a native or wild-type N domain or lobe. In a preferred embodiment, the C domain or lobe is modified so that it is not glycosylated and does not bind iron by substitution of the relevant C domain regions or amino acids to those present in the corresponding regions or sites of a native or wild-type N domain.

As used herein, a Tf moiety comprising "two N domains or lobes" includes a Tf molecule that is modified to replace the native C domain or lobe with a native or wild-type N domain or lobe or a modified N domain or lobe or contains a C domain that has been modified to function substantially like a wild-type or modified N domain.

Analysis of the two domains by overlay of the two domains (Swiss PDB Viewer 3.7b2, Iterative Magic Fit) and by direct amino acid alignment (ClustalW multiple alignment)
reveals that the two domains have diverged over time. Amino acid alignment shows 42% identity and 59% similarity between the two domains. However, approximately 80% of the N domain matches the C domain for structural equivalence. The C domain also has several extra disulfide bonds compared to the N domain.

Alignment of molecular models for the N and C domain reveals the following structural equivalents:

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</thead>
<tbody>
<tr>
<td>C domain (340-679)</td>
<td>34-0</td>
<td>36-5</td>
<td>41-7</td>
<td>43-9</td>
<td>47-1</td>
<td>47-9</td>
<td>49-7</td>
<td>50-5</td>
<td>54-7</td>
<td>55-5</td>
<td>59-5</td>
<td>60-5</td>
<td>61-4</td>
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<td>66-3</td>
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The disulfide bonds for the two domains align as follows:

<table>
<thead>
<tr>
<th>N</th>
<th>C</th>
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<tr>
<td>C339-C596</td>
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<tr>
<td>C9-C48</td>
<td>C345-C377</td>
</tr>
<tr>
<td>C19-C39</td>
<td>C355-C368</td>
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<td>C402-C674</td>
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<td></td>
<td>C418-C637</td>
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<tr>
<td>C118-C194</td>
<td>C450-C523</td>
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<tr>
<td>C137-C331</td>
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<td></td>
<td>C474-C665</td>
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<tr>
<td>C158-C174</td>
<td>C484-C498</td>
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<tr>
<td>C161-C179</td>
<td></td>
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<tr>
<td>C171-C177</td>
<td>C495-C506</td>
</tr>
<tr>
<td>C227-C241</td>
<td>C563-C577</td>
</tr>
<tr>
<td></td>
<td>C615-C620</td>
</tr>
</tbody>
</table>

**Bold** = aligned disulfide bonds  
**Italics** = bridging peptide
In one embodiment, the transferrin portion of the fusion protein includes at least two N terminal lobes of transferrin. In further embodiments, the transferrin portion of the transferrin fusion protein includes at least two N terminal lobes of transferrin derived from human serum transferrin.

In another embodiment, the transferrin portion of the fusion protein includes, comprises, or consists of at least two N terminal lobes of transferrin having a mutation in at least one amino acid residue selected from the group consisting of Asp63, Gly65, Tyr95, TyM88, and His249 of SEQ ID NO: 3.

In another embodiment, the transferrin portion of the fusion protein includes a recombinant human serum transferrin N-terminal lobe mutant having a mutation at Lys206 or His207 of SEQ ID NO: 3.

In another embodiment, the transferrin portion of the fusion protein includes, comprises, or consists of at least two C terminal lobes of transferrin. In further embodiments, the transferrin portion of the fusion protein includes at least two C terminal lobes of transferrin derived from human serum transferrin.

In a further embodiment, the C terminal lobe mutant further includes a mutation of at least one of Asn413 and Asn611 of SEQ ID NO: 3 which does not allow glycosylation.

In another embodiment, the transferrin portion of fusion protein includes at least two C terminal lobes of transferrin having a mutation in at least one amino acid residue selected from the group consisting of Asp392, Tyr426, Tyr514, Tyr517 and His585 of SEQ ID NO: 3, wherein the mutant retains the ability to bind metal. In an alternate embodiment, the transferrin portion of the fusion protein includes at least two C terminal lobes of transferrin having a mutation in at least one amino acid residue selected from the group consisting of Tyr426, Tyr514, Tyr517 and His585 of SEQ ID NO: 3, wherein the mutant has a reduced ability to bind metal. In another embodiment, the transferrin portion of the fusion protein includes at least two C terminal lobes of transferrin having a mutation in at least one amino acid residue selected from the group consisting of Asp392, Tyr426, Tyr517 and His585 of SEQ ID NO:3, wherein the mutant does not retain the ability to bind metal and functions substantially like an N domain.

In some embodiments, the Tf or Tf portion will be of sufficient length to increase the in vivo circulatory half-life, serum stability, in vitro solution stability or bioavailability of the melanocortin peptide or melanocortin receptor agonist or other peptide moiety of the invention to the in vivo circulatory half-life, serum stability, in vitro solution stability or bioavailability of the melanocortin peptide or melanocortin receptor agonist in an unfused state. Such an increase in stability, serum half-life or bioavailability may be about 5 fold, 10
fold, 50 fold, 100 fold, 200 fold, 300 fold, 400 fold, 500 fold, 600 fold, 700 fold, 800 fold, 900 fold, 1,000 fold, 5,000 fold, 10,000, 25,000 fold, 50,000 fold, 75,000 fold, or 100,000 fold or more compared to the unfused molecule. In some cases, the fusion proteins comprising transferrin and melanocortin and optionally a linker (e.g., substantially rigid linker) exhibit a serum half-life of about 3-6 hours, about 6-12 hours, about 12-24 hours, about 18-24 hours, about 1 day, about 30 hours, about 38 hours, about 40 hours, about 42 hours, about 45 hours, about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, about 7 days, about 8 days, about 9 days, about 10-20 or more days, about 12-18 days or about 14-17 days.

When the C domain of Tf is part of the fusion protein, the two N-linked glycosylation sites, amino acid residues corresponding to N413 and N611 of SEQ ID NO: 3 may be mutated for expression in a yeast system to prevent glycosylation or hypermannosylationn and extend the serum half-life of the fusion protein and/or therapeutic protein (to produce asialo-, or in some instances, monosialo-Tf or disialo-Tf). In addition to Tf amino acids corresponding to N413 and N611, mutations may be to the adjacent residues within the N-X-S/T glycosylation site to prevent or substantially reduce glycosylation. See U.S. Patent 5,986,067 of Funk et al. It has also been reported that the N domain of Tf expressed in Pichia pastoris becomes O-linked glycosylated with a single hexose at S32 which also may be mutated or modified to prevent such glycosylation.

Accordingly, in one embodiment of the invention, the fusion protein includes a modified transferrin molecule wherein the transferrin exhibits reduced glycosylation, including but not limited to asialo- monosialo- and disialo- forms of Tf. In another embodiment, the transferrin portion of the fusion protein includes a recombinant transferrin mutant that is mutated to prevent glycosylation. In another embodiment, the transferrin portion of the fusion protein includes a recombinant transferrin mutant that is fully glycosylated. In a further embodiment, the transferrin portion of the fusion protein includes a recombinant human serum transferrin mutant that is mutated to prevent N-linked glycosylation, wherein at least one of Asn413 and Asn611 of SEQ ID NO: 3 are mutated to an amino acid which does not allow glycosylation. In another embodiment, the transferrin portion of the transferrin fusion protein includes a recombinant human serum transferrin mutant that is mutated to prevent or substantially reduce glycosylation, wherein mutations may be to the adjacent residues within the N-X-S/T glycosylation site, for instance mutation of the S/T residues. Moreover, glycosylation may be reduced or prevented by mutating the serine or threonine residue. Further, changing the X to proline is known to inhibit glycosylation.
As discussed below in more detail, fusion proteins of the invention may also be engineered to not bind iron and/or bind the Tf receptor. In other embodiments of the invention, the iron binding is retained and the iron binding ability of Tf may be used to deliver a melanocortin peptide or melanocortin receptor agonist to the inside of a cell, across an epithelial or endothelial cell membrane and/or across the BBB. These embodiments that bind iron and/or the Tf receptor will often be engineered to reduce or prevent glycosylation to extend the serum half-life of the melanocortin peptide or melanocortin receptor agonist. The N domain alone will not bind to TfR when loaded with iron, and the iron bound C domain will bind TfR but not with the same affinity as the whole molecule.

In another embodiment, the transferrin portion of the fusion protein includes a recombinant transferrin mutant having a mutation wherein the mutant does not retain the ability to bind metal ions. In an alternate embodiment, the transferrin portion of the fusion protein includes a recombinant transferrin mutant having a mutation wherein the mutant has a weaker binding affinity for metal ions than wild-type serum transferrin. In an alternate embodiment, the transferrin portion of the fusion protein includes a recombinant transferrin mutant having a mutation wherein the mutant has a stronger binding affinity for metal ions than wild-type serum transferrin.

In another embodiment, the transferrin portion of the fusion protein includes a recombinant transferrin mutant having a mutation wherein the mutant does not retain the ability to bind to the transferrin receptor. For instance, the melanocortin and Tf fusion proteins of the invention may bind a cell surface melanocortin receptor but not a Tf receptor. Such fusion proteins can be therapeutically active at the cell surface, i.e., by not by entering the cell.

Such modifications can also be used to prevent the fusion protein from crossing the blood brain barrier. This may be especially for treatment of conditions affected, at least in part, by the interaction of a melanocortin peptide or melanocortin receptor agonist and a peripheral melanocortin receptor. Without wishing to be bound by any particular theory, it is believed that by not crossing the blood brain barrier, the effects of endogenous receptor antagonists that act on the nervous system are avoided or reduced.

In an alternate embodiment, the transferrin portion of the fusion protein includes a recombinant transferrin mutant having a mutation wherein the mutant has a weaker binding affinity for the transferrin receptor than wild-type serum transferrin. In an alternate embodiment, the transferrin portion of the fusion protein includes a recombinant transferrin mutant having a mutation wherein the mutant has a stronger binding affinity for the transferrin receptor than wild-type serum transferrin.
In another embodiment, the transferrin portion of the protein includes a recombinant transferrin mutant having a mutation wherein the mutant does not retain the ability to bind to carbonate ions. In an alternate embodiment, the transferrin portion of the fusion protein includes a recombinant transferrin mutant having a mutation wherein the mutant has a weaker binding affinity for carbonate ions than wild-type serum transferrin. In an alternate embodiment, the transferrin portion of the fusion protein includes a recombinant transferrin mutant having a mutation wherein the mutant has a stronger binding affinity for carbonate ions than wild-type serum transferrin.

In another embodiment, the transferrin portion of the fusion protein includes a recombinant human serum transferrin mutant having a mutation in at least one amino acid residue selected from the group consisting of Asp63, Gly65, Tyr95, TyM88, His249, Asp392, Tyr426, Tyr514, Tyr517 and His585 of SEQ ID NO: 3, wherein the mutant retains the ability to bind metal ions. In an alternate embodiment, a recombinant human serum transferrin mutant having a mutation in at least one amino acid residue selected from the group consisting of Asp63, Gly65, Tyr95, Tyr188, His249, Asp392, Tyr426, Tyr514, Tyr517 and His585 of SEQ ID NO: 3, wherein the mutant has a reduced ability to bind metal ions. In another embodiment, a recombinant human serum transferrin mutant having a mutation in at least one amino acid residue selected from the group consisting of Asp63, Gly65, Tyr95, TyM88, His249, Asp392, Tyr426, Tyr517 and His585 of SEQ ID NO: 3, wherein the mutant does not retain the ability to bind metal ions.

In another embodiment, the transferrin portion of the fusion protein includes a recombinant human serum transferrin mutant having a mutation at Lys206 or His207 of SEQ ID NO:3, wherein the mutant has a stronger binding affinity for metal ions than wild-type human serum transferrin (see U.S. Patent 5,986,067, which is herein incorporated by reference in its entirety). In an alternate embodiment, the transferrin portion of the fusion protein includes a recombinant human serum transferrin mutant having a mutation at Lys206 or His207 of SEQ ID NO:3, wherein the mutant has a weaker binding affinity for metal ions than wild-type human serum transferrin. In a further embodiment, the transferrin portion of the fusion protein includes a recombinant human serum transferrin mutant having a mutation at Lys206 or His207 of SEQ ID NO:3, wherein the mutant does not bind metal ions.

Any available technique may be used to produce the fusion proteins of the invention, including, but not limited to, molecular techniques commonly available, for instance, those disclosed in Sambrook et al. Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, 1989. When carrying out nucleotide substitutions using techniques for accomplishing site-specific mutagenesis that are well known in the art, the encoded amino acid changes are preferably of a minor nature, that is, conservative amino acids.
acid substitutions, although other, non-conservative, substitutions are contemplated as well, particularly when producing a modified transferrin portion of a fusion protein, e.g., a modified Tf protein exhibiting reduced glycosylation, reduced iron binding and the like. Specifically contemplated are amino acid substitutions, small deletions or insertions, typically of one to about 30 amino acids; insertions between transferrin domains; small amino- or carboxy-terminal extensions, such as an amino-terminal methionine residue, or small linker peptides of less than 50, 40, 30, 20 or 10 residues between transferrin domains or linking a transferrin protein and melanocortin peptide or a small extension that facilitates purification, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative amino acid substitutions are substitutions made within the same group such as within the group of basic amino acids (such as arginine, lysine, histidine), acidic amino acids (such as glutamic acid and aspartic acid), polar amino acids (such as glutamine and asparagine), hydrophobic amino acids (such as leucine, isoleucine, valine), aromatic amino acids (such as phenylalanine, tryptophan, tyrosine) and small amino acids (such as glycine, alanine, serine, threonine, methionine).

Non-conservative substitutions encompass substitutions of amino acids in one group by amino acids in another group. For example, a non-conservative substitution would include the substitution of a polar amino acid for a hydrophobic amino acid. For a general description of nucleotide substitution, see e.g. Ford et al. (1991), Prot. Exp. Pur. 2: 95-107. Non-conservative substitutions, deletions and insertions are particularly useful to produce fusion proteins of the invention that exhibit no or reduced binding of iron, no or reduced binding of the fusion protein to the Tf receptor and/or no or reduced glycosylation.

Iron binding and/or receptor binding may be reduced or disrupted by mutation, including deletion, substitution or insertion into, amino acid residues corresponding to one or more of Tf N domain residues Asp63, Tyr95, Tyr188, His249 and/or C domain residues Asp392, Tyr 426, Tyr 514 and/or His 585 of SEQ ID NO: 3. Iron binding may also be affected by mutation to amino acids Lys206, His207 or Arg632 of SEQ ID NO: 3. Carbonate binding may be reduced or disrupted by mutation, including deletion, substitution or insertion into, amino acid residues corresponding to one or more of Tf N domain residues Thr120, Arg124, Ala126, Gly 127 and/or C domain residues Thr 452, Arg 456, Ala 458 and/or Gly 459 of SEQ ID NO: 3. A reduction or disruption of carbonate binding may adversely affect iron and/or receptor binding.

Binding to the Tf receptor may be reduced or disrupted by mutation, including deletion, substitution or insertion into, amino acid residues corresponding to one or more of Tf N domain residues described above for iron binding.
As discussed above, glycosylation may be reduced or prevented by mutation, including deletion, substitution or insertion into, amino acid residues corresponding to one or more of Tf C domain residues around the N-X-S/T sites corresponding to C domain residues N413 and/or N611 (See U.S. Patent No. 5,986,067). For instance, the N413 and/or N611 may be mutated to Glu residues.

In instances where the fusion proteins of the invention are not modified to prevent glycosylation, iron binding, carbonate binding and/or receptor binding, glycosylation, iron and/or carbonate ions may be stripped from or cleaved off of the fusion protein. For instance, available deglycosylases may be used to cleave glycosylation residues from the fusion protein, in particular the sugar residues attached to the Tf portion, yeast deficient in glycosylation enzymes may be used to prevent glycosylation and/or recombinant cells may be grown in the presence of an agent that prevents glycosylation, e.g., tunicamycin.

The carbohydrates on the fusion protein may also be reduced or completely removed enzymatically by treating the fusion protein with deglycosylases. Deglycosylases are well known in the art. Examples of deglycosylases include, but are not limited to, galactosidase, PNGase A, PNGase F, glucosidase, mannosidase, fucosidase and Endo H deglycosylase.

Nevertheless, in certain circumstances, it may be preferable for oral delivery that the Tf portion of the fusion protein be fully glycosylated.

Additional mutations may be made with Tf to alter the three dimensional structure of Tf, such as modifications to the hinge region to prevent the conformational change needed for iron binding and Tf receptor recognition. For instance, mutations may be made in or around N domain amino acid residues 94-96, 245-247 and/or 316-318 as well as C domain amino acid residues 425-427, 581-582 and/or 652-658. In addition, mutations may be made in or around the flanking regions of these sites to alter Tf structure and function.

In one aspect of the invention, the fusion protein can function as a carrier protein to extend the half life or bioavailability of the melanocortin peptide or melanocortin receptor agonist as well as, in some instances, delivering the melanocortin peptide or melanocortin receptor agonist inside a cell and/or across the blood brain barrier. In an alternate embodiment, the fusion protein includes a modified transferrin molecule wherein the transferrin does not retain the ability to cross the blood brain barrier.

In another embodiment, the fusion protein includes a transferrin molecule wherein the transferrin molecule retains the ability to bind to the transferrin receptor and transport the therapeutic peptide inside cells. In an alternate embodiment, the fusion protein includes a
modified transferrin molecule wherein the transferrin molecule does not retain the ability to bind to the transferrin receptor and transport the therapeutic peptide inside cells.

In further embodiments, the fusion protein includes a transferrin molecule wherein the transferrin molecule retains the ability to bind to the transferrin receptor and transport the therapeutic peptide inside cells and retains the ability to cross the blood brain barrier. In an alternate embodiment, the transferrin fusion protein includes a modified transferrin molecule wherein the transferrin molecule retains the ability to cross the blood brain barrier, but does not retain the ability to bind to the transferrin receptor and transport the therapeutic peptide inside cells.

**Modified Transferrin Fusion Proteins**

The fusion of proteins of the invention may contain one or more copies of the melanocortin peptide or an analog thereof or melanocortin receptor agonist, melanocortin receptor antagonist or melanocortin receptor binding peptide attached to the N-terminus and/or the C-terminus of the Tf protein. In some embodiments, the melanocortin peptide, melanocortin receptor agonist, melanocortin receptor antagonist or melanocortin receptor binding peptide is attached to both the N- and C-terminal ends of the Tf protein. In other embodiments, the melanocortin peptide, melanocortin receptor agonist, melanocortin receptor antagonist or melanocortin receptor binding peptide is inserted into known domains of the Tf protein, for instance, into one or more of the loops of Tf (see Ali *et al*., 1999, *J. Biol. Chem.* 274(34): 24066-24073). In other embodiments, the melanocortin peptide, melanocortin receptor agonist, melanocortin receptor antagonist or melanocortin receptor binding peptide is inserted between the N and C domains of Tf. Alternatively, the melanocortin peptide, melanocortin receptor agonist, melanocortin receptor antagonist or melanocortin receptor binding peptide is inserted anywhere in the transferrin molecule.

The fusion protein of the present invention includes the use of a linker to separate the transferrin and melanocortin moieties of the protein. For instance, in one embodiment, melanocortin is attached to the N-terminus of Tf with an intervening linker peptide. In another embodiment, melanocortin is attached to the C-terminus of Tf with an intervening linker peptide. As can be appreciated by a skilled artisan, the invention envisions a linker being situated numerous ways in the molecule to separate melanocortin and transferrin. In one embodiment of the invention, the linker separating the melanocortin and Tf moieties is a substantially nonhelical linker.

Generally, the fusion protein of the invention may have one transferrin region and one melanocortin peptide, melanocortin receptor agonist, melanocortin receptor antagonist
or melanocortin receptor binding peptide region. Multiple regions of each protein, however, may be used to make a fusion protein of the invention.

Similarly, more than one therapeutic peptide may be used to make a transferrin fusion protein of the invention, thereby producing a multi-functional modified fusion protein. The second therapeutic peptide may act synergistically with the melanocortin peptide, melanocortin receptor agonist, melanocortin receptor antagonist or melanocortin receptor binding protein to treat a disease or condition. In one embodiment, the melanocortin peptide or melanocortin receptor binding peptide acts to direct the fusion protein, and thus the second therapeutic, to a particular cell type.

The invention includes a fusion protein comprising a transferrin region, a GLP-1 receptor agonist region (e.g., GLP-1 peptide or exendin peptide or variant thereof) and a melanocortin peptide region. A fusion protein comprising a GLP-1 peptide or exendin peptide or analog or derivative thereof can contain one or more modifications including, but not limited to, modifications disclosed in U.S. patent application 11/367,692 and U.S. provisional patent application 60/832,582, both of which are herein incorporated by reference in their entirety.

