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(57) Abstract

The invention relates to a novel bifunctional molecule comprising a first binding moiety which binds to a surface molecule on a target cell; and a second binding moiety which binds to a surface molecule on a viral vector. The bifunctional molecule targets the viral vector to the target cell with improved infectivity and selectivity. The molecule can be used, for example, in *in vitro* and *in vivo* gene delivery methods.

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BIFUNCTIONAL POLYPEPTIDES FOR CELL-SPECIFIC VIRAL TARGETING

RELATED APPLICATION

This application is a continuation-in-part of U.S. Serial No. 08/844,359, filed April 18, 1997, the teachings of which are incorporated herein by reference, in their entirety.

GOVERNMENT FUNDING

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BACKGROUND OF THE INVENTION

Many clinical trials for gene delivery have been and are presently employing viral vectors which include in their genome a nucleic acid sequence encoding a desired protein. Viral vectors which have been developed for gene delivery include retroviruses, adenoviruses and adeno-associated viruses. In recent years, significant advances have occurred in the development of viral vectors for gene delivery to increase infectivity and decrease their ability to replicate *in vivo*, for example.

It has been recognized that it is desirable in some instances to target the viral vector to a specific cell type. Cell-specific targeting has been suggested for retroviral vectors. These viruses contain two envelope glycoprotein subunits designated surface (SU) and

transmembrane (TM) which form an oligomeric complex on the viral surface and mediate viral entry. The SU protein contains the viral receptor binding determinants whereas the TM protein contains a hydrophobic transmembrane region and a separate hydrophobic segment that mediates virus-cell membrane fusion (Weiss, R.A., 1993, "Cellular receptors and viral glycoproteins involved in retrovirus entry," p. 1-107, in J.A. Levy (ed.), *The Retroviridae*, Vol. 2. Plenum press, New York).

To date, there have been several reports of cell type-specific retrovirus targeting. In one case, retroviral particles were chemically modified with lactose, a procedure which resulted in specific infection of human cells that expressed the asialoglycoprotein receptor (Neda, H., 1991, *J. Biol. Chem.* *266*:14143-14149). In another case, a low level of cell type-specific viral infection was achieved by forming an antibody bridge between a retroviral SU protein and specific host cell surface proteins (Roux, P., 1989, *Proc. Natl. Acad. Sci. USA 86*:9079). An alternative strategy that has been employed reengineered retroviral SU proteins to contain cell type-specific ligands or single chain antibodies that bind to specific cell surface proteins (Valsesia-Wittmann, S., 1994, *J. Virol.* *68*:4609-4619; Kasahara, Y., 1994, *Science 266*:1373-1376; Han, X., 1995, *Proc. Natl. Acad. Sci. USA 92*:9747-9751; Matano, T., 1995, *J. Gen. Virol. 76*:3165-3169; Valsesia-Wittmann, 1996, *J. Virol. 70*:2059-2064; Cosset, F-L., 1995, *J. Virol. 69*:6314-6322; Nilson, B.H.K., 1996, *Gene Therapy 3*:280-286; Etienne-Julan, M., 1992, *J. Virol.* *69*:2659-2663; Somia, N., 1995, *Proc. Natl. Acad. Sci USA*

92:7570-7574; Russell, S.J., 1993, *Nucleic Acids Res.* *21*:1081-1085; Marin, M., 1996, *J. Virol.* *70*:2957-2962). This latter approach requires a) the synthesis of recombinant envelope-ligand proteins in virus producing cells, b) the incorporation of the recombinant ligand-envelope fusion proteins (and in some cases also wild-type envelope proteins) onto viral surfaces, c) binding of the recombinant ligand-envelope fusion to a specific cell surface receptor, and d) fusion of the viral membrane with the host cell membrane to allow infection.