In one embodiment of the invention, the GLP-1 receptor agonist is GLP-1 and comprises GLP-1 (7-37) (SEQ ID NO.: 6) or GLP-1 (7-36) (amino acids 1-30 of SEQ ID NO.: 6). The GLP-1 peptide may be modified to prevent dipeptidyl peptidase cleavage. In one embodiment, GLP-1 is modified by mutating the Ala at position 2 in SEQ ID NO.: 6 (equivalent to A8 using GLP-1 (7-37) or GLP-1 (7-36) nomenclature). For instance, the Ala amino acid at position 2 can be substituted with Gly, Ser or Val. In another embodiment, the GLP-1 peptide contains a modification at the Lys amino acid at position 28 in SEQ ID NO.: 6 (equivalent to K34 using GLP-1 (7-37) or GLP-1 (7-36) nomenclature). For instance, the Lys amino acid at position 28 can be substituted with an Ala, Asn or Gln amino acid. In another embodiment, the GLP-1 peptide contains a mutation at both positions 2 and 28 of SEQ ID NO.: 6. For instance, the GLP-1 (7-37) or GLP-1 (7-36) peptide may contain the amino acid substitutions A8G and K34A.

The GLP-1 receptor agonist may also be an exendin peptide such as exendin-4 or exendin-3 or an analog or derivative thereof. In one embodiment, the exendin peptide is the peptide of SEQ ID NO.: 130. In another embodiment, the exendin peptide is the first thirty amino acids or first thirty-one amino acids of SEQ ID NO.: 130.

A GLP-1 receptor agonist, melanocortin and Tf fusion protein can be administered to a subject to treat a variety of diseases including, but not limited to, type 2 diabetes, obesity,
inflammatory bowel disease (IBD), and heart-related diseases and conditions such as congestive heart failure, myocardial infarction, ischemia and reperfusion injury.

In yet another embodiment, the fusion protein comprises a transferrin region, an interferon (IFN) region and a melanocortin region. The interferon can be, for example, IFNα (SEQ ID NO.: 268) or IFNβ (SEQ ID NO.: 267).

In one embodiment, the interferon is IFNβ. The IFNβ peptide may contain one or more modifications including, but not limited to, modifications disclosed in U.S. application 10/231,494 and U.S. provisional application 60/837,323, which are herein incorporated by reference in their entirety. For instance, the initial methionine residue of IFNβ may be deleted. The IFNβ peptide may also contain a modification at amino acid C17. For instance, the IFNβ peptide may contain the amino acid substitution C17S.

In one embodiment of the invention, an IFNβ peptide, melanocortin peptide and Tf fusion protein may contain an IFNβ peptide that exhibits reduced or no glycosylation. In one embodiment of the invention, the IFNβ contains an amino acid deletion or substitution at amino acid T82 (e.g., T82A amino acid substitution). In another embodiment of the invention, the IFNβ peptide contains an amino acid deletion or substitution within or adjacent to the glycosylation site NET.

In another embodiment of the invention, the fusion protein comprises an IFNβ peptide and IFNα hybrid peptide such as an IFNβ peptide wherein amino acids 34-47 are replaced with amino acids 34-46 of IFNα or an IFNβ peptide wherein amino acids 79-89 are replaced with amino acids 77-87 of IFNα.

An interferon and melanocortin fusion protein can be used for multiple therapeutic purposes. Such a fusion protein can also be used for the treatment of a disease or condition that can be treated or ameliorated by administration of interferon-β or interferon-α such as multiple sclerosis and other autoimmune diseases. In one embodiment of the invention, a melanocortin peptide, IFNβ and Tf fusion protein can be used to target IFNβ to cancer cells such as melanoma cells.

In a further embodiment, the fusion protein comprises a transferrin region, a melanocortin peptide region and a melanocortin receptor agonist region. The fusion protein may also comprise a transferrin region, a melanocortin peptide region and a melanocortin receptor binding region.

In one embodiment, the fusion protein of the invention contains a melanocortin peptide, melanocortin receptor agonist, melanocortin receptor antagonist or melanocortin receptor binding peptide fused to a transferrin molecule or portion thereof. In another
embodiment, the fusion protein of the invention contains a melanocortin peptide, melanocortin receptor agonist, melanocortin receptor antagonist or melanocortin receptor binding peptide fused to the N terminus of a transferrin molecule. In an alternate embodiment, the fusion protein of the invention contains a melanocortin peptide, melanocortin receptor agonist, melanocortin receptor antagonist or melanocortin receptor binding peptide fused to the C terminus of a transferrin molecule. In a further embodiment, the fusion protein of the invention contains a transferrin molecule fused to the N terminus of a melanocortin therapeutic peptide, melanocortin receptor agonist, melanocortin receptor antagonist or melanocortin receptor binding peptide. In an alternate embodiment, the fusion protein of the invention contains a transferrin molecule fused to the C terminus of a melanocortin peptide, melanocortin receptor agonist, melanocortin receptor antagonist or melanocortin receptor binding peptide.

In other embodiments, the transferrin fusion protein of the inventions contains a melanocortin peptide, melanocortin receptor agonist, melanocortin receptor antagonist or melanocortin receptor binding peptide fused to both the N-terminus and the C-terminus of modified transferrin. In an alternate embodiment, the therapeutic proteins fused at the N- and C-termini include one or more melanocortin peptide, melanocortin receptor agonist, melanocortin receptor antagonist or melanocortin receptor binding peptide and one or more different therapeutic proteins which may be used to treat or prevent disease or disorders which are known in the art to be treatable with melanocortin. In an alternate embodiment, the therapeutic proteins fused at the N- and C-termini include one or more melanocortin peptides or melanocortin receptor binding peptides and one or more different therapeutic proteins which may be used to target the fusion protein to cells expressing a high level of melanocortin receptor. In another embodiment, the therapeutic proteins fused at the N- and C-termini are one or more melanocortin peptides, melanocortin receptor agonists or melanocortin receptor antagonists and one or more different therapeutic proteins which may be used to treat or prevent diseases or disorders which are known in the art to commonly occur in patients simultaneously.

In addition to fusion protein of the invention in which the transferrin portion is fused to the N terminal and/or C-terminal of the melanocortin peptide or melanocortin receptor agonist, the fusion protein of the invention may also be produced by inserting the melanocortin peptide, melanocortin receptor agonist, melanocortin receptor antagonist or melanocortin receptor binding peptide (or a fragment or variant thereof) into an internal region of transferrin. Internal regions of transferrin include, but are not limited to, the iron binding sites, the hinge regions, the bicarbonate binding sites or the receptor binding domain.
Within the protein sequence of the transferrin molecule a number of loops or turns exist, which are stabilized by disulfide bonds. These loops are useful for the insertion, or internal fusion, of therapeutically active peptides particularly those requiring a secondary structure to be functional, or therapeutic proteins to generate a modified transferrin molecule with specific biological activity.

When therapeutic proteins are inserted into or replace at least one loop of a Tf molecule, insertions may be made within any of the surface exposed loop regions, in addition to other areas of Tf. For instance, insertions may be made within the loops comprising Tf amino acids 32-33, 74-75, 166-167, 256-257, 279-280 and 288-289 (Ali et al., supra) (See Figure 1). As previously described, insertions may also be made within other regions of Tf such as the sites for iron and bicarbonate binding, hinge regions and the receptor binding domain as described in more detail below. The loops in the Tf protein sequence that are amenable to modification/replacement for the insertion of proteins or peptides may also be used for the development of a screenable library of random peptide inserts. Any procedures may be used to produce nucleic acid inserts for the generation of peptide libraries, including available phage, yeast and bacterial display systems, prior to cloning into a Tf domain and/or fusion to the ends of Tf.

The N-terminus of Tf is free and points away from the body of the molecule. Fusions of a melanocortin peptide or melanocortin receptor agonist on the N-terminus may therefore be a preferred embodiment. Such fusions may include a substantially non-helical linker to separate the melanocortin peptide or melanocortin receptor agonist from Tf.

The C-terminus of Tf appears to be more buried and secured by a disulfide bond six amino acids from the C-terminus. In human Tf, the C-terminal amino acid is a proline which, depending on the way that it is orientated, will either point a fusion away or into the body of the molecule. Any melanocortin peptide described herein may be may be used in the fusion proteins, methods and various other aspects of the invention. There is also a proline near the N-terminus. In one aspect of the invention, the proline at the N- and/or the C- termini may be substituted with another amino acid. In another aspect of the invention, the C-terminal disulfide bond may be eliminated to untether the C-terminus. In one embodiment of the invention, expression of the fusion protein is increased by fusing a melanocortin peptide, melanocortin receptor binding peptide or melanocortin receptor agonist to the C-terminus of Tf.

A modified transferrin protein may be used with any of the fusion proteins, methods and various other aspects of the invention. In one embodiment, the modified transferrin protein contains modifications within or adjacent to one or two N-linked glycosylation sites.
(e.g., N-X-S/T). For instance, the invention includes fusion proteins wherein the Tf moiety contains mutations at serine and/or threonine amino acids within the N-linked glycosylation site. In one embodiment, the modified transferrin protein contains mutations at S415 and T613. For instance, the invention includes fusion proteins comprising a modified Tf protein with the mutations S415A and T613A.

In one embodiment of the invention, an α-MSH peptide comprising the amino acid sequence of SEQ ID NO.: 215 or variant or fragment thereof is linked to the N-terminus of a modified transferrin protein comprising mutations at S415 and T613 of SEQ ID NO.: 3. In one embodiment of the invention, an α-MSH peptide comprising the amino acid sequence of SEQ ID NO.: 215 or variant or fragment thereof is linked to the C-terminus of a modified transferrin protein comprising mutations at S415 and T613 of SEQ ID NO.: 3. In another embodiment, an α-MSH peptide comprising the amino acid sequence of SEQ ID NO.: 215 or variant or fragment thereof is inserted within a modified transferrin protein comprising mutations at S415 and T613 of SEQ ID NO.: 3.

In one embodiment of the invention, a β-MSH peptide comprising the amino acid sequence of SEQ ID NO.: 217 or variant or fragment thereof is linked to the N-terminus of a modified transferrin protein comprising mutations at S415 and T613 of SEQ ID NO.: 3. In one embodiment of the invention, a β-MSH peptide comprising the amino acid sequence of SEQ ID NO.: 217 or variant or fragment thereof is linked to the C-terminus of a modified transferrin protein comprising mutations at S415 and T613 of SEQ ID NO.: 3. In another embodiment, a β-MSH peptide comprising the amino acid sequence of SEQ ID NO.: 217 or variant or fragment thereof is inserted within a modified transferrin protein comprising mutations at S415 and T613 of SEQ ID NO.: 3.

In one embodiment of the invention, a γ-MSH peptide comprising the amino acid sequence of SEQ ID NO.: 216 or variant or fragment thereof is linked to the N-terminus of a modified transferrin protein comprising mutations at S415 and T613 of SEQ ID NO.: 3. In one embodiment of the invention, an γ-MSH peptide comprising the amino acid sequence of SEQ ID NO.: 216 or variant or fragment thereof is linked to the C-terminus of a modified transferrin protein comprising mutations at S415 and T613 of SEQ ID NO.: 3. In another embodiment, an γ-MSH peptide comprising the amino acid sequence of SEQ ID NO.: 216 or variant or fragment thereof is inserted within a modified transferrin protein comprising mutations at S415 and T613 of SEQ ID NO.: 3.

In one embodiment of the invention, an ACTH peptide comprising the amino acid sequence of SEQ ID NO.: 218 or variant or fragment thereof is linked to the N-terminus of a modified transferrin protein comprising mutations at S415 and T613 of SEQ ID NO.: 3.
one embodiment of the invention, an ACTH peptide comprising the amino acid sequence of
SEQ ID NO.: 218 or variant or fragment thereof is linked to the C-terminus of a modified
transferrin protein comprising mutations at S415 and T613 of SEQ ID NO.: 3. In another
embodiment, an ACTH peptide comprising the amino acid sequence of SEQ ID NO.: 218 or
variant or fragment thereof is inserted within a modified transferrin protein comprising
mutations at S415 and T613 of SEQ ID NO.: 3.

A fusion protein comprising a melanocortin moiety fused to the N-terminus or C-
terminus end or within a modified transferrin protein comprising mutations at S415 and T613
of SEQ ID NO.: 3 may further comprise a leader sequence, a linker sequence such as
(PEAPTD)₂, and a second therapeutic peptide such as a GLP-1 receptor agonist (e.g., GLP-
1 or an exendin peptide) or an interferon (e.g., IFNβ or IFNα).

Melanocortin Therapeutic Proteins and Peptides

The melanocortin family of peptides is made up of α-melanocyte stimulating hormone
(α-MSH) (SEQ ID NO.: 215), β-melanocyte stimulating hormone (β-MSH) (SEQ ID NO.: 217), γ-melanocyte-stimulating hormone (γ-MSH) (SEQ ID NO.: 216) and
adrenocorticotropic hormone (ACTH) (SEQ ID NO.: 218). The melanocortins are relatively short peptides ranging in size from 11 amino acids (γ-MSH) to 39 amino acids (ACTH) and are posttranslational products of proopiomelanocortin (POMC) (Eberle, 1988, The Melanocortins, Karger, Basel Switzerland and Hadley and Haskell-Luevano, 1999, Ann. NY Acad. Sci. 885: 1-21). Specifically, melanocortin peptides are formed as the result of intracellular cleavage of POMC by several proprotein convertases (Seidah and Chretien, 1994, Methods Enzymol. 244: 175-188). As a result of the cleavage process, α-MSH shares the sequence of ACTH (1-13), although α-MSH is acetylated at the N terminus and amidated at the C terminus. All melanocortins share an invariant sequence of four amino acids, His-Phe-Arg-Trp (SEQ ID NO.: 219).

Some melanocortins exist naturally in multiple forms in the body. For instance, β-
MSH (1-22) was first isolated from human pituitary tissue as a 22-amino acid peptide. Later, it was demonstrated that a shorter human β-MSH (5-22) peptide is produced by the hypothalamus (Hsiung et al., 2005, Peptides. 26(10): 1988-1996). α-MSH has also been shown to exist in multiple forms in the body. Three different types of α-MSH that differ in the acetyl status of the N-terminal serine have been found in the neurointermediate lobe of the pituitary gland and in the brain (Rudman et al., 1983, Am. J. Physiol. 245(1): E47-54).

The effects of melanocortins are disparate and range from modulation of fever and inflammation to control of food intake, autonomic functions and exocrine secretion. The
various activities of melanocortin peptides stem from activation of at least five G protein-coupled melanocortin receptors, which are referred to as melanocortin 1 receptor (MC1R), melanocortin 2 receptor (MC2R), melanocortin 3 receptor (MC3R), melanocortin 4 receptor (MC4R) and melanocortin 5 receptor (MC5R). The five known melanocortin receptors are all functionally coupled to an adenylyl cyclase and mediate their effects primarily by activating a cAMP-dependent signaling pathway.

The five known melanocortin receptors are differentially expressed throughout the body. For instance, MC1R is expressed in peripheral tissues including melanocytes, macrophages, monocytes, lymphocytes, neutrophils, endothelial cells and fibroblasts and in scattered neurons of periaqueductal gray substance in the brain (Xia et al., 1995, Neuroreport. 6: 2193-2196). MC2R is expressed on the adrenal cortex. MC3R is expressed in the brain, placenta, gut, heart and monocytes (Gantz et al., 1993, J. Biol. Chem. 268: 15174-15179; Chhajlani, 1996, Biochem. Mol. Biol. Int. 38: 73-80; and Taherzadeh et al., 1999, Am. J. Physiol. 276: R1289-R1294). MC4R is found primarily in the brain and has not been detected in peripheral tissues (Gantz et al., 1993, J. Biol. Chem. 268: 15174-15479 and Chhajlani, 1996, Biochem. Mol. Biol. Int. 38: 73-80). MC5R, the most recently cloned of the MC receptors, is ubiquitously expressed in peripheral tissues (Catania et al., 2004, Pharmacol. Rev. 56: 1-29).

The five melanocortin receptors also have differing affinities for the melanocortins. For instance, the order of potency for activation of MC1R is $\alpha$-MSH $\geq$ ACTH $\geq$ $\gamma$-MSH; of MC3R is $\gamma$-MSH = ACTH $\geq$ $\alpha$-MSH; of MC4R is $\alpha$-MSH = ACTH $\geq$ $\gamma$-MSH; and of MC5R is $\alpha$-MSH $\geq$ ACTH $>\gamma$-MSH (Chhajlani and Wikberg, 1992, FEBS Lett. 309: 417-420; Suzuki et al., 1996, Endocrinology. 137: 1627-1633; and Roselli-Rehfuss et al., 1993, Proc. Natl. Acad. Sci. USA. 90: 8856-8860). ACTH is the only melanocortin that interacts with MC2R.

$\alpha$-MSH has been perhaps the most studied melanocortin. $\alpha$-MSH and MC1R interactions contribute to the regulation of skin physiology and melanogenesis (Slominski et al., 2000, Endocr. Rev. 21: 457-487). The binding of $\alpha$-MSH to MC1R in melanocytes initiates a signal cascade that activates adenylyl cyclase, increases intracellular cAMP and induces activity of tyrosinase. The intracellular signal cascade increases the formation of melanin, a molecule that guards against the photodamaging effects of UV radiation by acting as a filter that limits the penetration of UV rays into the epidermal layers of the skin and acts as a scavenger of UV-induced reactive oxygen species that cause lipid peroxidation and damage proteins and DNA (Kaidbey et al., 1979, J. Am. Acad. Dermatol. 1: 249-260; Kobayashi et al., 1998, J. Invest. Dermatol. 110: 806-810; and Bustamante et al., 1993, Pigment Cell Res. 6: 348-353).
Melanocortin analogs have been developed for the treatment of skin diseases caused or exacerbated by exposure to UV radiation as well as for cosmetic tanning (U.S. Patent 4,457,864; U.S. Patent 4,485,039; U.S. Patent 4,866,038; U.S. Patent 4,918,055; 5,674,839; U.S. Patent 5,683,981; and U.S. Patent 5,714,576). For instance, Melanotan-1 ([Nle4-D-Phe7] α-MSH) is a chemical analog of α-MSH that is reported to exhibit increased potency and increased half-life compared to α-MSH. The plasma half-life of Melanotan-1 following subcutaneous dosing has been reported as ranging from 0.07 to 0.79 hours for the absorption phase and from 0.8 to 1.7 hours for the beta-phase (Ugwa et al., 1997, BioPharm. Drug Dispos. 18(3): 259-269). Melanotan-1 (marketed as CUV1647 by Clinuvel Pharmaceuticals) is currently being evaluated for prophylactic treatment of a number of photodermatoses such as polymorphous light eruption and actinic keratosis.

α-MSH and other melanocortins are also capable of exerting anti-inflammatory influences through inhibition of inflammatory mediator production and inflammatory cell migration. Although activation of MC1R by α-MSH and ACTH has been shown to have significant anti-inflammatory effects, there is evidence that MC3R and MC5R may also contribute to the anti-inflammatory effects of melanocortins. Further, the C-terminal tripeptide Lys-Pro-Val of α-MSH (α-MSH 11-13), which has been shown to share the anti-inflamatory and antipyretic effects of α-MSH, does not recognize any of the known melanocortin receptors (Catania et al., 2004, Pharmacol. Rev. 56: 1-29).

One mechanism by which α-MSH is known to inhibit inflammatory response is through interaction with MC1R. MC1R expression occurs mainly in peripheral tissues, including, for instance, in macrophages and monocytes, lymphocytes with antigen-presenting and cytotoxic functions and neutrophils (Star et al., 1995, Proc. Natl. Acad. Sci. USA. 92: 8016-8020; Neumann-Anderson et al., 2001, Clin. Exp. Immunol. 126: 441-446; and Catania et al., 1996, Peptides. 17: 675-679). These cells are significant targets for the anti-inflammatory effects of α-MSH. For instance, α-MSH has been shown to down-regulate CD86, a major T-cell costimulatory molecule in LPS-stimulated monocytes (Bhardwaj et al., 1997, J. Immunol. 158: 3378-3384). In human peripheral blood monocytes and cultured human monocytes, α-MSH has been shown to increase production and expression of IL-10 (Bhardwaj et al., 1996, J. Immunol. 156: 2517-2521). Because IL-10 reduces proinflammatory cytokine production in macrophages, its up regulation can have anti-inflammatory effects.

α-MSH has also been shown to reduce the production of inflammatory mediators such as cytokines TNF-α, IL-1 and IL-6 (Catania et al., 2004, Pharmacol. Rev. 56: 1-29). Overexpression of proinflammatory cytokines can result in inflammatory diseases and has been linked to treatment failure (Kulmatycki and Jamali, 2005, J. Pharm. Pharmaceut. Sci.
8(3): 602-625). Diseases associated with overexpression of TNF-α, IL-1 and/or IL-6, include, but are not limited to, AIDS, acute infections, progression and metastasis of prostate cancer, acute myocardial infarction, congestive heart failure, fever, Alzheimer's disease, cerebral ischemia, multiple sclerosis, obesity, schizophrenia and rheumatoid arthritis (Kulmatycki and Jamali, supra).

TNF-α is a cytokine that is produced in response to many inflammatory stimuli and participates in tissue damage. α-MSH has been shown to reduce production of TNF-α in vitro and in vivo. For instance, the addition of small concentrations of α-MSH to LPS-stimulated whole blood samples has been shown to inhibit TNF-α and IL-1β production by 30-40% (Catania et al., 2000, Crit. Care Med. 28: 1403-1407). α-MSH has also been shown to inhibit production of TNF-α and IL-1β induced by HIV envelope glycoprotein 120 in peripheral blood mononuclear cells and inhibit TNF-α in whole blood from HIV-positive patients stimulated with exotoxin (Catania et al., 1998, Peptides. 19: 1099-1104). Administration of α-MSH has been shown to reduce the production of TNF-α in a murine colitis model for inflammatory bowel disease (Rajora et al., 1997, Peptides. 18(3): 381-385).

In addition to inhibiting TNF-α in peripheral tissue, a single central injection or multiple intraperitoneal injections of α-MSH have been found to inhibit TNF-α formation in the brain. The presence of TNF-α in the brain has been linked to multiple sclerosis, HIV infection of the CNS, Alzheimer's disease, meningitis and acute brain injury as a result of ischemia and reperfusion or trauma (Rajora et al., 1997, J. of Neurosci. 17(6): 2181-2186).

For instance, TNF-α occurs in abundance in lesions of multiple sclerosis, which, with its capacity to promote myelin destruction and to increase adhesion molecule expression, makes it a prime suspect in the etiology of the leions (Raine, C.S., 1994, Ann. Neurol. 36: 561-572). Experimental multiple sclerosis is improved by anti-TNF-α antibodies and by soluble TNF-α receptors (Selmaj and Raine, 1995, Neurology. 45: S44-49). It has been suggested that if TNF-α causes or promotes pathology of a CNS disorder, including those described above, agents that inhibit its production such as α-MSH could preserve neurological function (Rajora et al., 1997, J. of Neurosci. 17(6): 2181-2186).

Nitric oxide production is also reduced by α-MSH. α-MSH has been shown to inhibit nitric oxide induced by LPS and interferon-γ (IFN-γ) in RAW264.7 mouse macrophages (Star et al., 1995, Proc. Natl. Acad. Sci. USA. 92: 8016-8020).

The remarkably broad effects of α-MSH on inflammatory mediator production were puzzling to researchers until the discovery that α-MSH inhibits activation of nuclear factor-κB (NF-κB) (Manna and Aggarwal, 1998, J. Immunol. 161 :2873-2880). NF-κB induces transcription of many molecules involved in the inflammatory process, including cytokines.
and iNOS. Found in virtually all cell types, NF-κB is retained in an inactive form in the
cytoplasm bound to members of the IκB inhibitory protein family until phosphorylation of IκB
by various agents such as drugs, cytokines, bacterial products and viruses. Upon
phosphorylation and subsequent degradation of IκB, free NF-κB is translocated into the
nucleus where it binds to sequences of DNA encoding NF-κB-responsive elements and
triggers transcription of target genes.

α-MSH and amino acids derived from α-MSH, such as C-terminal peptide fragments,
have also been reported to possess anti-microbial effects. See U.S. Patent 6,887,846 which
is herein incorporated by reference in its entirety. Such peptides act against both eukaryotic
and prokaryotic microorganisms such as C. albicans and S. aureus. Disclosed anti-microbial
peptides include peptides consisting of or containing the amino acid sequences KPV and
MEHFRWG.

Melanocortins can also affect food intake and obesity by interacting with MC3R and
MC4R. Mice in which MC4R has been knocked-out exhibit weight gain (Huszar et al., 1997,
Cell. 88: 131-141). Further, it has been shown that injection of synthetic peptides that mimic
melanocortins and bind MC4R cause suppressed feeding in normal and mutant obese mice

Not surprising, much attention has been focused on the study of MC3R and MC4R
agonists and antagonists and their use for treating body weight disorders, such as obesity
and anorexia. Although the focus of much of this research has been on the development of
MC4R agonists for appetite and weight reduction, MC3 receptor agonists and MC4 receptor
antagonists have been targeted as promising drug candidates for treating anorexia and
weight loss associated with wasting diseases, for example, cancer and HIV (Mayorov et al.,
2006, J. Med. Chem. 49(6): 1946-1952). For instance, it has been shown that MC3R
receptor agonist d-Trp(8)-γ-MSH stimulates feeding when injected peripherally (Marks et al.,
2006, Peptides. 27(2): 259-264). It has also been shown that MC4 receptor antagonist 14c
stimulates feeding when injected in mice by intracerebroventricular administration (Pontillo et
al., 2005, Bioorg. Med. Chem. Lett. 15(10): 2541-2546). Also see, for example, PCT
application WO 00/74679; PCT application WO 99/64002; U.S. Patent 6,127,381; PCT
application WO 01/91752; PCT application WO 01/70708, PCT application WO 01/70337;
PCT application WO 02/00654; PCT application WO 00/58361; and PCT application WO
99/54358.

In addition to appetite stimulating effects, γ-MSH has also been linked to
steroidogenic effects. Pro-γ-MSH (16k fragment) and Lys-γ-MSH have both been shown to
potentiate the steroidogenic action of corticotrophin (ACTH) on the adrenal cortex. It has
been reported that MC3R is not involved in mediating the potentiation effect, suggesting that another melanocortin receptor may exist (Harmer and Bicknell, 2004, Endocrin. Res. 30(4): 629-635).

Y-MSH has also been reported to have effects on the cardiovascular and renal systems (Harmer and Bicknell, 2005, Peptides. 26(10): 1944-1951). γ-MSH has been reported to exhibit potent pressor and cardioaccelerator effects (Li et al., 1996, J. Neurosci. 16(16): 5182-5188). It is unclear how γ-MSH exerts these effects. Although α-MSH exerts hypotension and bradycardia effects as a result of interactions with MC3R and MC4R, data suggest that these receptors are not involved in the pressor and tachycardia effects of Y-MSH (Li et al., supra and Van Bergen et al., 1997, Br. J. Pharmacol. 120(8): 1561-1567). It has been postulated that amino acids Arg-Trp (positions 7 and 8) and Arg-Phe (positions 10 and 11) are responsible for enhancing cardiovascular activity of γ-MSH (Gruber and Callahan, 1989, Am. J. Physiol. 257: R681-R694).

It has also been reported that melanocortin peptides, including γ(1)-MSH, are able to exert a protective effect in rats subjected to myocardial ischemia and reperfusion by stimulating MC3 receptors (Mioni et al., 2003, Eur. J. Pharmacol. 477(3): 227-234). It has been suggested that melanocortins such as γ-MSH can be used to prevent myocardial reperfusion injury (Mioni et al., supra).

Melanocortins have also been reported to act as potent initiators of penile erection and have been reported to increase libido. Administration of Melanotan II, a cyclic heptapeptide analog of α-MSH, has been reported to result in a 75% response rate when injected intramuscularly or subcutaneously to males with psychogenic erectile dysfunction. When administered subcutaneously, the duration of action of the drug is approximately 2.5 hours. Adverse reactions observed include nausea (which was reported as extreme when the drug was administered in high doses), yawning and stretching (Dorr et al., 1996, Life Sci. 58(20): 1777-1784 and Wessells et al., 2000, Int. J. Impot. Res. 12 Suppl. 4: S74-79).

Recently, it has been reported that melanocortin receptor agonists, in particular, agonists of human melanocortin-4 receptor can be used for treatment of diabetes. See U.S. Published Patent Application 20020004512.

Any melanocortin therapeutic molecule may be used as the fusion partner to Tf according to the methods and compositions of the present invention. As used herein, a "therapeutic molecule" or a "melanocortin therapeutic" is a melanocortin peptide or variant or analog thereof capable of exerting a beneficial biological effect in vitro or in vivo. For instance, a beneficial effect as related to a disease state includes any effect that is advantageous to the treated subject, including disease prevention, disease stabilization, the
lessening or alleviation of disease symptoms or a modulation, alleviation or cure of the underlying defect to produce an effect beneficial to the treated subject.

Although melanocortins are naturally melanocortin receptor agonists, it is known that melanocortin receptor antagonists may also exert beneficial effects (e.g., use of MC4R antagonists for weight gain). In addition to fusion proteins comprising a melanocortin receptor agonist fused to Tf, the present invention also envisions the fusion of a melanocortin receptor antagonist peptide to Tf.

The present invention also includes fusion proteins comprising a melanocortin receptor binding peptide fused to Tf. In this embodiment, it is not necessary for the peptide to be a receptor agonist or receptor antagonist. Rather, it is only necessary that the peptide bind a melanocortin receptor such as MC1R, MC2R, MC3R, MC4R or MC5R. By binding to one or more melanocortin receptors, the melanocortin binding receptor peptide is capable of targeting the fusion protein to cells that express the receptor. In one embodiment, the peptide binds specifically to one or more melanocortin receptors (e.g., binds only to MC1R, binds only to MC3R, binds only to MC4R, binds only to MC1R and MC3R, etcetera). In another embodiment, the peptide binds specifically to melanocortin receptors present in a particular part of the body, for instance, the peptide may bind to only peripheral melanocortin receptors or may bind to only central nervous system melanocortin receptors. The invention also includes fusion proteins that can be used to target the fusion protein to a particular cell type, for instance, melanoma cells. Such fusion proteins may also comprise one or more additional therapeutic peptides, for instance, interferon-β or a toxin.

A fusion protein of the invention includes at least a fragment or variant of a melanocortin peptide, melanocortin receptor agonist, melanocortin receptor antagonist or melanocortin receptor binding peptide and at least a fragment or variant of transferrin, which are associated with one another, preferably by genetic fusion.

The fusion proteins can contain melanocortin peptides, melanocortin receptor agonists, melanocortin receptor antagonists or melanocortin receptor binding peptides that can be peptide fragments or peptide variants at least about 4, at least about 5, at least about 6, at least about 7, at least about 8, at least about 9, at least about 10, at least about 11, at least about 12, at least about 13, at least about 14, at least about 15, at least about 16, at least about 17, at least about 18, at least about 19, at least about 20, at least about 21, at least about 22, at least about 23, at least about 24, at least about 25, at least about 26, at least about 27, at least about 28, at least about 29, at least about 30, or at least about 31 amino acids in length fused to the N and/or C termini, inserted within, or inserted into a loop of transferrin.
The fusion proteins of the present invention may contain one or more peptides. Increasing the number of peptides may enhance the function of the peptides fused to transferrin and the function of the entire fusion protein. The peptides may be used to make a bi- or multi-functional fusion protein by including peptide or protein domains with multiple functions. For instance, a multi-functional fusion protein can be made with a melanocortin peptide, melanocortin receptor agonist, melanocortin receptor antagonist or melanocortin receptor binding peptide and a second therapeutic peptide. In one embodiment of the invention, the second peptide is a GLP agonist such as GLP-1 or exendin-4. Such fusion proteins can be administered for treatment of diseases and conditions, including, but not limited to, congestive heart failure, myocardial infarction, cardiac ischemia and reperfusion injury, obesity and diabetes.

In another embodiment of the invention, the second peptide is IFN-β. Such fusion proteins can be administered for the treatment of diseases and conditions, including, but not limited to, cancers such as melanoma and autoimmune diseases such as multiple sclerosis.

The fusion protein of the present invention may comprise a linker linking the transferrin to the melanocortin peptide, melanocortin receptor agonist, melanocortin receptor antagonist or melanocortin receptor binding peptide. Preferably, the linker is the sequence PEAPTD (SEQ ID NO.: 13) in one or more copies. There may be one or more melanocortin peptides at the amino terminus of the fusion protein.

In another embodiment, the fusion molecules may contain a melanocortin peptide or melanocortin receptor agonist or antagonist having one or more residues deleted from the amino terminus of the amino acid sequence.

In another embodiment, the fusion molecules may contain a melanocortin peptide or melanocortin receptor agonist or antagonist having one or more residues deleted from the carboxy terminus of the amino acid sequence.

In another embodiment, the modified transferrin fusion molecules contain a melanocortin peptide or melanocortin receptor agonist or antagonist that has one or more amino acids deleted from both the amino and the carboxy termini.

In another embodiment, the fusion molecules contain a melanocortin peptide or melanocortin receptor agonist that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to a reference melanocortin peptide sequence set forth herein, or fragments thereof. In further embodiments, the transferrin fusion molecules contain a melanocortin peptide or melanocortin receptor agonist that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to reference polypeptides having the amino acid sequence of N- and C-terminal deletions as described above.
In another embodiment, the fusion molecules contain a melanocortin peptide or melanocortin receptor agonist that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, identical to, for example, the native or wild-type amino acid sequence of a melanocortin peptide. Fragments, of these polypeptides are also provided.

The invention also includes fusion proteins which contain a melanocortin hybrid peptide, many of which are known in the art. For instance, the invention includes fusion proteins which comprise an α-MSH and γ-MSH hybrid peptide. In one embodiment of the invention, the fusion protein comprises a γ-MSH peptide wherein the C-terminus has been replaced with one or more C-terminal amino acids of α-MSH.

In one embodiment of the invention, the melanocortin peptide or melanocortin receptor agonist or antagonist portion of the fusion protein is modified by the attachment of one or more oligosaccharide groups. The modification referred to as glycosylation can significantly affect the physical properties of proteins and can be important in protein stability, secretion, and localization. In another embodiment, the melanocortin peptide or melanocortin receptor agonist or antagonist portion of the fusion protein is modified so that glycosylation at one or more sites is altered as a result of manipulation(s) of their nucleic acid sequence by the host cell in which they are expressed, or due to other conditions of their expression. For example, glycosylation isomers may be produced by abolishing or introducing glycosylation sites, e.g., by substitution or deletion of amino acid residues, such as substitution of glutamine for asparagine, or unglycosylated recombinant proteins may be produced by expressing the proteins in host cells that will not glycosylate them, e.g. in glycosylation-deficient yeast. These approaches are known in the art.

Melanocortin peptides, melanocortin receptor agonists, melanocortin receptor antagonists and melanocortin receptor binding peptides and their nucleic acid sequences are well known in the art and available in public databases such as Chemical Abstracts Services Databases (e.g., the CAS Registry), GenBank, and GenSeq.

In other embodiments, the fusion proteins of the invention are capable of a therapeutic activity and/or biologic activity, corresponding to the therapeutic activity and/or biologic activity of the melanocortin peptide or melanocortin receptor agonist. In further embodiments, the therapeutically active protein portions of the fusion proteins of the invention are fragments or variants of the reference sequences cited herein.

The present invention is further directed to fusion proteins comprising fragments of the melanocortin peptides and melanocortin receptor agonists herein described. Even if deletion of one or more amino acids from the N-terminus of a protein results in modification or loss of one or more biological functions of the therapeutic protein portion, other
therapeutic activities and/or functional activities (e.g., ability to multimerize, ability to bind a melanocortin receptor) may still be retained. For example, the ability of polypeptides with N-terminal deletions to induce and/or bind to antibodies which recognize the complete or mature forms of the polypeptides generally will be retained with less than the majority of the residues of the complete polypeptide removed from the N-terminus. Whether a particular polypeptide lacking N-terminal residues of a complete polypeptide retains such immunologic activities can be assayed by routine methods described herein and otherwise known in the art. It is not unlikely that a mutant with a large number of deleted N-terminal amino acid residues may retain some biological or immunogenic activities. In fact, peptides composed of as few as six amino acid residues may often evoke an immune response.

Also as mentioned above, even if deletion of one or more amino acids from the N-terminus or C-terminus of a melanocortin peptide, melanocortin receptor agonist or antagonist results in modification or loss of one or more biological functions of the protein, other functional activities (e.g., ability to multimerize, ability to bind a melanocortin receptor) and/or therapeutic activities may still be retained. For example, the ability of polypeptides with C-terminal deletions to induce and/or bind to antibodies which recognize the complete or mature forms of the polypeptide generally will be retained when less than the majority of the residues of the complete or mature polypeptide are removed from the C-terminus. Whether a particular polypeptide lacking the N-terminal and/or, C-terminal residues of a reference polypeptide retains therapeutic activity can readily be determined by routine methods described herein and/or otherwise known in the art.

Peptide fragments of the melanocortin peptide or melanocortin receptor agonist or antagonist can be fragments comprising, or alternatively, consisting of, an amino acid sequence that displays a therapeutic activity and/or functional activity (e.g., biological activity) of the polypeptide sequence of the therapeutic protein of which the amino acid sequence is a fragment.

The peptide fragments of the melanocortin peptide or melanocortin receptor agonist or antagonist may comprise only the N- and C- termini of the protein, i.e., the central portion of the therapeutic protein has been deleted. Alternatively, the peptide fragments may comprise non-adjacent and/or adjacent portions of the central part of the therapeutic protein.

Generally, variants of melanocortin peptides, melanocortin receptor agonists or melanocortin receptor antagonists are overall very similar, and, in many regions, identical to the amino acid sequence of the melanocortin peptide, melanocortin receptor agonist or melanocortin receptor antagonist, respectively. Nucleic acids encoding these variants are also encompassed by the invention.
Further therapeutic polypeptides that may be used in the invention are polypeptides encoded by polynucleotides which hybridize to the complement of a nucleic acid molecule encoding an amino acid sequence of a melanocortin peptide or melanocortin receptor agonist under stringent hybridization conditions which are known to those of skill in the art. (see, for example, Ausubel, F.M. et al., eds., 1989 Current protocol in Molecular Biology, Green Publishing Associates, Inc., and John Wiley & Sons Inc., New. York). Polynucleotides encoding these polypeptides are also encompassed by the invention.

By a polypeptide-having an amino acid sequence at least, for example, 95% "identical" to a query amino acid sequence of the present invention, it is intended that the amino acid sequence of the subject polypeptide is identical to the query sequence except that the subject polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the query amino acid sequence. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a query amino acid sequence, up to 5% of the amino acid residues in the subject sequence may be inserted, deleted, or substituted with another amino acid. These alterations of the reference sequence may occur at the amino- or carboxy-terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence, or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular polypeptide is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequence of a fusion protein of the invention or a fragment thereof (such, as the melanocortin peptide, melanocortin receptor agonist, melanocortin receptor antagonist or melanocortin receptor binding peptide portion of the fusion protein or the transferrin portion of the fusion protein), can be determined conventionally using known computer programs. A preferred method for determining the best overall match between a query sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, can be determined using the FASTDB computer program based on the algorithm of Brufiag et al. (Comp. App. Biosci 245 (1990)).

The polynucleotide variants of the invention may contain alterations in the coding regions, non-coding regions, or both. Polynucleotide variants containing alterations which produce silent substitutions, additions or deletions, but do not alter the properties or activities of the encoded polypeptide may be used to produce the fusion proteins. Nucleotide variants produced by silent substitutions due to the degeneracy of the genetic code can be utilized. Moreover, polypeptide variants in which less than about 50, less than 40, less than 30, less than 20, less than 10, or 5-50, 5-25, 5-10, 1-5, or 1-2 amino acids are substituted, deleted, or
added in any combination can also be utilized. Polynucleotide variants can be produced for a variety of reasons, for example, to optimize codon expression for a particular host (change codons in the human mRNA to those preferred by a host, such as, yeast or E. coli as described above).

In other embodiments, the melanocortin peptide moiety, melanocortin receptor agonist or melanocortin receptor antagonist moiety has conservative substitutions compared to the wild-type sequence. By "conservative substitutions" is intended swaps within groups such as replacement of the aliphatic or hydrophobic amino acids Ala, Val, Leu and Ile; replacement of the hydroxyl residues Ser and Thr; replacement of the acidic residues Asp and Glu; replacement of the amide residues Asn and Gln, replacement of the basic residues Lys, Arg, and His; replacement of the aromatic residues Phe, Tyr, and Trp, and replacement of the small-sized amino acids Ala, Ser, Thr, Met, and Gly. Guidance concerning how to make phenotypically silent amino acid substitutions is provided, for example, in Bowie et al., "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," Science 247:1306-1310 (1990). In specific embodiments, the polypeptides of the invention comprise, or alternatively, consist of, fragments or variants of the amino acid sequence of a therapeutic protein described herein and/or serum transferrin, and/or modified transferrin protein of the invention, wherein the fragments or variants have 1-5, 5-10, 5-25, 5-50, 10-50 or 50-150 amino acid residue additions, substitutions, and/or deletions when compared to the reference amino acid sequence. In further embodiments, the amino acid substitutions are conservative. Nucleic acids encoding these polypeptides are also encompassed by the invention.