The current strategies for retroviral vector targeting suffer from a number of limitations which severely restrict the utility of these vectors for a number of gene therapy approaches. These problems are discussed in Cosset, F-L and Russell, S.J., 1996, *Gene Therapy* *3*:946-956. These problems include the fact that it is difficult to manipulate these proteins "without impairing some critical envelope functions;" "Only a few variable peptides or loops in the SU glycoprotein have been found to tolerate substitution by foreign peptides;" in some cases the need for co-expressed wild-type envelope proteins "has not been adequately explained and greatly complicates the process whereby targeted vector particles are generated;" in general these strategies give rise to viruses that infect cells with a low efficiency (Cosset, 1996, *Gene Therapy* *3*:946-956).

Thus, there is a continued need to develop new and improved methods for targeting viral vectors to specific cell types.

SUMMARY OF THE INVENTION

This invention is based upon the discovery that bifunctional molecules, e.g., polypeptides, comprising a specific binding and entry moiety, or partner, to a viral vector and a specific binding moiety, or partner, to a target cell results in efficient binding and infection of the cell and, thereby, gene delivery. Thus the invention relates to a bifunctional molecule comprising a first binding moiety which binds to a surface protein on a target cell, a second binding moiety which binds to a surface protein on a viral vector and activates viral entry and, optionally, a linking moiety. In a preferred embodiment, the first binding polypeptide is a ligand to a cell-type specific cellular receptor and/or the second binding polypeptide is a polypeptide which has an amino acid sequence which is the same or substantially the same as an amino acid sequence of at least the viral-binding portion or fragment of a native extracellular domain of a viral cellular receptor.

In another embodiment the first binding moiety binds to a cell surface molecule on a target cell and the second binding moiety to a viral surface molecule. The target cell surface molecule can be a peptide, sugar, lipid, anion or cation, or any combination thereof which are required for specific, high affinity binding and activate viral entry into the target cell.

The invention also includes nucleic acid molecules which encode, independently or together, polypeptide binding moieties of the above bifunctional molecule. Also envisioned are host cells which comprise the nucleic acid molecules of the invention and express the

polypeptide binding moieties separately or together as a bifunctional molecule.

In yet another embodiment, the invention includes methods for preparing the bifunctional molecules of the invention which includes maintaining one or more recombinant host cells which express the bifunctional molecule (e.g., which contain one or more nucleic acid molecules encoding the bifunctional molecule) under conditions suitable for expression. Alternatively, the bifunctional molecules can be prepared via chemical syntheses, such as conjugating the two binding moieties.

The invention also includes methods for delivering a viral vector, such as a retroviral vector, to a cell, *in vitro* or *in vivo*, comprising contacting the cell with a bifunctional molecule, as described herein. The retroviral vector typically encodes a heterologous protein.

In yet another embodiment, the invention relates to methods of screening ligand and ligand-cell specific receptor binding pairs using bifunctional molecules.

The inventions which are described herein provide alternative and improved mechanisms for the successful gene delivery to specific cell types via viral vectors. In contrast to the prior art, the present invention does not require the careful incorporation of a cellular binding partner into the viral particle without disturbing the conformation of a viral protein (e.g. the env protein) required for proper binding and cell entry. Neither does it require the specific cloning and elucidation of the viral protein required for binding and the mechanisms for infection. The invention thereby

offers greater opportunities in cell-type specific targeting than previously offered in the prior art.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 schematically illustrates the use of the bifunctional molecule in binding and inducing infection of a cell.

Figure 2 is a bar graph which illustrates TVA-EGF binding to mouse L cells expressing EGF receptors lacking kinase activity (M5) and wild type EGF receptors (T23) but not to mouse L cells lacking EGF receptors (B82).

Figure 3 is a line graph which illustrates the EGF receptor specificity of TVA-EGF cell surface binding to M5 cells.

Figure 4 is a three dimensional scatter diagram which illustrates differences in the time required for a bifunctional molecule containing TVA and EGF to be cleared from the cell surface of M5 and T23 cells.