The fusion proteins of the present invention can be composed of amino acids joined to each other by peptide bonds or modified peptide bonds and may contain amino acids other than the 20 gene-encoded amino acids. The polypeptides may be modified by either natural processes, such as post-translational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. For instance, the invention includes, but is not limited to, fusion proteins comprising modified amino acids such as those described in U.S. Patent 7,083,970 and by Deiters et al., 2003, J. Am. Chem. Soc. 125: 11782-11783; Chin et al., 2003, Science. 301: 964-967; Wang et al., 2001, Science. 292: 498-500; and Zhang et al., 2004, Science. 303: 371-373.

Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxy termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications.
Polypeptides may be branched, for example, as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched, and branched cyclic polypeptides may result from posttranslation natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristylation, oxidation, pegylation, proteolytic processing, phosphorylation, prenylation, racemization, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. (See, for instance, PROTEINS - STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York (1993); POST-TRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C. Johnson, Ed., Academic Press, New York, pgs. 1-12 (1983); Seifter et al. (1990) Meth. Enzymol. 182:626-646; Rattan et al., Ann. N.Y. Acad. Sci 663:48-62.

Nucleic Acids

The present invention also provides nucleic acid molecules encoding fusion proteins comprising a transferrin protein or a portion of a transferrin protein covalently linked or joined to a melanocortin peptide, melanocortin receptor agonist, melanocortin receptor antagonist or melanocortin receptor binding peptide. The fusion protein may further comprise a second therapeutic peptide, for instance, a GLP-1 receptor agonist or an interferon such as IFNβ. The fusion protein may also further comprise a linker region, for instance a linker less than about 50, 40, 30, 20, or 10 amino acid residues. The linker can be covalently linked to and between the transferrin protein or portion thereof and the melanocortin peptide or melanocortin receptor agonist. Nucleic acid molecules of the invention may be purified or not.

Host cells and vectors for replicating the nucleic acid molecules and for expressing the encoded fusion proteins are also provided. Any vectors or host cells may be used, whether prokaryotic or eukaryotic, but eukaryotic expression systems, in particular yeast expression systems, may be preferred. Many vectors and host cells are known in the art for such purposes. It is well within the skill of the art to select an appropriate set for the desired application.

DNA sequences encoding a melanocortin peptide or melanocortin receptor agonist or transferrin may be cloned from a variety of genomic or cDNA libraries known in the art. The
techniques for isolating such DNA sequences using probe-based methods are conventional techniques and are well known to those skilled in the art. Probes for isolating such DNA sequences may be based on published DNA or protein sequences (see, for example, Baldwin, G.S. (1993) Comparison of Transferrin Sequences from Different Species. Comp. Biochem. Physiol. 106B/1, 203-218 and all references cited therein, which are hereby incorporated by reference in their entirety). Alternatively, the polymerase chain reaction (PCR) method disclosed by Mullis et al. (U.S. Pat. No. 4,683,195) and Mullis (U.S. Pat. No. 4,683,202), incorporated herein by reference may be used. The choice of library and selection of probes for the isolation of such DNA sequences is within the level of ordinary skill in the art.

As known in the art "similarity" between two polynucleotides or polypeptides is determined by comparing the nucleotide or amino acid sequence and its conserved nucleotide or amino acid substitutes of one polynucleotide or polypeptide to the sequence of a second polynucleotide or polypeptide. Also known in the art is "identity" which means the degree of sequence relatedness between two polypeptide or two polynucleotide sequences as determined by the identity of the match between two strings of such sequences. Both identity and similarity can be readily calculated (Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991).


Preferred methods to determine identity are designed to give the largest match between the two sequences tested. Methods to determine identity and similarity are codified in computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, GCG program package.
(Devereux, et al., 1984, Nucl. Acid Res. 12(1):387), BLASTP, BLASTN, FASTA (Atschul, et al., 1990, J. Mol. Biol. 215:403). The degree of similarity or identity referred to above is determined as the degree of identity between the two sequences, often indicating a derivation of the first sequence from the second. The degree of identity between two nucleic acid sequences may be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman and Wunsch, 1970, J. Mol. Biol. 48:443-453). For purposes of determining the degree of identity between two nucleic acid sequences for the present invention, GAP is used with the following settings: GAP creation penalty of 5.0 and GAP extension penalty of 0.3.

15 Codon Optimization

The degeneracy of the genetic code permits variations of the nucleotide sequence of a transferrin protein, melanocortin peptide or melanocortin receptor agonist or antagonist, while still producing a polypeptide having the identical amino acid sequence as the polypeptide encoded by the native DNA sequence. The procedure, known as "codon optimization" (described in U.S. Patent 5,547,871 which is incorporated herein by reference in its entirety) provides one with a means of designing such an altered DNA sequence. The design of codon optimized genes should take into account a variety of factors, including the frequency of codon usage in an organism, nearest neighbor frequencies, RNA stability, the potential for secondary structure formation, the route of synthesis and the intended future DNA manipulations of that gene. In particular, available methods may be used to alter the codons encoding a given fusion protein with those most readily recognized by yeast when yeast expression systems are used.

The degeneracy of the genetic code permits the same amino acid sequence to be encoded and translated in many different ways. For example, leucine, serine and arginine are each encoded by six different codons, while valine, proline, threonine, alanine and glycine are each encoded by four different codons. However, the frequency of use of such synonymous codons varies from genome to genome among eukaryotes and prokaryotes. For example, synonymous codon-choice patterns among mammals are very similar, while evolutionarily distant organisms such as yeast (such as S. cerevisiae), bacteria (such as E. coli) and insects (such as D. melanogaster) reveal a clearly different pattern of genomic codon use frequencies (Grantham, R., et al., Nucl. Acid Res., 8, 49-62 (1980); Grantham, R., et al., Nucl. Acid Res., 9, 43-74 (1981); Maroyama, T., et al., Nucl. Acid Res., 14, 151-197 (1986); Aota, S., et al., Nucl. Acid Res., 16, 315-402 (1988); Wada, K., et al., Nucl. Acid Res., 19 Supp., 1981-1985 (1991); Kurland, C. G., FEBS Lett., 285, 165-169 (1991)). These differences in codon-choice patterns appear to contribute to the overall expression levels of

The preferred codon usage frequencies for a synthetic gene should reflect the codon usages of nuclear genes derived from the exact (or as closely related as possible) genome of the cell/organism that is intended to be used for recombinant protein expression, particularly that of yeast species. As discussed above, in one embodiment the human Tf sequence is codon optimized, before or after modification as herein described for yeast expression as may be the therapeutic protein nucleotide sequence(s).

Vectors

Expression units for use in the present invention will generally comprise the following elements, operably linked in a 5' to 3' orientation: a transcriptional promoter, a secretory signal sequence, a DNA sequence encoding a fusion protein comprising transferrin protein or a portion of a transferrin protein joined to a DNA sequence encoding a melanocortin peptide or melanocortin receptor agonist or antagonist and a transcriptional terminator. As discussed above, any arrangement of the melanocortin peptide portion and Tf portion may be used in the vectors of the invention. The selection of suitable promoters, signal sequences and terminators will be determined by the selected host cell and will be evident to one skilled in the art and are discussed more specifically below.

In one embodiment of the invention, the fusion protein further comprises a linker. In this embodiment, expression units will generally comprise the following elements, operably linked in a 5' to 3' orientation: a transcriptional promoter, a secretory signal sequence, a DNA sequence encoding a fusion protein comprising transferrin protein or a portion of a transferrin protein joined to a DNA sequence encoding a linker and a melanocortin peptide or melanocortin receptor agonist or antagonist and a transcriptional terminator.

available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (Yips) and incorporate the yeast selectable markers HIS3, TRP1, LEU2 and URA3. Plasmid pRS413-41.6 are Yeast Centromere plasmids (YCps).

Such vectors will generally include a selectable marker, which may be one of any number of genes that exhibit a dominant phenotype for which a phenotypic assay exists to enable transformants to be selected. Preferred selectable markers are those that complement host cell auxotrophy, provide antibiotic resistance or enable a cell to utilize specific carbon sources, and include LEU2 (Broach et al., ibid.), URA3 (Botstein et al., Gene 8: 17, 1979), HIS3 (Struhl et al., ibid.) or POT1 (Kawasaki and Bell, EP 171,142). Other suitable selectable markers include the CAT gene, which confers chloramphenicol resistance on yeast cells. Promoters for use in yeast include promoters from yeast glycolytic genes (Hitzeman et al., J Biol. Chem. 225: 12073-12080, 1980; Alber and Kawasaki, J. Mol. Appl. Genet. 1: 419-434, 1982; Kawasaki, U.S. Pat. No. 4,599,311) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals, Hollaender et al., (eds.), p. 355, Plenum, N.Y., 1982; Ammerer, Meth. Enzymol. 101: 192-201, 1983). Other promoters include the TPH promoter (Kawasaki, U.S. Pat. No. 4,599,311) and the ADH2-4 promoter (U.S. Patent 6,291,212 and Russell et al., Nature 304: 652-654, 1983). The expression units may also include a transcriptional terminator. For instance, the TPH terminator can be used (Alber and Kawasaki, 1982, J. Mol. Appl. Genet. 1: 419-434). Other vectors and components such as promoters and terminators of a yeast expression system that can be used are disclosed in European Patents EP 0258067, EP 0286424, EP0317254, EP 0387319, EP 0386222, EP 0424117, EP 0431880, and EP 1002095; European Patent Publications EP 0828759, EP 0764209, EP 0749478, and EP 0889949; PCT Publication WO 00/44772 and WO 94/04687; and U.S. Patents 5,739,007; 5,637,504; 5,302,697; 5,260,202; 5,667,986; 5,728,553; 5,783,423; 5,965,386; 6150,133; 6,379,924; and 5,714,377, which are herein incorporated by reference in their entirety.

In addition to yeast, fusion proteins of the present invention can be expressed in filamentous fungi, for example, strains of the fungi Aspergillus. Examples of promoters include those derived from Aspergillus nidulans glycolytic genes, such as the adh3 promoter (McKnight et al., 1985, EMBO J. 4: 2093-2099) and the tpiA promoter. An example of a suitable terminator is the adh3 terminator (McKnight et al., supra.). The expression units utilizing such components may be cloned into vectors that are capable of insertion into the chromosomal DNA of Aspergillus, for example.

Mammalian expression vectors for use in carrying out the present invention will include a promoter capable of directing the transcription of the fusion protein. Promoters
include viral promoters and cellular promoters. For instance, viral promoters include the major late promoter from adenovirus 2 (Kaufman and Sharp, 1982, Mol. Cell. Biol. 2: 1304-13199) and the SV40 promoter (Subramani et al., 1981, Mol. Cell. Biol. 1: 854-864). Cellular promoters include the mouse metallothionein 1 promoter (Palmiter et al., 1983, Science 222: 809-814) and a mouse V6 promoter (U.S. Patent 6,291,212 and Grant et al., 1987, Nuc. Acids Res. 15: 5496). The invention also includes the use of a mouse $V_H$ promoter (U.S. Patent 6,291,212). Such expression vectors may also contain a set of RNA splice sites located downstream from the promoter and upstream from the DNA sequence encoding the transferrin fusion protein. For instance, RNA splice sites may be obtained from adenovirus and/or immunoglobulin genes.

Also contained in the expression vectors is a polyadenylation signal located downstream of the coding sequence of interest. Polyadenylation signals include the early or late polyadenylation signals from SV40 (Kaufman and Sharp, 1982, Mol. Cell. Biol. 2: 1304-13199), the polyadenylation signal from the adenovirus 5 E1B region and the human growth hormone gene terminator (DeNoto et al., 1981, Nucl. Acid Res. 9: 3719-3730). A polyadenylation signal that can be used with the fusion protein of the invention is the $V_H$ gene terminator (see U.S. Patent 6,291,212). The expression vectors may include a noncoding viral leader sequence, such as the adenovirus 2 tripartite leader, located between the promoter and the RNA splice sites. Vectors may also include enhancer sequences, such as the SV40 enhancer (U.S. Patent 6,291,212 and Gillies, 1983, Cell 33: 717-728).

Expression vectors may also include sequences encoding the adenovirus VA RNAs.

**Transformation**

Techniques for transforming fungi are well known in the literature, and have been described, for instance, by Beggs (*ibid.*), Hinnen et al. (1978, Proc. Natl. Acad. Sci. USA 75: 1929-1933), Yelton et al. (1984, Proc. Natl. Acad. Sci. USA 81: 1740-1747), and Russell (1983, Nature 301: 167-169). Other techniques for introducing cloned DNA sequences into fungal cells, such as electroporation (Becker and Guarente, 1991, Methods in Enzymol. 194: 182-187) may be used. The genotype of the host cell will generally contain a genetic defect that is complemented by the selectable marker present on the expression vector. Choice of a particular host and selectable marker is well within the level of ordinary skill in the art.

Cloned DNA sequences comprising fusion proteins of the invention may be introduced into cultured mammalian cells by, for example, calcium phosphate-mediated transfection (Wigler et al., 1978, Cell 14: 725; Corsaro and Pearson, 1981, Somatic Cell Genetics 7: 603; and Graham and Van der Eb, 1973, Virology 52: 456). Other techniques for introducing cloned DNA sequences into mammalian cells, such as electroporation
(Neumann et al., 1982, EMBO J. 1: 841-845) or lipofection may also be used. In order to identify cells that have integrated the cloned DNA, a selectable marker is generally introduced into the cells along with the gene or cDNA of interest. For instance, selectable markers for use in cultured mammalian cells include genes that confer resistance to drugs, such as neomycin, hygromycin, and methotrexate. The selectable marker may be an amplifiable selectable marker, for instance, the DHFR or DHFR\(^{-}\)cDNA (see U.S. Patent 6,291,212 and Simonsen and Levinson, 1983, Proc. Natl. Acad. Sci. USA 80: 2495-2499).

Examples of selectable markers are provided by Thilly (Mammalian Cell Technology, Butterworth Publishers, Stoneham, Mass.) and the choice of selectable markers is well within the level of ordinary skill in the art.

**Host Cells**

The present invention also includes a cell, preferably a yeast cell transformed to express a fusion protein of the invention. In addition to the transformed host cells themselves, the present invention also includes a culture of those cells, preferably a monoclonal (clonally homogeneous) culture, or a culture derived from a monoclonal culture, in a nutrient medium. If the polypeptide is secreted, the medium will contain the polypeptide, with the cells, or without the cells if they have been filtered or centrifuged away.

Host cells for use in practicing the present invention include eukaryotic cells, and in some cases prokaryotic cells, capable of being transformed or transfected with exogenous DNA and grown in culture, such as cultured mammalian, insect, fungal, plant and bacterial cells.

Fungal cells, including species of yeast (e.g., *Saccharomyces* spp., *Schizosaccharomyces* spp., *Pichia* spp.) may be used as host cells within the present invention. Examples of fungi including yeasts contemplated to be useful in the practice, of the present invention as hosts for expressing the, transferrin fusion protein of the inventions are *Pichia* (some species of which were formerly classified as *Hansenula*), *Saccharomyces*, *Kluyveromyces*, *Aspergillus*, *Candida*, *Torulopsis*, *Torulaspora*, *Schizosaccharomyces*, *Citeromyces*, *Pachysolen*, *Zygosaccharomyces*, *Debaromyces*, *Trichoderma*, *Cephalosporium*, *Humicola*, *Mucor*, *Neurospora*, *Yarrowia*, *Metschunikowia*, *Rhodosporidium*, *Leucosporidium*, *Botryoascus*, *Sporidiobolus*, *Endomycopsis*, and the like. Examples of *Saccharomyces* spp. are *S. cerevisiae*, *S. italicus* and *S. rouxii*. Examples of *Kluyveromyces* spp. are *K. fragilis*, *K. lactis* and *K. marxianus*. A suitable *Torulaspora* species is *T. delbrueckii*. Examples of *Pichia* spp. are *P. angusta* (formerly *H. polymorpha*), *P. anomala* (formerly *H. anomala*) and *P. pastoris*.
In one embodiment of the invention, fusion proteins of the invention are produced in *Pichia pastoris* (Steinlein et al., 1995, Protein Express. Purif. 6:619-624). *Pichia pastoris* has been developed to be an outstanding host for the production of foreign proteins since its alcohol oxidase promoter was isolated and cloned; its transformation was first reported in 1985. *P. pastoris* can utilize methanol as a carbon source in the absence of glucose. The *P. pastoris* expression system can use the methanol-induced alcohol oxidase (AOX1) promoter, which controls the gene that codes for the expression of alcohol oxidase, the enzyme which catalyzes the first step in the metabolism of methanol. This promoter has been characterized and incorporated into a series of *P. pastoris* expression vectors. Since the proteins produced in *P. pastoris* are typically folded correctly and secreted into the medium, the fermentation of genetically engineered *P. pastoris* provides an excellent alternative to *E. coli* expression systems. A number of proteins have been produced using this system, including tetanus toxin fragment, *Bordatella pertussis* pertactin, human serum albumin and lysozyme.

In another embodiment, the host is *Saccharomyces cerevisiae*. The yeast cell, or more specifically, a *Saccharomyces cerevisiae* host cell may be modified to contain a genetic deficiency in a gene required for asparagine-linked glycosylation of glycoproteins is used. *S. cerevisiae* host cells having such defects may be prepared using standard techniques of mutation and selection, although many available yeast strains have been modified to prevent or reduce glycosylation or hypermannosylation. Ballou et al. (1980, J. Biol. Chem. 255: 5986-5991) have described the isolation of mannoprotein biosynthesis mutants that are defective in genes which affect asparagine-linked glycosylation. Gentzsch and Tanner (1997, Glycobiology 7:481-486) have described a family of at least six genes (*PMT1-6*) encoding enzymes responsible for the first step in O-glycosylation of proteins in yeast. Mutants defective in one or more of these genes show reduced O-linked glycosylation and/or altered specificity of O-glycosylation.

In one embodiment, the host is a *S. cerevisiae* strain described in WO 05/061718, which is herein incorporated by reference in its entirety. For instance, the host can contain a pSAC35 based plasmid carrying a copy of the *PDH* gene or any other chaperone gene in a strain with the host version of *PDH* or other chaperone knocked out, respectively. Such a construct confers enhanced stability.

To optimize production of the heterologous proteins, the host strain may also carry a mutation, such as the *S. cerevisiae* pep4 mutation (Jones, 1977, Genetics 85: 23-33), which results in reduced proteolytic activity. Host strains containing mutations in other protease encoding regions are particularly useful to produce large quantities of the fusion proteins of the invention.
Host cells containing DNA constructs of the present invention are grown in an appropriate growth medium. As used herein, the term "appropriate growth medium" means a medium containing nutrients required for the growth of cells. Nutrients required for cell growth may include a carbon source, a nitrogen source, essential amino acids, vitamins, minerals and growth factors. The growth medium will generally select for cells containing the DNA construct by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker on the DNA construct or co-transfected with the DNA construct. Yeast cells, for example, are preferably grown in a chemically defined medium, comprising a carbon source, e.g. sucrose, a non-amino acid nitrogen source, inorganic salts, vitamins and essential amino acid supplements. The pH of the medium is preferably maintained at a pH greater than 2 and less than 8, preferably at pH 5.5-6.5. Methods for maintaining a stable pH include buffering and constant pH control. Preferred buffering agents include succinic acid and Bis-Tris (Sigma Chemical Co., St. Louis, Mo.). Yeast cells having a defect in a gene required for asparagine-linked glycosylation are preferably grown in a medium containing an osmotic stabilizer. A preferred osmotic stabilizer is sorbitol supplemented into the medium at a concentration between 0.1 M and 1.5 M, preferably at 0.5 M or 1.0 M.

Cultured mammalian cells are generally grown in commercially available serum-containing or serum-free media. Selection of a medium appropriate for the particular cell line used is within the level of ordinary skill in the art. Transfected mammalian cells are allowed to grow for a period of time, typically 1-2 days, to begin expressing the DNA sequence(s) of interest. Drug selection is then applied to select for growth of cells that are expressing the selectable marker in a stable fashion. For cells that have been transfected with an amplifiable selectable marker the drug concentration may be increased in a stepwise manner to select for increased copy number of the cloned sequences, thereby increasing expression levels.

Baculovirus/insect cell expression systems may also be used to produce the fusion proteins of the invention. The BacPAK™ Baculovirus Expression System (BD Biosciences (Clontech)) expresses recombinant proteins at high levels in insect host cells. The target gene is inserted into a transfer vector, which is cotransfected into insect host cells with the linearized BacPAK6 viral DNA. The BacPAK6 DNA is missing an essential portion of the baculovirus genome. When the DNA recombines with the vector, the essential element is restored and the target gene is transferred to the baculovirus genome. Following recombination, a few viral plaques are picked and purified, and the recombinant phenotype is verified. The newly isolated recombinant virus can then be amplified and used to infect insect cell cultures to produce large amounts of the desired protein.
Fusion proteins of the present invention may also be produced using transgenic plants and animals. For example, sheep and goats can make the melanocortin peptide in their milk or tobacco plants can include the protein in their leaves. Both transgenic plant and animal production of proteins comprises adding a new gene coding the fusion protein into the genome of the organism. Not only can the transgenic organism produce a new protein, but it can also pass this ability on to its offspring.

**Secretory Signal Sequences**

The terms "secretory signal sequence" or "signal sequence" or "secretion leader sequence" are used interchangeably and are described, for example in U.S. Patent 6,291,212 and U.S. Patent 5,547,871, both of which are herein incorporated by reference in their entirety. Secretory signal sequences or signal sequences or secretion leader sequences encode secretory peptides. A secretory peptide is an amino acid sequence that acts to direct the secretion of a mature polypeptide or protein from a cell. Secretory peptides are generally characterized by a core of hydrophobic amino acids and are typically (but not exclusively) found at the amino termini of newly synthesized proteins. Very often the secretory peptide is cleaved from the mature protein during secretion. Secretory peptides may contain processing sites that allow cleavage of the signal peptide from the mature protein as it passes through the secretory pathway. Processing sites may be encoded within the signal peptide or may be added to the signal peptide by, for example, *in vitro* mutagenesis.

Secretory peptides may be used to direct the secretion of the fusion proteins of the invention. One such secretory peptide that may be used in combination with other secretory peptides is the alpha mating factor leader sequence. Secretory signal sequences or signal sequences or secretion leader sequences are required for a complex series of post-translational processing steps which result in secretion of a protein. If an intact signal sequence is present, the protein being expressed enters the lumen of the rough endoplasmic reticulum and is then transported through the Golgi apparatus to secretory vesicles and is finally transported out of the cell. Generally, the signal sequence immediately follows the initiation codon and encodes a signal peptide at the amino-terminal end of the protein to be secreted. In most cases, the signal sequence is cleaved off by a specific protease, called a signal peptidase. Preferred signal sequences improve the processing and export efficiency of recombinant protein expression using viral, mammalian or yeast expression vectors.

In one embodiment, the native Tf signal sequence may be used to express and secrete fusion proteins of the present invention. Since transferrin molecules exist in various
types of secretions such as blood, tears, and milk, there are many different transferrin signal peptides. For example, the transferrin signal peptide could be from serum transferrin, lactotransferrin or melanotransferrin. The native transferrin signal peptide also could be from various species such as insects, mammals, fish, frog, duck, chicken or other species. Preferably, the signal peptide is from a mammalian transferrin molecule. More preferably, the signal peptide is from human serum transferrin. The table below summarizes the signal peptide sequences from various mammalian transferrin molecules.

**Signal Peptide Sequences (from GenBank entries)**

<table>
<thead>
<tr>
<th>Species</th>
<th>Type</th>
<th>Sequence</th>
<th>Location in immature protein, SEQ ID NO:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mammals</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bos taurus</em> (cow)</td>
<td>serum</td>
<td>MRPAVRALLA CAVGLGLCLA</td>
<td>1-19, SEQ ID NO: 97</td>
</tr>
<tr>
<td><em>Equus caballus</em> (horse)</td>
<td>serum</td>
<td>MRLAIRALLA CAVGLGLCLA</td>
<td>1-19, SEQ ID NO: 98</td>
</tr>
<tr>
<td><em>Homo sapiens</em> (human)</td>
<td>serum</td>
<td>MRLAVGALLV CAVGLGLCLA</td>
<td>amino acids 1-19 of SEQ ID NO: 2</td>
</tr>
<tr>
<td><em>Mus musculus</em> (mouse)</td>
<td>serum</td>
<td>MRLTVGALLA CAALGLCLCA</td>
<td>1-19, SEQ ID NO: 99</td>
</tr>
<tr>
<td><em>Oryctolagus cuniculus</em> (rabbit)</td>
<td>serum</td>
<td>MRLAAGALLA CAALGLCLCA</td>
<td>1-19, SEQ ID NO: 100</td>
</tr>
<tr>
<td><em>Rattus norvegicus</em> (rat)</td>
<td>serum</td>
<td>MRFAVGALLA CAALGLCLCA</td>
<td>1-19, SEQ ID NO: 101</td>
</tr>
<tr>
<td><em>Sus scrofa</em> (pig)</td>
<td>serum</td>
<td>missing in sequence?</td>
<td>NA*</td>
</tr>
<tr>
<td><em>Bos taurus</em> (cow)</td>
<td>lacto</td>
<td>MKLFVPALLS LGALGLCLCA</td>
<td>1-19, SEQ ID NO: 102</td>
</tr>
<tr>
<td><em>Bubalus bubalis</em> (buffalo)</td>
<td>lacto</td>
<td>MKLFVPALLS LGALGLCLCA</td>
<td>1-19, SEQ ID NO: 103</td>
</tr>
<tr>
<td><em>Camelus dromedaries</em> (camel)</td>
<td>lacto</td>
<td>MKLFPALLS LGALGLCLCA</td>
<td>1-19, SEQ ID NO: 104</td>
</tr>
<tr>
<td><em>Capra hircus</em> (goat)</td>
<td>lacto</td>
<td>MKLFVPALLS LGALGLCLCA</td>
<td>1-19, SEQ ID NO: 105</td>
</tr>
<tr>
<td><em>Equus caballus</em> (horse)</td>
<td>lacto</td>
<td>LGLCLA (rest missing?)</td>
<td>1-6, SEQ ID NO: 106</td>
</tr>
<tr>
<td><em>Mus musculus</em> (mouse)</td>
<td>lacto</td>
<td>MRLLIPSIF LEALGLCLCA</td>
<td>1-19, SEQ ID NO: 107</td>
</tr>
<tr>
<td><em>Sus scrofa</em> (pig)</td>
<td>lacto</td>
<td>MKLFIPALLF LGTLGLCLCA</td>
<td>1-19, SEQ ID NO: 108</td>
</tr>
<tr>
<td><em>Sus scrofa</em> (pig)</td>
<td>ica</td>
<td>MRLAFCVLLC AGSLGLCLCA</td>
<td>1-19, SEQ ID NO: 109</td>
</tr>
<tr>
<td><em>Homo sapiens</em> (human)</td>
<td>melano</td>
<td>MRGPGSALWL LLALRTVLG</td>
<td>1-19, SEQ ID NO: 110</td>
</tr>
<tr>
<td><em>Mus musculus</em> (mouse)</td>
<td>melano</td>
<td>MRLLSVTFWL LLSLRTVVC</td>
<td>1-19, SEQ ID NO: 111</td>
</tr>
<tr>
<td><em>Oryctolagus cuniculus</em> (rabbit)</td>
<td>melano</td>
<td>MRCRSAAMWI FLALRTALG (by inference)</td>
<td>NA*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1-19, SEQ ID NO: 112</td>
</tr>
</tbody>
</table>

*NA: Not available in GenBank description; sequence shown was inferred from relatives and multiple sequence alignment and checked against SignalP.

"Missing in Sequence": signal peptide not included in published sequence data.
In another embodiment, the signal peptides are from variant or modified transferrin molecules that have functionally active signal peptides. In yet another embodiment, the signal peptides are variant or modified forms of transferrin signal peptides that retain the ability to transport a transferrin fusion protein of the present invention across the cell membrane and then to process the fusion protein.

In another embodiment, the transferrin derived signal sequence may be used to secrete a heterologous protein, for instance, any protein of interest that is heterologous to the Tf signal sequence may be expressed and secreted using a Tf signal. In particular, a Tf signal sequence may be used to secrete proteins from recombinant yeast. Preferably, the signal peptide is from human serum transferrin (nl_, amino acids 1-19 of SEQ ID NO: 2).

In order to ensure efficient removal of the signal sequence, in some cases it may be preferable to include a short pro-peptide sequence between the signal sequence and the mature protein in which the C-terminal portion of the pro-peptide comprises a recognition site for a protease, such as the yeast kex2p protease. Preferably, the pro-peptide sequence is about 2-12 amino acids in length, more preferably about 4-8 amino acids in length.

Examples of such pro-peptides are Arg-Ser-Leu-Asp-Lys-Arg (SEQ ID NO: 113), Arg-Ser-Leu-Asp-Arg-Arg (SEQ ID NO: 114), Arg-Ser-Leu-Glu-Lys-Arg (SEQ ID NO: 115), and Arg-Ser-Leu-Glu-Arg-Arg (SEQ ID NO: 116).

Linkers

The fusion proteins of the invention may include a linker peptide of various lengths to provide greater physical separation and allow more spatial mobility between Tf and the therapeutic peptide and thus maximize the accessibility of the therapeutic protein, for instance, for binding to its cognate receptor. The linker can be less than about 50, 40, 30, 20, 10 or 5 amino acid residues. The linker can be covalently linked to and between the transferrin protein or portion thereof and the melanocortin peptide or melanocortin receptor agonist or portion thereof. These linkers may be used to link melanocortin to transferrin.

In the preferred embodiment of the invention, melanocortin is linked to Tf via a substantially non-helical linker. Examples of such rigid linkers include PE, PEA, PEAPTD (SEQ ID NO.: 13), (PEAPTD)\(_2\) (SEQ ID NO.: 10), (PEAPTD)\(_3\) (SEQ ID NO.: 14), or (PEAPTD)\(_n\), wherein n is an integer. The present invention also provides the IgG hinge linker, the CEx linker (SSGAPPPS; SEQ ID NO.: 15 (C-terminal extension to Exendin-4)), the IgG hinge linker in conjunction with the PEAPTD linker (SEQ ID NOS.: 118-123 and 126-129) and the IgG hinge linker in conjunction with the CEx linker (SEQ ID NOS.:220-229).
Detection of melanocortin/Tf Fusion Proteins

Assays for detection of biologically active fusion protein may include Western transfer, protein blot or colony filter as well as activity based assays that detect the fusion protein comprising transferrin and therapeutic protein. A Western transfer filter may be prepared using the method described by Towbin et al. (Proc. Natl. Acad. Sci. USA 76: 4350-4354, 1979). Briefly, samples are electrophoresed in a sodium dodecylsulfate polyacrylamide gel. The proteins in the gel are electrophoretically transferred to nitrocellulose paper. Protein blot filters may be prepared by filtering supernatant samples or concentrates through nitrocellulose filters using, for example, a Minifold (Schleicher & Schuell, Keene, N.H.). Colony filters may be prepared by growing colonies on a nitrocellulose filter that has been laid across an appropriate growth medium. In this method, a solid medium is preferred. The cells are allowed to grow on the filters for at least 12 hours. The cells are removed from the filters by washing with an appropriate buffer that does not remove the proteins bound to the filters. In one embodiment, the buffer comprises 25 mM Tris-base, 19 mM glycine, pH 8.3, 20% methanol.

Fusion proteins of the present invention may be labeled with a radioisotope or other imaging agent and used for in vivo diagnostic purposes. Radioisotope imaging agents include, for instance, iodine-125 and technetium-99. Methods for producing protein-isotope conjugates are well known in the art, and are described by, for example, Eckelman et al. (U.S. Pat. No. 4,652,440), Parker et al. (WO 87/05030) and Wilber et al. (EP 203,764).

Alternatively, the fusion proteins may be bound to spin label enhancers and used for magnetic resonance (MR) imaging. Suitable spin label enhancers include stable, sterically hindered, free radical compounds such as nitroxides. Methods for labeling ligands for MR imaging are disclosed by, for example, Coffman et al. (U.S. Pat. No. 4,656,026).

The iron binding site of the transferrin moiety can also be used to incorporate metal ions such as gallium or technetium ions. The incorporation of such metal ions can be for diagnostic as well as therapeutic purposes. For instance, in one embodiment of the invention, the fusion protein can be labeled with radioactive metals such as $^{67}$Ga, $^{68}$Ga, $^{99}$Tc or $^{57}$Fe for diagnostic imaging methods such as PET and MRI. In one embodiment, melanoma cells are imaged using the imaging methods of the invention.

Detection of a fusion protein of the present invention can be facilitated by coupling (i.e., physically linking) the fusion protein to a detectable substance. In one embodiment, the melanocortin peptide, melanocortin receptor agonist, melanocortin receptor antagonist or melanocortin receptor binding peptide moiety is bound to a detectable substance. In another embodiment, transferrin moiety is bound to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent
materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include $^{125}$I, $^{131}$I, $^{35}$S or $^3$H.

In one embodiment where one is assaying for the ability of a fusion protein of the invention to bind or compete with an antigen for binding to an antibody, various immunoassays known in the art can be used, including but not limited to, competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), sandwich immunoassays, immunoradiometric assays, gel diffusion precipitation reactions, immunodiffusion assays, in situ immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, precipitation reactions, agglutination assays (e.g., gel agglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and Immunelectrophoresis assays, etc. In one embodiment, the binding of the fusion protein is detected by detecting a label on the fusion protein. In another embodiment, the fusion protein is detected by detecting binding of a secondary antibody or reagent that interacts with the fusion protein. In a further embodiment, the secondary antibody or reagent is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention.

Fusion proteins of the invention may also be detected by assaying for the activity of the melanocortin peptide or melanocortin receptor agonist. Specifically, fusion proteins of the invention may be assayed for functional activity (e.g., biological activity or therapeutic activity) using assays known to one of ordinary skill in the art. Additionally, one of skill in the art may routinely assay fragments of a melanocortin peptide or melanocortin receptor agonist for activity using well-known assays. Further, one of skill in the art may routinely assay fragments of a transferrin protein for activity using assays known in the art.

For example, in one embodiment where one is assaying for the ability of a fusion protein of the invention to bind or compete with a therapeutic protein for binding to an anti-therapeutic polypeptide antibody and/or anti-transferrin antibody, various immunoassays known in the art can be used, including but not limited to, competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), sandwich immunoassays, immunoradiometric assays, gel diffusion
precipitation reactions, immunodiffusion assays, in situ immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, precipitation reactions, agglutination assays (e.g., gel agglutination assays), complement fixation assays, immunofluorescence assays, protein A assays and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody.

In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention.

In a further embodiment, where a binding partner (e.g., a receptor or a ligand) of a melanocortin peptide or melanocortin receptor agonist is identified, binding to that binding partner by a fusion protein of the invention can be assayed, for instance, by means well-known in the art, such as, for example, reducing and non-reducing gel chromatography, protein affinity chromatography, and affinity blotting. Other methods will be known to the skilled artisan and are within the scope of the invention.

Production of Fusion Proteins

The present invention further provides methods for producing a fusion protein of the invention using nucleic acid molecules herein described. In general terms, the production of a recombinant form of a protein typically involves the following steps.

A nucleic acid molecule is first obtained that encodes a fusion protein of the invention. The nucleic acid molecule is then preferably placed in operable linkage with suitable control sequences, as described above, to form an expression unit containing the protein open reading frame. The expression unit is used to transform a suitable host and the transformed host is cultured under conditions that allow the production of the recombinant protein. Optionally the recombinant protein is isolated from the medium or from the cells; recovery and purification of the protein may not be necessary in some instances where some impurities may be tolerated.

Each of the foregoing steps can be accomplished in a variety of ways. For example, the construction of expression vectors that are operable in a variety of hosts is accomplished using appropriate replicons and control sequences, as set forth above. The control sequences, expression vectors, and transformation methods are dependent on the type of host cell used to express the gene and were discussed in detail earlier and are otherwise known to persons skilled in the art. Suitable restriction sites can, if not normally available, be added to the ends of the coding sequence so as to provide an excisable gene to insert into
these vectors. A skilled artisan can readily adapt any host/expression system known in the art for use with the nucleic acid molecules of the invention to produce a desired recombinant protein.

As discussed above, any expression system may be used, including yeast, bacterial, animal, plant, eukaryotic and prokaryotic systems. In some embodiments, yeast, mammalian cell culture and transgenic animal or plant production systems are preferred. In other embodiments, yeast systems that have been modified to reduce native yeast glycosylation, hyper-glycosylation or proteolytic activity may be used.

**Isolation/Purification of Modified Transferrin Fusion Proteins**

Secreted, biologically active fusion proteins may be isolated from the medium of host cells grown under conditions that allow the secretion of the biologically active fusion proteins. The cell material is removed from the culture medium, and the biologically active fusion proteins are isolated using isolation techniques known in the art. Suitable isolation techniques include precipitation and fractionation by a variety of chromatographic methods, including gel filtration, ion exchange chromatography and affinity chromatography.

A particularly preferred purification method is affinity chromatography on an iron binding or metal chelating column or an immunoaffinity chromatography using an antigen directed against the transferrin or therapeutic protein of the polypeptide fusion. The antigen is preferably immobilized or attached to a solid support or substrate. A particularly preferred substrate is CNBr-activated Sepharose (Pharmacia LKB Technologies, Inc., Piscataway, N.J.). By this method, the medium is combined with the antigen/substrate under conditions that will allow binding to occur. The complex may be washed to remove unbound material, and the transferrin fusion protein is released or eluted through the use of conditions unfavorable to complex formation. Particularly useful methods of elution include changes in pH, wherein the immobilized antigen has a high affinity for the transferrin fusion protein at a first pH and a reduced affinity at a second (higher or lower) pH; changes in concentration of certain chaotropic agents; or through the use of detergents.

**Delivery of a Drug or Therapeutic Protein to the inside of a Cell and/or across the Blood Brain Barrier (BBB)**

Within the scope of the invention, the fusion proteins may be used as a carrier to deliver a molecule or small molecule therapeutic complexed to the ferric ion of transferrin to the inside of a cell or across the blood brain barrier or other barriers including across the cell membrane of any cell type that naturally or engineered to express a Tf receptor. In these
embodiments, the fusion protein will typically be engineered or modified to inhibit, prevent or remove glycosylation to extend the serum half-life of the fusion protein and/or therapeutic protein portion.

The invention contemplates the ability to direct melanocortin fusion proteins to peripheral melanocortin receptors by modifying the Tf moiety to substantially prevent the ability of the fusion protein to cross the blood brain barrier. Such a fusion protein, by design, would be unable to activate MC4R. For receptors expressed in peripheral tissues and in the brain such as MC1R and MC3R, such a fusion protein would only be targeted to the peripheral receptors. A fusion protein targeted to peripheral receptors would not be subject to endogenous receptor antagonists present in the nervous system (e.g., AgRP) (Catania et al., 2004, Pharmacol. Rev. 56: 1-29).

The addition of a targeting peptide is specifically contemplated to further target the fusion protein to a particular cell type, for instance, a cancer cell.

In one embodiment, the iron-containing, anti-anemic drug, ferric-sorbitol-citrate complex is loaded onto a fusion protein of the invention. Ferric-sorbitol-citrate (FSC) has been shown to inhibit proliferation of various murine cancer cells in vitro and cause tumor regression in vivo, while not having any effect on proliferation of non-malignant cells (Poljak-Blazi et al. (June 2000) Cancer Biotherapy and Radiopharmaceuticals (United States), 153:285-293).

In another embodiment, the antineoplastic drug Adriamycin® (doxorubicin) and/or the chemotherapeutic drug bleomycin, both of which are known to form complexes with ferric ion, is loaded on to a fusion protein of the invention. In other embodiments, a salt of a drug, for instance, a citrate or carbonate salt, may be prepared and complexed with the ferric iron that is then bound to Tf. As tumor cells often display a higher turnover rate for iron; transferrin modified to carry at least one anti-tumor agent, may provide a means of increasing agent exposure or load to the tumor cells. (Demant, E.J., 1983, Eur. J. Biochem. 137/(1-2):1 13-1 18 and Padbury et a./., 1985, J. Biol. Chem. 260/13:7820-7823).

Pharmaceutical Formulations and Treatment Methods

In one aspect of the present invention, the pharmaceutical compositions comprising the melanocortin peptide, melanocortin receptor agonist, melanocortin receptor antagonist or melanocortin receptor binding peptide and Tf fusion proteins may be formulated by any of the established methods of formulating pharmaceutical compositions, e.g. as described in Remington’s Pharmaceutical Sciences, 1985. The composition may be in a form suited for systemic injection or infusion and may, as such, be formulated with a suitable liquid vehicle.
such as sterile water or an isotonic saline or glucose solution. The compositions may be sterilized by conventional sterilization techniques which are well known in the art. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with the sterile aqueous solution prior to administration. The composition may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as buffering agents, tonicity adjusting agents and the like, for instance sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, etcetera.

The fusion proteins of the present invention may also be adapted for oral, nasal, transdermal, pulmonal or rectal administration (see PCT/US03/26778, which is herein incorporated by reference in its entirety). The pharmaceutically acceptable carrier or diluent employed in the composition may be any conventional solid carrier. Examples of solid carriers are lactose, terra alba, sucrose, talc, gelatin, agar, pectin, acacia, magnesium stearate and stearic acid. Similarly, the carrier or diluent may include any sustained release material known in the art, such as glyceryl monostearate or glyceryl distearate, alone or mixed with a wax.

It may be of particular advantage to provide the composition of the invention in the form of a sustained release formulation. As such, the composition may be formulated as microcapsules or microparticles containing the fusion protein encapsulated by or dispersed in a suitable pharmaceutically acceptable biodegradable polymer such as polylactic acid, polyglycolic acid or a lactic acid/glycolic acid copolymer.

For nasal administration, the preparation may contain the fusion protein dissolved or suspended in a liquid carrier, in particular an aqueous carrier, for aerosol application. The carrier may contain additives such as solubilizing agents, e.g. propylene glycol, surfactants, absorption enhancers such as lecithin (phosphatidylcholine) or cyclodextrin, or preservatives such as parabens.

Generally, the compounds of the present invention are dispensed in unit dosage form comprising 0.5-500 mg of the fusion protein together with a pharmaceutically acceptable carrier per unit dosage.

Moreover, the present invention contemplates the use of the fusion protein for the manufacture of a medicinal product which can be used in the treatment of diseases associated with melanocortin activities, including, but not limited to the activation of the melanocortin receptors. In one embodiment, the present invention contemplates the use of fusion protein of the invention for treatment or prevention of diseases or conditions associated with inflammation such as inflammatory bowel disease, asthma, allergic
inflammation, autoimmune uveoretinitis, gouty arthritis, rheumatoid arthritis, brain
inflammation, septic shock, systemic vasculitis, acute respiratory distress syndrome,
hemorrhagic shock, ischemia and reperfusion injury, organ transplantation and infections.

The present invention includes the use of a melanocortin and Tf fusion protein for
treatment or prevention of diseases or conditions caused by or exacerbated by inflammatory
mediator production and inflammatory cell migration. For instance, the fusion protein of the
invention can be used to treat or prevent diseases or conditions associated with increased
production of nitric oxide, increased activation of NF-κB, increased expression of TNF-α,
increased expression of IL-1β, increased expression of VACM-1, and/or increased
expression of E-selectin.

The present invention also includes the administration of a melanocortin and Tf
fusion protein to treat or prevent various cardiovascular diseases and conditions, including,
but not limited to, low blood pressure, myocardial ischemia and reperfusion injury. The
invention also includes the prophylactic administration of a melanocortin and Tf fusion
protein to patients during and after an organ transplant, for instance, heart transplant.

In one embodiment of the invention, a melanocortin and Tf fusion protein can be
administered to a subject for treatment or prevention of a skin disease or disorder associated
with UV radiation, including, but not limited to, skin cancer (squamous cell carcinoma, basal
cell carcinoma, melanoma), polymorphous light eruption and actinic keratosis. The invention
can also be used to induce therapeutic or non-therapeutic tanning in an individual.

In another embodiment of the invention, a melanocortin and Tf fusion protein can be
administered to a subject for treatment or prevention of a condition associated with activation
of the MC4 receptor. For instance, such a fusion protein can be administered to treat or
prevent obesity, diabetes and sexual dysfunction (erectile dysfunction, female sexual
dysfunction, improve libido).

The present invention also includes the use of a melanocortin and Tf fusion protein to
increase appetite and induce weight gain. For instance, a γ-MSH and Tf fusion protein or
MC4R antagonist and Tf fusion protein can be administered to patients with chronic wasting
diseases such as HIV and cancer to reduce weight loss.

In another embodiment of the invention, a melanocortin and Tf fusion protein is
administered to a patient for treatment of a microbial infection. It has been reported that
melanocortins exhibit anti-microbial effects. Such a fusion protein can be administered to a
patient suffering from sepsis.
The fusion proteins of the invention may be administered to a patient in need thereof using standard administration protocols. For instance, the fusion proteins of the present invention can be provided alone, or in combination, or in sequential combination with other agents that modulate a particular pathological process. As used herein, two agents are said to be administered in combination when the two agents are administered simultaneously or are administered independently in a fashion such that the agents will act at the same or near the same time.

The fusion proteins of the present invention can be administered via parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal and buccal routes. For example, an agent may be administered locally to a site of injury via microinfusion. Alternatively, or concurrently, administration may be noninvasive by either the oral, inhalation, nasal, or pulmonary route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

While any method of administration may be used to deliver the fusion proteins of the invention, administration or delivery orally may be a preferred embodiment for certain classes of fusion proteins or to treat certain conditions.

The present invention further provides compositions containing one or more fusion proteins of the invention. While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages comprise about 1 µg/kg to about 150 mg/kg body weight. In one embodiment, dosages for systemic administration comprise about 100 ng/kg to about 100 mg/kg body weight. In another embodiment, doses range from 300 µg/kg to 900 µg/kg. The present invention also includes dosing weekly at a total dose of about 50 mg, 100 mg, or 150 mg. Other dosages for direct administration to a site via microinfusion comprise about 1 ng/kg to about 1 mg/kg body weight. When administered via direct injection or microinfusion, modified fusion proteins of the invention may be engineered to exhibit reduced or no binding of iron to prevent, in part, localized iron toxicity.

In addition to the pharmacologically active fusion protein, the compositions of the present invention may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries that facilitate processing of the active compounds into preparations which can be used pharmaceutically for delivery to the site of action. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable
lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension and include, for example, sodium carboxymethyl cellulose, sorbitol and dextran. Optionally, the suspension may also contain stabilizers. Liposomes can also be used to encapsulate the agent for delivery into the cell.

The pharmaceutical formulation for systemic administration according to the invention may be formulated for enteral, parenteral or topical administration. Indeed, all three types of formulations may be used simultaneously to achieve systemic administration of the active ingredient. Suitable formulations for oral administration include hard or soft gelatin capsules, pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release forms thereof.

The pharmaceutical composition of the present invention can be in unit dosage form, e.g. as tablets or capsules. In such form, the composition is sub-divided in unit dose containing appropriate quantities of the active ingredient; the unit dosage forms can be packaged compositions, for example, packeted powders, vials, ampoules, prefilled syringes or sachets containing liquids. The unit dosage form can be, for example, a capsule or tablet itself, or it can be the appropriate number of any such compositions in package form. The dosage to be used in the treatment must be subjectively determined by the physician.

In practicing the methods of this invention, the fusion proteins of this invention may be used alone or in combination, or in combination with other therapeutic or diagnostic agents. In certain preferred embodiments, the compounds of this invention may be co-administered along with other compounds typically prescribed for these conditions according to generally accepted medical practice. The compounds of this invention can be utilized in vivo, ordinarily in mammals, such as humans, sheep, horses, cattle, pigs, dogs, cats, rats and mice, or in vitro.

In the present invention, fusion proteins may be formulated for oral delivery. In particular, certain fusion proteins of the invention that are used to treat certain classes of diseases or medical conditions may be particularly amenable for oral formulation and delivery. Such classes of diseases or conditions include, but are not limited to, acute, chronic and recurrent diseases. Chronic or recurrent diseases include, but are not limited to, viral disease or infections, cancer, a metabolic diseases, obesity, autoimmune diseases, inflammatory diseases, sexual dysfunction, systemic microbial infection, cardiovascular disease, genetic diseases, neurodegenerative diseases, diseases of the endocrine system or gastrointestinal diseases. Examples of these classes of disease include skin cancer,
inflammatory bowel disease, diabetes, multiple sclerosis, asthma, arthritis, erectile dysfunction and Alzheimer's disease. In many chronic diseases, oral formulations of fusion proteins of the invention and methods of administration are particularly useful because they allow long-term patient care and therapy via home oral administration without reliance on injectable treatment or drug protocols.

Oral formulations and delivery methods comprising fusion proteins of the invention take advantage of, in part, transferrin receptor mediated transcytosis across the gastrointestinal (GI) epithelium. The Tf receptor is found at a very high density in the human GI epithelium, transferrin is highly resistant to tryptic and chymotryptic digestion and Tf chemical conjugates have been used to successfully deliver proteins and peptides across the GI epithelium (Xia et al., (2000) J. Pharmacol. Experiment. Therap., 295:594-600; Xia et al. (2001) Pharmaceutical Res., 18(2): 191-195; and Shah et al. (1996) J. Pharmaceutical ScL, 85(12): 1306-1311, all of which are herein incorporated by reference in their entirety). Once transported across the GI epithelium, fusion proteins of the invention exhibit extended half-life in serum, that is, the melanocortin peptide or melanocortin receptor agonist attached or inserted into Tf exhibits an extended serum half-life compared to the peptide in its non-fused state.

Oral formulations of fusion proteins of the invention may be prepared so that they are suitable for transport to the GI epithelium and protection of the fusion protein component and other active components in the stomach. Such formulations may include carrier and dispersant components and may be in any suitable form, including aerosols (for oral or pulmonary delivery), syrups, elixirs, tablets, including chewable tablets, hard or soft capsules, troches, lozenges, aqueous or oily suspensions, emulsions, cachets or pellets granulates, and dispersible powders. Preferably, fusion protein formulations are employed in solid dosage forms suitable for simple, and preferably oral, administration of precise dosages. Solid dosage forms for oral administration are preferably tablets, capsules or the like.

For oral administration in the form of a tablet or capsule, care should be taken to ensure that the composition enables sufficient active ingredient to be absorbed by the host to produce an effective response. Thus, for example, the amount of fusion protein may be increased over that theoretically required or other known measures such as coating or encapsulation may be taken to protect the polypeptides from enzymatic action in the stomach.

Traditionally, peptide and protein drugs have been administered by injection because of the poor bioavailability when administered orally. These drugs are prone to chemical and
conformational instability and are often degraded by the acidic conditions in the stomach, as well as by enzymes in the stomach and gastrointestinal tract. In response to these delivery problems, certain technologies for oral delivery have been developed, such as encapsulation in nanoparticles composed of polymers with a hydrophobic backbone and hydrophilic branches as drug carriers, encapsulation in microparticles, insertion into liposomes in emulsions, and conjugation to other molecules. All of which may be used with the fusion molecules of the present invention.


Drug delivery particles composed of alginate and pectin, strengthened with polylysine, are relatively acid and base resistant and can be used as a carrier for drugs. These particles combine the advantages of bioadhesion, enhanced absorption and sustained release (Liu et al., J. Pharm. Pharmacol. 51(2): 141-149, 1999).

Additionally, lipoamino acid groups and liposaccharide groups conjugated to the N- and C-termini of peptides such as synthetic somatostatin, creating an amphipathic surfactant, were shown to produce a composition that retained biological activity (Toth et al., J. Med. Chem. 42(1 9):401 0-401 3, 1999).

Examples of other peptide delivery technologies include carbopol-coated mucoadhesive emulsions containing the peptide of interest and either nitroso-N-acetyl-D,L-penicillamine and carbopol or taurocholate and carbopol. These were shown to be effective when orally administered to rats to reduce serum calcium concentrations (Ogiso et al., Biol. Pharm. Bull. 24(6):656-661, 2001). Phosphatidylethanol, derived from phosphatidylcholine, was used to prepare liposomes containing phosphatidylethanol as a carrier of insulin. These liposomes, when administered orally to rats, were shown to be active (Kisel et al., Int. J. Pharm. 216(1-2):105-1 4, 2001).

The fusion protein may also be formulated in polyvinyl alcohol)-gel spheres, optionally containing a protease inhibitor. The glucose-lowering properties of these insulin gel spheres have been demonstrated in rats, where insulin is released largely in the lower intestine (Kimura et al., Biol. Pharm. Bull. 19(6):897-900, 1996).

In other methods, the N- and C-termini of a peptide are linked to polyethylene glycol and then to allyl chains to form conjugates with improved resistance to enzymatic degradation and improved diffusion through the GI wall (www.nobexcorp.com).

BioPORTER® is a cationic lipid mixture, which interacts non-covalently with peptides to create a protective coating or layer. The peptide-lipid complex can fuse to the plasma membrane of cells, and the peptides are internalized into the cells (www.genetherapysystems.com).

In a process using liposomes as a starting material, cochleate-shaped particles have been developed as a pharmaceutical vehicle. A peptide is added to a suspension of liposomes containing mainly negatively charged lipids. The addition of calcium causes the collapse and fusion of the liposomes into large sheets composed of lipid bilayers, which then spontaneously roll up or stack into cochleates (U.S. Patent 5,840,707; www.biodeliverysciences.com).

Compositions comprising fusion protein intended for oral use may be prepared according to any method known to the art for the manufacture of pharmaceutical compositions and such compositions may contain one or more agents selected from the group consisting of sweetening agents in order to provide a pharmaceutically elegant and palatable preparation. For example, to prepare orally deliverable tablets, a fusion protein is mixed with at least one pharmaceutical excipient, and the solid formulation is compressed to form a tablet according to known methods for delivery to the gastrointestinal tract. The tablet composition is typically formulated with additives such as a saccharide or cellulose carrier, a binder such as starch paste or methyl cellulose, a filler, a disintegrator, or other additives typically usually used in the manufacture of medical preparations. To prepare orally deliverable capsules, DHEA is mixed with at least one pharmaceutical excipient, and the solid formulation is placed in a capsular container suitable for delivery to the gastrointestinal tract. Compositions comprising fusion proteins may be prepared as described generally in Remington's Pharmaceutical Sciences, 18th Ed. 1990 (Mack Publishing Co. Easton Pa. 18042) at Chapter 89, which is herein incorporated by reference.

As described above, many of the oral formulations of the invention may contain inert ingredients which allow for protection against the stomach environment, and release of the biologically active material in the intestine. Such formulations, or enteric coatings, are well
known in the art. For example, tablets containing fusion protein in admixture with non-toxic pharmaceutically acceptable excipients which are suitable for manufacture of tablets may be used. These excipients may be inert diluents, such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, for example, maize starch, gelatin or acacia, and lubricating agents, for example, magnesium stearate, stearic acid, or talc.

The tablets may be uncoated or they may be coated with known techniques to delay disintegration and absorption in the gastrointestinal track and thereby provide a sustained action over a longer period of time. For example, a time delay material such as glyceryl monostearate or glyceryl distearate alone or with a wax may be employed.

Formulations for oral use may also be presented as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate, or kaolin or as soft gelatin capsules wherein the active ingredient is mixed with an aqueous or an oil medium, for example, arachis oil, peanut oil, liquid paraffin or olive oil.

Aqueous suspensions may contain fusion protein in the admixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients are suspending agents, for example, sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents may be a naturally occurring phosphatide, for example, lecithin, or condensation products of an alkylene oxide with fatty acids, for example, polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example, heptadecylhexyloxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyoxyethylene sorbitan monooleate. The aqueous suspensions may also contain one or more preservatives for example, ethyl or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents and one or more sweetening agents such as sucrose or saccharin.

Oily suspensions may be formulated by suspending the active ingredient in a vegetable oil, for example, arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin. The oil suspensions may contain a thickening agent, for example, beeswax, hard paraffin or cetyl alcohol. Sweetening agents, such as those set forth above, and flavoring agents may be added to provide a palatable oral preparation. These compositions may be preserved by the addition of an antioxidant such as ascorbic acid.
Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active ingredient and admixture with dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents and suspending agents are exemplified by those already mentioned above. Additional excipients, for example, sweetening, flavoring and coloring agents, may also be present.

The pharmaceutical compositions containing fusion proteins may also be in the form of oil-in-water emulsions. The oil phase may be a vegetable oil, for example, olive oil or arachis oil, or a mineral oil for example, gum acacia or gum tragacanth, naturally-occurring phosphotides, for example soybean lecithin, and esters or partial esters derived from fatty acids and hexitol anhydrides, for example, sorbitan monooleate, and condensation products of the same esters with ethylene oxide, for example, polyoxyethylene sorbitan monooleate. The emulsions may also contain sweetening and flavoring agents.

Syrups and elixirs containing fusion proteins may be formulated with sweetening agents, for example, glycerol, sorbitol or sucrose. Such formulations may also contain a demulcent, a preservative and flavoring and coloring agents. The pharmaceutical compositions may be in the form of a sterile injectable preparation, for example, as a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents which have been mentioned above. The sterile injectable preparations may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvate, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this period any bland fixed oil may be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

Pharmaceutical compositions may also be formulated for oral delivery using polyester microspheres, zein microspheres, proteinoid microspheres, polycyanoacrylate microspheres, and lipid-based systems (see, for example, DiBase and Morrel, *Oral Delivery of Microencapsulated Proteins*, in Protein Delivery: Physical Systems, Sanders and Hendren (eds.), pages 255-288 (Plenum Press 1997)).

The proportion of pharmaceutically active fusion protein to carrier and/or other substances may vary from about 0.5 to about 100 wt. % (weight percent). For oral use, the pharmaceutical formulation will generally contain from about 5 to about 100% by weight of
the active material. For other uses, the formulation will generally have from about 0.5 to about 5 wt. % of the active material.

Fusion protein formulations employed in the invention provide an effective amount of fusion protein upon administration to an individual. As used in this context, an "effective amount" of fusion is an amount that is effective to ameliorate a symptom of a disease.

The fusion protein composition of the present invention may be, though not necessarily, administered daily, in an effective amount to ameliorate a symptom. Generally, the total daily dosage will be at least about 50 mg, preferably at least about 100 mg, and more preferably at least about 200 mg, and preferably not more than 500 mg per day, administered orally, e.g., in 4 capsules or tablets, each containing 50 mg fusion protein.

Capsules or tablets for oral delivery can conveniently contain up to a full daily oral dose, e.g., 200 mg or more.

In a particularly preferred embodiment, oral pharmaceutical compositions comprising fusion protein are formulated in buffered liquid form which is then encapsulated into soft or hard-coated gelatin capsules which are then coated with an appropriate enteric coating. For the oral pharmaceutical compositions of the invention, the location of release may be anywhere in the GI system, including the small intestine (the duodenum, the jejunum or the ileum), or the large intestine.

In other embodiments, oral compositions of the invention are formulated to slowly release the active ingredients, including the fusion proteins of the invention, in the GI system using known delayed release formulations.

The fusion proteins of the invention for oral delivery are capable of binding the receptor found in the GI epithelium. To facilitate this binding and receptor mediated transport, fusion proteins of the invention are typically produced with iron and in some instances carbonate, bound to the moiety. Processes and methods to load the moiety of the fusion protein compositions of the invention with iron and carbonate are known in the art.

In some pharmaceutical formulations of the invention, the TF moiety of the fusion protein may be modified to increase the affinity or affinity of the moiety to iron. Such methods are known in the art. For instance, mutagenesis can be used to produce mutant transferrin moieties that bind iron more avidly than natural transferrin. In human serum transferrin, the amino acids which are ligands for metal ion chelation include, but are not limited to N lobe amino acids Asp63, Tyr 95, TyM88, Lys206, His207 and His249; and C lobe amino acids Asp392, Tyr426, Tyr517 and His585 of SEQ ID NO: 3 (the number beside the amino acid indicates the position of the amino acid residue in the primary amino acid sequence where the valine of the mature protein is designated position 1). See U.S. Patent
5,986,067, which is herein incorporated by reference. In one embodiment, the Lys206 and His207 residues within the N lobe are replaced with Gln and Glu, respectively.

In some pharmaceutical formulations of the invention, the fusion protein is engineered to contain a cleavage site between the therapeutic protein or peptide and the Tf moiety. Such cleavable sites or linkers are known in the art.


In preferred embodiments of the invention, oral pharmaceutical formulations include fusion proteins comprising a modified Tf moiety exhibiting reduced or no glycosylation fused at the N terminal end to a melanocortin protein or peptide as described above.

The effective dose of fusion protein may be measured in a numbers of ways, including dosages calculated to alleviate symptoms associated with a specific disease state in a patient, such as the symptoms of inflammation. In other instances, the pharmaceutical compositions are formulated and methods of administration modified to detect an increase in the activity level of the melanocortin peptide or melanocortin receptor agonist. Such formulations and methods may deliver between about 1 µg to about 150 mg/kg body weight of fusion protein, about 100 ng to about 100 µg/kg body weight of fusion protein, about 100 µg/kg to about 100 mg/kg body weight of fusion protein, about 1 µg to about 1 g of fusion protein, about 10 µg to about 100 mg of fusion protein or about 10 mg to about 50 mg of fusion protein. In one embodiment, the effective dose is 300 µg/kg to 900 µg/kg. In another embodiment, the effective dose is administered weekly for a total dose of about 50 mg, 100 mg, or 150 mg.

Formulations for effective dose may also be calculated using a unit measurement of therapeutic protein activity, such as about 5 to about 500 units of human α-MSH or about 10 to about 100 units of human α-MSH. The measurements by weight or activity can be calculated using known standards for each therapeutic protein or peptide fused to Tf.

The invention also includes methods of orally administering the pharmaceutical compositions of the invention. Such methods may include, but are not limited to, steps of orally administering the compositions by the patient or a caregiver. Such administration steps may include administration on intervals such as once or twice per day depending on the fusion protein, disease or patient condition or individual patient. Such methods also include the administration of various dosages of the individual fusion protein. For instance,
the initial dosage of a pharmaceutical composition may be at a higher level to induce a
desired effect. Subsequent dosages may then be decreased once a desired effect is
achieved. These changes or modifications to administration protocols may be done by the
attending physician or health care worker.

The invention also includes methods of producing oral compositions or medicant
compositions of the invention comprising formulating a fusion protein of the invention into an
orally administerable form. In other instances, the invention includes methods of producing
compositions or medicant compositions of the invention comprising formulating a fusion
protein of the invention into a form suitable for oral administration.

Moreover, the present invention includes pulmonary delivery of the fusion protein
formulations. Pulmonary delivery is particularly promising for the delivery of macromolecules
which are difficult to deliver by other routes of administration. Such pulmonary delivery can
be effective both for systemic delivery and for localized delivery to treat diseases of the
lungs, since drugs delivered to the lung are readily absorbed through the alveolar region
directly into the blood circulation.

The present invention provides compositions suitable for forming a drug dispersion
for oral inhalation (pulmonary delivery) to treat various conditions or diseases, including, but
not limited to, asthma. The fusion protein formulation could be delivered by different
approaches such as liquid nebulizers, aerosol-based metered dose inhalers (MDI's), and dry
powder dispersion devices. In formulating compositions for pulmonary delivery,
pharmaceutically acceptable carriers including surface active agents or surfactants and bulk
carriers are commonly added to provide stability, dispersibility, consistency, and/or bulking
characteristics to enhance uniform pulmonary delivery of the composition to the subject.

Surface active agents or surfactants promote absorption of polypeptide through
mucosal membrane or lining. Useful surface active agents or surfactants include fatty acids
and salts thereof, bile salts, phospholipid, or an alkyl saccharide. Examples of fatty acids
and salts thereof include sodium, potassium and lysine salts of caprylate (C\textsubscript{8}), caprate (C\textsubscript{10}),
laurate (C\textsubscript{12}) and myristate (C\textsubscript{14}). Examples of bile salts include cholic acid,
chenodeoxycholic acid, glycocholic acid, taurocholic acid, glycochenodeoxycholic acid,
taurochenodeoxycholic acid, deoxycholic acid, glycodeloxycholic acid, taurodeoxycholic acid,
lithocholic acid, and ursodeoxycholic acid.

Examples of phospholipids include single-chain phospholipids, such as
lysophosphatidylcholine, lysophosphatidylglycerol, lysophosphatidylethanolamine,
lysophosphatidylinositol and lysophosphatidylserine; or double-chain phospholipids, such as
diacylphosphatidylcholines, diacylphosphatidylglycerols, diacylphosphatidylethanolamines,
diacylphosphatidylinositols and diacylphosphatidylserines. Examples of alkyl saccharides include alkyl glucosides or alkyl maltosides, such as decyl glucoside and dodecyl maltoside.

Pharmaceutical excipients that are useful as carriers include stabilizers such as human serum albumin (HSA); bulking agents such as carbohydrates, amino acids and polypeptides; pH adjusters or buffers; salts such as sodium chloride; and the like. These carriers may be in a crystalline or amorphous form or may be a mixture of the two.

Examples of carbohydrates for use as bulking agents include monosaccharides such as galactose, D-mannose, sorbose, and the like; disaccharides, such as lactose, trehalose, and the like; cyclodextrins, such as 2-hydroxypropyl-beta-cyclodextrin; and polysaccharides, such as raffinose, maltodextrins, dextran; and the like; alditols, such as mannitol, xylitol, and the like. Examples of polypeptides for use as bulking agents include aspartame. Amino acids include alanine and glycine, with glycine being preferred.

Additives, which are minor components of the composition, may be included for conformational stability during spray drying and for improving dispersibility of the powder. These additives include hydrophobic amino acids such as tryptophan, tyrosine, leucine, phenylalanine, and the like.

Suitable pH adjusters or buffers include organic salts prepared from organic acids and bases, such as sodium citrate, sodium ascorbate, and the like; sodium citrate is preferred.

The fusion compositions for pulmonary delivery may be packaged as unit doses where a therapeutically effective amount of the composition is present in a unit dose receptacle, such as a blister pack, gelatin capsule, or the like. The manufacture of blister packs or gelatin capsules is typically carried out by methods that are generally well known in the packaging art.

U.S. Patent 6,524,557 discloses a pharmaceutical aerosol formulation comprising (a) a HFA propellant; (b) a pharmaceutically active polypeptide dispersible in the propellant; and (c) a surfactant which is a C₈-C₁₆ fatty acid or salt thereof, a bile salt, a phospholipid, or an alkyl saccharide, which surfactant enhances the systemic absorption of the polypeptide in the lower respiratory tract. The invention also provides methods of manufacturing such formulations and the use of such formulations in treating patients.

One approach for the pulmonary delivery of dry powder drugs utilizes a hand-held device with a hand pump for providing a source of pressurized gas. The pressurized gas is abruptly released through a powder dispersion device, such as a venturi nozzle, and the dispersed powder made available for patient inhalation.
Dry powder dispersion devices are described in several patents. U.S. Pat. No. 3,921,637 describes a manual pump with needles for piercing through a single capsule of powdered medicine. The use of multiple receptacle disks or strips of medication is described in European Patent Application No. EP 0 467 172; International Patent Publication Nos. WO 91/02558; and WO 93/09832; U.S. Pat. Nos. 4,627,432; 4,811,731; 5,035,237; 5,048,514; 4,446,862; 5,048,514, and 4,446,862.


The present invention provides formulating fusion protein for oral inhalation. The formulation comprises fusion protein and suitable pharmaceutical excipients for pulmonary delivery. The present invention also provides administering the fusion protein composition via oral inhalation to subjects in need thereof.

The invention also includes the administration of labeled fusion proteins to a subject. For instance, the invention includes the administration of a fusion protein labeled with a radioactive metal such as $^{67}$Ga, $^{68}$Ga, $^{99}$Tc or a paramagnetic metal such as $^{57}$Fe for treatment of diseases associated with cells expressing or over-expressing melanocortin receptors.

**Transgenic Animals**

The production of transgenic non-human animals that contain a fusion construct with increased serum half-life increased serum stability or increased bioavailability of the instant invention is contemplated in one embodiment of the present invention. In some embodiments, lactoferrin may be used as the Tf portion of the fusion protein so that the fusion protein is produced and secreted in milk.

The successful production of transgenic, non-human animals has been described in a number of patents and publications, such as, for example U.S. Patent 6,291,740 (issued September 18, 2001); U.S. Patent 6,281,408 (issued August 28, 2001); and U.S. Patent 6,271,436 (issued August 7, 2001) the contents of which are hereby incorporated by reference in their entireties.

The ability to alter the genetic make-up of animals, such as domesticated mammals including cows, pigs, goats, horses, cattle, and sheep, allows a number of commercial applications. These applications include the production of animals which express large quantities of exogenous proteins in an easily harvested form (e.g., expression into the milk
or blood), the production of animals with increased weight gain, feed efficiency, carcass composition, milk production or content, disease resistance and resistance to infection by specific microorganisms and the production of animals having enhanced growth rates or reproductive performance. Animals which contain exogenous DNA sequences in their genome are referred to as transgenic animals.


An alternative means for infecting embryos with retroviruses is the injection of virus or virus-producing cells into the blastocoele of mouse embryos (Jahner, D. et al., 1982, Nature 298: 623). The introduction of transgenes into the germline of mice has been reported using intrauterine retroviral infection of the midgestation mouse embryo (Jahner et al., supra). Infection of bovine and ovine embryos with retroviruses or retroviral vectors to create transgenic animals has been reported. These protocols involve the micro-injection of retroviral particles or growth arrested (i.e., mitomycin C-treated) cells which shed retroviral particles into the perivitelline space of fertilized eggs or early embryos (PCT International Application WO 90/08832 and Haskell and Bowen, 1995, Mol. Reprod. Dev., 40: 386). PCT International Application WO 90/08832 describes the injection of wild-type feline leukemia virus B into the perivitelline space of sheep embryos at the 2 to 8 cell stage. Fetuses derived from injected embryos were shown to contain multiple sites of integration.

U.S. Patent 6,291,740 (issued September 18, 2001) describes the production of transgenic animals by the introduction of exogenous DNA into pre-maturation oocytes and mature, unfertilized oocytes (i.e., pre-fertilization oocytes) using retroviral vectors which transduce dividing cells (e.g., vectors derived from murine leukemia virus [MLV]). This patent also describes methods and compositions for cytomegalovirus promoter-driven, as well as mouse mammary tumor LTR expression of various recombinant proteins.

U.S. Patent 6,281,408 (issued August 28, 2001) describes methods for producing transgenic animals using embryonic stem cells. Briefly, the embryonic stem cells are used in a mixed cell co-culture with a morula to generate transgenic animals. Foreign genetic
material is introduced into the embryonic stem cells prior to co-culturing by, for example, electroporation, microinjection or retroviral delivery. ES cells transfected in this manner are selected for integrations of the gene via a selection marker such as neomycin.

U.S. Patent 6,271,436 (issued August 7, 2001) describes the production of transgenic animals using methods including isolation of primordial germ cells, culturing these cells to produce primordial germ cell-derived cell lines, transforming both the primordial germ cells and the cultured cell lines, and using these transformed cells and cell lines to generate transgenic animals. The efficiency at which transgenic animals are generated is greatly increased, thereby allowing the use of homologous recombination in producing transgenic non-rodent animal species.

Gene Therapy

The use of fusion constructs for gene therapy wherein a Tf or mTf domain is joined to a melanocortin peptide or melanocortin receptor agonist is contemplated in one embodiment of this invention. The fusion constructs with increased serum half-life or serum stability of the instant invention are ideally suited to gene therapy treatments.

The successful use of gene therapy to express a soluble fusion protein has been described. Briefly, gene therapy via injection of an adenovirus vector containing a gene encoding a soluble fusion protein consisting of cytotoxic lymphocyte antigen 4 (CTLA4) and the Fc portion of human immunoglobulin G1 was recently shown in Ijima et al. (June 10, 2001) Human Gene Therapy (United States) 12/9: 1063-77. In this application of gene therapy, a murine model of type II collagen-induced arthritis was successfully treated via intraarticular injection of the vector.

Gene therapy is also described in a number of U.S. patents including U.S. Pat. 6,225,290 (issued May 1, 2001); U.S. Pat. 6,187,305 (issued February 13, 2001); and U.S. Pat. 6,140,111 (issued October 31, 2000).

U.S. Patent 6,225,290 provides methods and constructs whereby intestinal epithelial cells of a mammalian subject are genetically altered to operatively incorporate a gene which expresses a protein which has a desired therapeutic effect. Intestinal cell transformation is accomplished by administration of a formulation composed primarily of naked DNA, and the DNA may be administered orally. Oral or other intragastrointestinal routes of administration provide a simple method of administration, while the use of naked nucleic acid avoids the complications associated with use of viral vectors to accomplish gene therapy. The expressed protein is secreted directly into the gastrointestinal tract and/or blood stream to obtain therapeutic blood levels of the protein thereby treating the patient in need of the
protein. The transformed intestinal epithelial cells provide short or long term therapeutic
cures for diseases associated with a deficiency in a particular protein or which are amenable
to treatment by overexpression of a protein.

U.S. Pat. 6,187,305 provides methods of gene or DNA targeting in cells of vertebrate,
particularly mammalian, origin. Briefly, DNA is introduced into primary or secondary cells of
vertebrate origin through homologous recombination or targeting of the DNA, which is
introduced into genomic DNA of the primary or secondary cells at a preselected site.

U.S. Pat. 6,140,111 (issued October 31, 2000) describes retroviral gene therapy
vectors. The disclosed retroviral vectors include an insertion site for genes of interest and
are capable of expressing high levels of the protein derived from the genes of interest in a
wide variety of transfected cell types. Also disclosed are retroviral vectors lacking a
selectable marker, thus rendering them suitable for human gene therapy in the treatment of
a variety of disease states without the co-expression of a marker product, such as an
antibiotic. These retroviral vectors are especially suited for use in certain packaging cell
lines. The ability of retroviral vectors to insert into the genome of mammalian cells has made
them particularly promising candidates for use in the genetic therapy of genetic diseases in
humans and animals. Genetic therapy typically involves (1) adding new genetic material to
patient cells in vivo, or (2) removing patient cells from the body, adding new genetic material
to the cells and reintroducing them into the body, i.e., in vitro gene therapy. Discussions of
how to perform gene therapy in a variety of cells using retroviral vectors can be found, for
example, in U.S. Pat. Nos. 4,868,116, issued Sep. 19, 1989, and 4,980,286, issued Dec. 25,
378,576 published Jul. 25, 1990 (fibroblast cells), and WO 89/05345 published Jun. 15, 1989
and WO/90/06997, published Jun. 28, 1990 (endothelial cells), the disclosures of which are
incorporated herein by reference.

Kits Containing Fusion Proteins

In a further embodiment, the present invention provides kits containing Tf and
melanocortin fusion proteins, which can be used, for instance, for therapeutic or non-
therapeutic applications. The kit comprises a container with a label. Suitable containers
include, for example, bottles, vials, and test tubes. The containers may be formed from a
variety of materials such as glass or plastic. The container holds a composition which
includes a fusion protein that is effective for therapeutic or non-therapeutic applications, such
as described above. The active agent in the composition is the therapeutic protein. The
label on the container indicates that the composition is used for a specific therapy or non-
therapeutic application, and may also indicate directions for either in vivo or in vitro use, such as those described above.

The kit of the invention will typically comprise the container described above and one or more other containers comprising materials desirable from a commercial and user standpoint, including buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

Without further description, it is believed that a person of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the present invention and practice the claimed methods. For example, a skilled artisan would readily be able to determine the biological activity, both in vitro and in vivo, for the fusion protein constructs of the present invention as compared with the comparable activity of the therapeutic moiety in its unfused state. Similarly, a person skilled in the art could readily determine the serum half life and serum stability of constructs according to the present invention. The following working examples therefore, specifically point out the preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

EXAMPLES

**Example 1: α-MSH (PEAPTD)_2 mTf Fusion Protein**

A melanocortin and transferrin fusion protein was prepared containing α-MSH and modified transferrin and a (PEAPTD)_2 linker (SEQ ID NO.: 10). Primers were designed, P1999 and P2000, to insert the α-MSH sequence along with the (PEAPTD)_2 linker sequence (SEQ ID NO.: 10) between the signal sequence (nl_) and mTf sequence by single overlap extension (SOE) PCR.

```
P0177 >
GC GATAAAAGAC GCCATIG
661 tgcgggagtgcgggggcaa acacaccgc gcataagagc gcagatgata taahaagggg
cgcctcac cgccccttgc tgtggtggtcg ctatttcg ctatttcgctatttccttatttttttc
35
AFIII
721 ccaatgttaccttgattgc tttccattaca aacttaagag tcaatattgc
gtattcag tgcctgctc aacccctgag aaggtgttgc tgaattctc aggttaacg
40
781 ttcagccgta ataaaaaac aagctaaaac ttaatttaca aaccaagat gaggctgccc
aagtagccgt tatattttttg ttcgatttgc attaaatgtg tgcttttcac ctccgagccc
```
m r l a

841 gtggagcccc tgtggtcctg cgccgtcctg gggcgtctta ttctatggaa
caccctcggg agccagagc'gccccgagc cccagacacgiccctgagat aagatacctt

SCAGGA C GCCGACAgAIi GCCGAGAATI AAGATACCTT

P2000

v g a M v c a v l g c l a s y m e

P1999

GAT1 GGGGTAAACC AGTTCCAGAA GCTCCACTCT AGTCCAGAAGC

901 cattttgat gggtcataa agttccagaa gttcacaactg tccaactgt

gttaacta ccctattgtgagctt cagagctt taggttctcg agagctacga

GTAAAGTIAI UCCCCATTIGG II CMMAGFCT II CGAG

P2000

......alpha-MSH ......» " ........(PEAPTID)2 .........»
h f r w g k p v e a p t d p e a p t d

P1999 >

GTAI 'O/GATA AAACCT/C/GAG A/G

961 gtacctgata aacatgtag atgttgctga gttgccgac ataggccag taagtgccag
catgactat ttgacactc taccagctg taccagctg attcagcgtc

» "...................mTf. .....................»

v p d k t v r w c a v s e h a t k c q

30

1021 agtttcccgcc acatataa acagctcatt ccaatcggac atggactgtc tccaagggc cggattactt tggatcagc cagggact cacaagacac

>...........................mTf. .....................»

s f r d h m k s v i p s d g p s v a c v

35

1081 aagaaagcct cctaccttgga tgtcattcag gcctattcgg gaagaagcg ggtgtctgtg
tcttctgc ggatggaaact aagctgtcct cggtagccaag cccattcacc cccacacac

>...........................mTf. .....................»

k k a s y l d c i r a i a a n e a d a v

40

1141 acatgtggct caggtttgcgt ttgatcagct tacatggtct ccaataaccg aagggtcttg
tgtcttctc gcacagacca cataactcg atggcgc ggtttta ctcgacacac

>...........................mTf. .....................»

t l d a g l v d y d a y l a p n l k p v

45

BamHI

1201 gtggcgagtgt cttatttgct aaaaagaggt ccaacagactt cttatatgc tggctgttg
caccgtcctga agataacccct ttttctctca ggtgtgctga aagataatacg aacaacagc

>...........................mTf. .....................»

va e f y g s k e d p q f t f y y a v a v

1261 gttgagaagc atagttgcct ccaatgacaac cagccgctgct gcaagaagct ctcgccacacg
cacattcc tcccaacag ccacgacagc ggttatcgtc gttcctgac cagggcgtgc

>...........................mTf. .....................»

v k k d s g f a m n q l r g k k s c h t

1321 ggtcttaggca ggtccgctgg gttggaacatc ccctaggtct tacatcactc
tcagctcgtt ccacgcgacc caccttgtag ggttatcpga atgaaatgac actgaatgga
Initially two PCR products were created using a primer 5' of the AfIW site (P0177) with P2000 or a primer 3' of the SamHI site (P0014) with P1999. The plasmid pREX0549 was used as the template. The products from these reactions were gel purified and joined using only the outer primers, P0177 and P0014, in a second round of PCR.

The product from this second reaction was gel purified and digested with the restriction enzymes AfIW and SamHI, as was the plasmid pREX0549. The appropriate products from these reactions were ligated together to give pREX1075, which was DNA sequenced between the AfIW and SamHI sites to confirm correct insertion of the α-MSH sequence. The expression cassette was recovered from pREX1075 by restriction enzyme digestion with NotI and ligated into NotI-digested, alkaline phosphatase-treated pSAC35 to give pREX1097.

Example 2: mTf (PEAPTD)_2 α-MSH Fusion Protein

Oligonucleotides P2001 and P2002 were designed to insert the α-MSH sequence along with the (PEAPTD)_2 linker sequence (SEQ ID NO.: 10) at the C-terminus of mTf by
annealing of the oligonucleotides and ligation of the resulting product into Sa/I/H/ndlll restriction enzyme-digested pREX0549. When annealed, the double stranded DNA had 5' and 3' ends compatible with Sa/I and H/ndlll sites. The resultant plasmid pREX1095 was DNA sequenced between the Sa/I and Hindlll sites to confirm correct insertion of the α-MSH sequence. The expression cassette was recovered from pREX1095 by restriction enzyme digestion with NotI and ligated into Xofl-digested, alkaline phosphatase-treated pSAC35 to give pREX1096.

P2001

| TCGACCTCCAGAAGCTCCAACTGATCCAGAAGCTCCAACTGATTCTTATTCTATGGAACATTTTAGATGGGGTAAACCAGTTTAATA |
|---------------------------------------------|---------------------------------------------|

DNA sequence (SEQ ID NO.: 236)
Amino acid sequence (SEQ ID NO.: 237)

P2001 (SEQ ID NO.: 238)
TCGACCTCCAGAAGCTCCAACTGATCCAGAAGCTCCAACTGATTCTTATTCTATGGAACATTTTAGATGGGGTAAACCAGTTTAATA
Example 3: γ₂ Fusion Proteins

Constructs were also generated for N- and C-terminal γ₂-MSH fusions using the same procedures as described above and the following primers.

**γ-MSH (PEAPTD)₂ mTf**

P2003 (SEQ ID NO.: 240)
GATGGGATAGATTTGGTCCAGAAGCTCCAACTGATCCAGAAGCTCCAACTGATG
TACCTGATAAAACTGTGAGATG

P2004 (SEQ ID NGv. 241)
GAGCTTCTGGACCAAATCTATCCCATCTAAAATGACCCATAACATACGCCAGAC
ACAGCCCCAGGACG

**mTf (PEAPTD)₂ γ-MSH (pREX1078/pREX1080)**

P2005 (SEQ ID NO.: 242)
TCGACCTCCAGAAGCTCCAACTGATCCAGAAGCTCCAACTGATTATGTTATGGG
TCATTTTAGATGGGATAGATTTGGTTAATA

P2006 (SEQ ID NO.: 243)
AGCTTATTAAACTGGTTTACCCCATCTAAAATGTTCCATAGAATAAGAATCAGTT
GGAGCTTCTGGATCAGTTGGAGCTTCTGGAGG

**N-Terminal γ-MSH Fusion Protein**

Briefly, primers P2003 and P2004 were designed to insert the γ-MSH sequence along with the (PEAPTD)₂ linker sequence (SEQ ID NO.: 10) between the signal sequence (nl_) and mTf sequence by single overlap extension (SOE) PCR. The plasmid pREX0549 was used as the template. The products from these reactions were gel purified and joined using only the outer primers, P0177 and P0014, in a second round of PCR.

The product from this second reaction was gel purified and digested with the restriction enzymes AfiI and BamHI, as was the plasmid pREX0549. The appropriate products from these reactions were ligated together to give pREX1076, which was DNA sequenced between the AfiI and BamYV sites to confirm correct insertion of the γ-MSH sequence. The expression cassette was recovered from pREX1076 by restriction enzyme digestion with NofI and ligated into NofI-digested, alkaline phosphatase-treated pSAC35 to give pREX1 102.

**C-Terminal γ-MSH Fusion Protein**
Oligonucleotides P2005 and P2006 were designed to insert the γ-MSH sequence along with the (PEAPTD)_2 linker sequence (SEQ ID NO.: 10) at the C-terminus of mTf by annealing of the oligonucleotides and ligation of the resulting product into Sa/I/HndIII restriction enzyme-digested pREX0549. When annealed, the double stranded DNA had 5' and 3' ends compatible with Sa/I and HndIII sites. The resultant plasmid pREX1078 was DNA sequenced between the Sa/I and HindWl sites to confirm correct insertion of the γ-MSH sequence. The expression cassette was recovered from pREX1078 by restriction enzyme digestion with NotI and ligated into NotI-digested, alkaline phosphatase-treated pSAC35 to give pREX1080.

Example 4: Fusion Proteins with αMSH inserted in mTf

mTfaMSH 279

A fusion protein was prepared by inserting α-MSH at amino acid 279 of mTf. Using pREX0549 as a template the primers P0177 and P2095 or P2096 and P0012 were used to generate PCR products. These products were then joined together in a subsequent PCR reaction using just the outer primers P0177 and P0012.

The resulting PCR product (Figure 2) and pREX0549 were then digested with the restriction enzymes SamHI and EcoRI, and the PCR product ligated into the backbone of the pREX0549 plasmid to give pREX1230. The plasmid was DNA sequenced between the βSamHI and EcoRI sites to confirm correct insertion and the absence of PCR induced errors. The NotI expression was recovered from pREX1230 and ligated into pSAC35 digested with NotI and treated with alkaline phosphatase to give the plasmid pREX1199.

P0177 (SEQ ID NO.: 234)
GCGATAAAAGAGCGCGATG

P2095 (SEQ ID NO: 244)
CATCTAAAATGTTCCATAGAATAAGATTTGTCTTTGCCAAAATGTTCC

P2096 (SEQ ID NO.: 245)
CTATGGAACATTTAGATGGGGTAAACCAGTTTCAAAAGAATTCCAACTATTC

P0012 (SEQ ID NO.: 246)
CATGATCTTGGCGATGCAGTC

DNA sequence (SEQ ID NO.: 247)
Amino acid sequence (SEQ ID NO.: 248)
mTfa-MSH 166

Using the same methods as described above, a fusion protein was created by
inserting α-MSH at position 166 of mTf using primers P2097 and P2097. The PCR product
was ligated between SamHI and EcoRI of pREX0549.

P2097 (SEQ ID NO.: 249)
CATCTAAAATGTTCATAGAATAAGACGTCCCATCCGCACAAGG

P2098 (SEQ ID NO.: 250)
CTATGGAACATTTAGATGGGGTAAACCAGTTCATGGGAAGGACCTGCTG

mTfa-MSH 289

A fusion protein was also created by inserting α-MSH at position 289 of mTf using
primers P2093 and P2094 as described above. The resulting PCR product was ligated
between EcoRI and BspEI of pREX0549.

P2093 (SEQ ID NO.: 251)
CATCTAAAATGTTCATAGAATAAGAAGGAGAGCTGAATAGTTGG

P2094 (SEQ ID NO.: 252)
CTATGGAACATTTAGATGGGGTAAACCAGTTCATGGGAAGGACCTGCTG

Combinations of Inserts

α-MSH can be inserted at more than one position in mTf using the primers and
methods described above. For example, a fusion protein containing α-MSH inserted at
positions 166 and 279 can be designed by using pREX01230 as the template for the PCR
reactions and by using the primers for the 166 insertion, i.e., P2097 and P2098.
Example 5: Fusion Proteins with γ-MSH inserted in mTf

mTf Y-MSH 279

A fusion protein was prepared by inserting γ-MSH at amino acid 279 of mTf. Using pREX0549 as a template the primers P0669 and P2210 or P2209 and P0012 were used to generate PCR products. These products were then joined together in a subsequent PCR reaction using just the outer primers P0669 and P0012.

The resulting PCR product and pREX0549 were then digested with the restriction enzymes AflII and EcoRI, and the PCR product ligated into the backbone of the pREX0549 plasmid to give pREX1219. The plasmid was DNA sequenced between the AflII and EcoRI sites to confirm correct insertion and the absence of PCR induced errors. The NotI expression was recovered from pREX1219 and ligated into pSAC35 digested with NotI and treated with alkaline phosphatase to give the plasmid pREX1229.

P0699 (SEQ ID NO.: 253)
CCGCGATAAAAGAGCGCGATG

P2209 (SEQ ID NO.: 254)
TATGTTATGGGTCAATTCTAGGGAGGATAGTTTGTAAGAGATTTCCAACATTACAGCTCT C

P2210 (SEQ ID NO.: 255)
ACCAAATCTATCCCATCTAAATGACCCATAACATATGATTTGTCTTTGCCAAAATGTTC CTG

P0012 (SEQ ID NO.: 246)
CATGATCTTGGCGATGCAGTC

DNA sequence (SEQ ID NO.: 256)

Amino acid sequence (SEQ ID NO.: 257)

961 aagtatgggc ggcaaggagg acttgatctg ggagctttct aaccaggccc aggaacattt

< P2210

> mTf .........................

r s m g k e d l i w e l l n q a q e h

EcoRI

P2209

TATGTTATGGGTCAATTCTAGGGAGGATAGTTTGTAAGAGATTTCCAACATTACAGCTCT

P2210

> mTf .........................

f g k d k s y v m g h f r w d r f g k e

" ...........279 g-MSH ..........."
Using the same methods as described above, a fusion protein was created by inserting γ-MSH at position 166 of mTf using primers P2207 and P2208. The PCR product was ligated between AfIW and EcoRI of pREX0549.

P2207 (SEQ ID NO.: 258)
TATGTTATGGGTCACTTTAGATGAGTAGATTGACTTCCCCAGCTGTTTAAGTG

P2208 (SEQ ID NO.: 259)
ACCAAATCTATCCCATCTAAAATGACCCATAACCATACGTCCCCATCCGCAACAGGGGCAC

A fusion protein was also created by inserting γ-MSH at position 289 of mTf using primers P2211 and P2212 as described above. The resulting PCR product was ligated between EcoRI and SspEl of pREX0549.

P2211 (SEQ ID NO.: 260)
TATGTTATGGGTCACTTTAGATGAGTAGATTGACTTCCCCAGCTGTTTAAGTG

P2212 (SEQ ID NO.: 261)
CCCATCTAAATGACCCATAACCATACGTGAGAGCTGAATAGTTGGAATTCTTTTG

Y-MSH can be inserted at more than one position in mTf using the primers and methods described above. For example, a fusion protein containing γ-MSH inserted at positions 166 and 279 can be designed by using pREX01219 as the template for the PCR reactions and by using the primers for the 166 insertion, i.e., P2207 and P2208.

Example 6: GLP-1 (7-37; A8G, K34A) (PEAPTD)_2 mTf (PEAPTD)_2 α-MSH Fusion Protein

To make a fusion comprising GLP-1 at the N-terminus of mTf and α-MSH at the C-terminus of mTf, the plasmids pREX0584 and pREX1095 were digested with the restriction enzymes EcoRI and C/al. The 5027bp fragment of pREX0584 was ligated with the 1641bp fragment of pREX1095 to give pREX1 148. The expression cassette was recovered from
pREX1 148 by restriction enzyme digestion with NotI and ligated into NotI-digested, alkaline phosphatase-treated pSAC35 to give pREX1 149.

In a similar fashion an exendin-4(1-39) (PEAPTD)₂ mTf (PEAPTD)₂ α-MSH construct could be made by ligation of the 5021 bp EcoRI/C/al fragment of pREX0935 with the 1641bp EcoRI/C/al fragment of pREX1095. Similarly, the 1638bp EcoRI/C/al fragment of pREX1078 could be used to make a version of GLP-1 or exendin-4 N-terminal mTf fusion with γ₂-MSH at the C-terminus.

Example 7: Activity of mTf-(PEAPTD)₂-αMSH in Melanin Biosynthesis Assay

Mouse melanoma B16-F10 cells were seeded at 2.5x10³ cells per well in medium lacking phenol red into a 96-well plate. On day 2, the medium was supplemented with fresh medium containing mTf-(PEAPTD)₂-αMSH fusion protein or α-MSH peptide. On day 4, the medium was removed and the accumulation of melanin pigment was determined by measuring the absorbance at 405 nm. The fusion protein showed a similar potency to α-MSH peptide in this assay (Figure 4).

Example 8: Activity of mTf-(PEAPTD)₂-αMSH in cAMP Assay

Mouse melanoma B16-F10 cells were seeded at 3x10⁴ cells per well into a 96-well plate. On day 2, medium was replaced with Krebs Ringer Buffer supplemented with glucose (KRBG) containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX). After 30 min, α-MSH peptide or mTf-(PEAPTD)₂-α-MSH in KRBG with 0.5 mM IBMX was added to the cells and incubated for an additional 30 min. Cells were then lysed and intracellular cAMP was assayed using the CatchPoint cAMP fluorescent assay (Molecular Devices Corporation) according to the manufacturer’s instructions. The fusion protein showed a similar potency to α-MSH peptide in this assay (Figure 5).

Example 9: IFNβ-1a mTf (PEAPTD)₂ α-MSH in cAMP Assay

Using pREX1095 as the template a PCR product was created using the primer P0007 and the mutagenic primer P2240. This was to modify the sequence 3’ of the α-MSH sequence for it to be inserted in the IFNβ-1a mTf construct in pREX1007. The PCR product and pREX1007 were both digested with the restriction enzymes SsO and EcoRV. The products of these reactions were ligated together to create pREX1 200.
P2240 (SEQ ID NO.: 262)
GGCCGCCACTGTGCTGGATATCTGCAGAATTGTCTTGACCCTTTTAAAC
TGGTTTACCCCATCTAAATG

P0007 (SEQ ID NO.: 263)
TGGCTCCACAAGATATTACG

DNA sequence (SEQ ID NO.: 264)

Amino acid sequence (SEQ ID NO.: 265)

3241 atcacgctgt ggtcacacgg aaagataagg aacgtgctgt ccacaagata ttacgtaac
3301 agcagcacct atttggaagg aacgtgctgg ggacactttgt tttgcttgcgt
3361 cggaaaccaa ggaccttctg tcagagatg acacgtaatg ttggccaaa ttgacgaca
3421 gaaacacata tggaaaaatc ttaggagaag aatgctgctg gcctttggg aacctgagaa
3481 aatgctccac ctcatcactc tggaaacgct gcactttcg ggacaccaact cggacggcag
3541 ctgatccaga agctccaaact gattccttt cttgacgagg agatgctgat cgggaagcag

EcoRV NotI XbaI

3601 taaaaggg tcaacaaat tttctgctgg tttggtctgg gcgttgctgg gcgttgctgg gcgttgctgg
Example 10: Therapeutic effect of MSH mTf in treating myocardial infarction

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>IA/RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A (control)</td>
<td>24</td>
<td>Infarct =58.4±1.1% of the area at risk</td>
</tr>
<tr>
<td>Group B (pre-ischemic Rx(GLP-1-Tf))</td>
<td>10</td>
<td>Infarct =45.7±1.9% of the area at risk</td>
</tr>
<tr>
<td>Group C (post-ischemic Rx(GLP-1-Tf))</td>
<td>10</td>
<td>Infarct =44.1±3.3% of the area at risk</td>
</tr>
<tr>
<td>Group D (post-ischemic Rx(MSH-Tf))</td>
<td>10</td>
<td>Infarct =44.8±2.2% of the area at risk</td>
</tr>
<tr>
<td>Group E (post-ischemic Rx(just Tf))</td>
<td>4</td>
<td>Infarct =59.7±2.0% of the area at risk</td>
</tr>
</tbody>
</table>

Group D - 10 mg/kg BRX1 164

Group E - 10 mg/kg transferrin

Although the present invention has been described in detail with reference to examples above, it is understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims. All cited patents, patent applications and publications referred to in this application are herein incorporated by reference in their entirety.
We Claim:

1. A fusion protein comprising a transferrin (Tf) protein and a melanocortin peptide.
2. The fusion protein of claim 1, wherein said melanocortin peptide is selected from the group consisting of alpha-melanocortin (α-MSH), beta-melanocortin (β-MSH), ACTH and gamma-melanocortin (γ-MSH) or a derivative or analog thereof.
3. The fusion protein of claim 2, wherein said melanocortin peptide is α-MSH.
4. The fusion protein of claim 2, wherein said melanocortin peptide is β-MSH.
5. The fusion protein of claim 2, wherein said melanocortin is gamma-melanocortin (γ-MSH).
6. The fusion protein of claim 1, wherein said Tf protein is modified to exhibit reduced glycosylation.
7. The fusion protein of claim 6, wherein the modification is within or adjacent to an N-linked glycosylation site comprising the sequence N-X-S/T which corresponds to amino acid N413 or N611 of SEQ ID NO: 3.
8. The fusion protein of claim 1, wherein the Tf protein has reduced affinity for a Tf receptor (TfR).
9. The fusion protein of claim 1, wherein the Tf protein is modified to exhibit reduced affinity for iron.
10. The fusion protein of claim 1, wherein the melanocortin peptide is fused to the N-terminal end and/or C-terminal end of Tf.
11. The fusion protein of claim 1, wherein the melanocortin peptide is inserted into at least one loop of the Tf.
12. The fusion protein of claim 1, wherein said melanocortin peptide is inserted between an N and a C domain of the Tf.
13. The fusion protein of claim 12, wherein the melanocortin peptide is inserted between one or more adjacent amino acids selected from the group consisting of amino acids 32-33, 74-75, 166-167, 256-257, 279-280 and 288-289 of SEQ ID NO: 3.
14. The fusion protein of claim 1, wherein the Tf has at least one amino acid substitution, deletion, or addition in the hinge region, wherein said hinge region is selected from the group consisting of about residue 94 to about residue 96, about residue 245 to about residue 247, about residue 316 to about residue 318, about residue 425 to about residue 427, about residue 581 to about residue 582 and about residue 652 to about residue 658 of SEQ ID NO: 3.
15. The fusion protein of claim 1, wherein said Tf protein has at least one amino acid substitution, deletion or addition at a position selected from the group consisting of Asp
63, Gly 65, Tyr 95, Tyr 188, Lys 206, His 207, His 249, Asp 392, Tyr 426, Tyr 514, Tyr 517, His 585, Thr 120, Arg 124, Ala 126, Gly 127, Thr 452, Arg 456, Ala 458 and Gly 459 of SEQ ID NO.: 3.

16. The fusion protein of claim 18, wherein the Tf comprises at least one amino acid substitution, deletion or addition at an amino acid residue corresponding to an amino acid selected from the group consisting of Asp 63, Gly 65, Tyr 95, Tyr 188, Lys 206, His 207, His 249, Asp 392, Tyr 426, Tyr 514, Tyr 517, His 585, Thr 120, Arg 124, Ala 126, Gly 127, Thr 452, Arg 456, Ala 458 and Gly 459 of SEQ ID NO.: 3.

17. The fusion protein of claim 1, further comprising a peptide linker.

18. The fusion protein of claim 17, wherein said peptide linker is a substantially non-helical peptide linker.

19. The fusion protein of claim 18, wherein the linker is selected from the group consisting of PEAPTD (SEQ ID NO.: 13), (PEAPTD)_{2} (SEQ ID NO.: 10), PEAPTD in combination with an IgG hinge linker (SEQ ID NOS.: 118-123 and 126-129), and (PEAPTD)_{2} in combination with an IgG hinge linker (SEQ ID NOS.: 205-214).

20. The fusion protein of claim 19, wherein the linker is (PEAPTD)_{n} (SEQ ID NO.: 266), wherein n is an integer from 1 to 15.

21. The fusion protein of claim 1, comprising at least two melanocortin peptides.

22. The fusion protein of claim 1, wherein the N-terminus of the fusion protein comprises a secretion signal sequence prior to cleavage.

23. The fusion protein of claim 22, wherein the signal sequence is a signal sequence from serum transferrin, lactoferrin, melanotransferrin, or a variant thereof.

24. The fusion protein of claim 22, wherein the signal sequence is selected from the group consisting of a HSA/MF-a hybrid leader sequence or a Tf signal sequence comprising amino acids 1-19 of SEQ ID NO: 2.

25. The fusion protein of claim 1, further comprising a GLP-1 receptor agonist selected from the group consisting of a GLP-1 peptide, a GLP-1 derivative or a GLP-1 analog.

26. The fusion protein of claim 25, wherein the GLP-1 peptide is GLP-1 (7-36) or GLP-1 (7-37).

27. The fusion protein of claim 25, wherein the GLP-1 peptide consists of the sequence: His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-Gly (SEQ ID NO: 6).

28. The fusion protein of claim 25, wherein the GLP-1 melanocortin peptide has been modified and the mutation in the modified GLP-1 is selected from the group consisting of mutating Ala at position 2 in SEQ ID NO: 6, mutating Ala at position 2 in SEQ ID NO: 6 to Gly, Ser, or Val, mutating Lys at position 28 in SEQ ID NO: 6, mutating Lys at position 28 in
SEQ ID NO: 6 to Ala, Asn, or Gin, mutating Ala at position 2 in SEQ ID NO: 6 to Gly and Lys at position 28 in SEQ ID NO: 6 to Ala, mutating GLP-1 (7-37) A8 to S, G, or V (A2 of SEQ ID NO 6 to S, G, or V), deleting from GLP-1 (7-37) V33 to G37 (V27 to G31 of SEQ ID NO. 6), deleting from GLP-1 A30 to G37 (A24 to G31 of SEQ ID NO. 6), modifying GLP-1 to GLP-1 (7-37; A8G.K34A), modifying GLP-1 (7-37) A8 to S, G, or V (K28 of SEQ ID NO. 6 to S, G, or N).  

29. The fusion protein of claim 1 further comprising interferon β (IFNβ).  
30. The fusion protein of claim 79, wherein IFNβ is selected from the group consisting of IFNβ-1a and IFNβ-1b or an analog or derivative thereof.  
31. A nucleic acid molecule encoding a fusion protein of any one of claims 1-30.  
32. A vector comprising a nucleic acid molecule of claim 30.  
33. A host cell comprising a vector of claim 32 or the nucleic acid molecule of claim 31.  
34. A method of expressing a fusion protein comprising culturing a host cell of claim 33 under conditions which express the encoded fusion protein.  
35. The host cell of claim 33, wherein the cell is prokaryotic or eukaryotic.  
36. The host cell of claim 35, wherein the cell is a yeast cell.  
37. A pharmaceutical composition comprising the fusion protein of any one of claims 1-30 and a carrier.  
38. A method of treating or preventing a disease or condition in a subject in need thereof comprising administering to the subject a therapeutically effective amount of a fusion protein of any one of claims 1-30.  
39. A method of treating or preventing a myocardial disease or condition in a subject comprising administering to said subject a therapeutically effective amount of any one of claims 1-30.
FIGURE 2
FIGURE 3
FIGURE 4

Activity of mTf-(PEAPT1)2-αMSH in melanin biosynthesis assay
FIGURE 5

Activity of mTf-(PEAPTID)$_2$-aMSH in cAMP assay