Figure 5 is a bar graph which illustrates the avian leukosis viral (ALV) infection of M5 cells which express the epidermal growth factor EGF receptor with a bifunctional molecule containing TVA and EGF (TVA-EGF).

Figure 6 is a bar graph which illustrates TVA-EGF dependent ALV-A infection of M5 cells, which express the EGF receptor, but not B82 cells, which do not express the EGF receptor.

Figure 7 is a bar graph which illustrates TVA-EGF dose dependent effects on ALV-A infectivity of T23 and M5 cells but not in B82 cells.

Figure 8 is a bar graph which illustrates the EGF receptor specificity of TVA-EGF mediated ALV-A infection in M5 cells.

Figure 9 schematically illustrates binding of the TVA-EGF bifunctional molecule to the EGF receptor on a target cell (EGF binding moiety) and the Env protein of a viral vector (TVA binding moiety).

DETAILED DESCRIPTION OF THE INVENTION

As set forth above, the present invention relates to the discovery that viral vectors can be targeted to specific cell-types by contacting the viral vectors and cells with bifunctional molecules that possess moieties which bind to surface proteins of both the vector and cell. The bifunctional molecule thus comprises a first binding moiety which binds to a surface protein on a target cell and a second binding moiety which binds to a surface protein on a viral vector and induces viral entry.

Viral vectors include retrovirus, adenovirus, parvovirus (e.g., adeno-associated viruses), coronavirus, negative strand RNA viruses such as orthomyxovirus (e.g., influenza virus), rhabdovirus (e.g., rabies and vesicular stomatitis virus), paramyxovirus (e.g. measles and Sendai), positive strand RNA viruses such as picornavirus and alphavirus, and double stranded DNA viruses including adenovirus, herpesvirus (e.g., Herpes Simplex virus types 1 and 2, Epstein-Barr virus, cytomegalovirus), and poxvirus (e.g., vaccinia, fowlpox and canarypox). Other viruses include Norwalk virus, togavirus, flavivirus, reoviruses, papovavirus, hepadnavirus, and hepatitis

virus, for example. Examples of retroviruses include: avian leukosis-sarcoma, mammalian C-type, B-type viruses, D-type viruses, HTLV-BLV group, lentivirus, spumavirus (Coffin, J.M., 1996, Retroviridae: The viruses and their replication. in Fundamental Virology, Third Edition, edited by B.N. Fields, D.M. Knipe, P.M. Howley, et al. Lippincott-RavenPublishers, Philadelphia). Other examples include murine leukemia viruses, murine sarcoma viruses, mouse mammary tumor virus, bovine leukemia virus, feline leukemia virus, feline sarcoma virus, avian leukemia virus, human T-cell leukemia virus, baboon endogenous virus, Gibbon ape leukemia virus, Mason Pfizer monkey virus, simian immunodeficiency virus, simian sarcoma virus, Rous sarcoma virus and lentiviruses.

Retroviruses are a family of RNA viruses which infect cells in a two step mechanism. The first step of infection is the binding of the viral particle via the surface protein of the retrovirus envelope (env) protein and viral and cellular membrane fusion for viral uptake via the transmembrane protein of the env protein. This is discussed in more detail above. The env protein is largely responsible for the specificity (between cell-types and between species) of the infectivity of retroviral vectors.

Adenoviruses have a linear double-stranded DNA genome. Adenoviruses infect cells by a two step mechanism. First a viral surface fiber protein binds specifically to a cell surface receptor. In the case of human HeLa cells, the receptor for adenoviruses 2 and 5 is designated CAR, a member of the immunoglobulin protein superfamily, which also serves as a cellular

receptor for coxsackie B viruses (Bergelson, 1996, *Science*, *275*:1320-1323). However, other viral receptors have been described. Following receptor binding, adenoviruses are taken up into the cell by receptor-mediated endocytosis and interaction between the viral penton base protein and cellular integrins is necessary for viral entry (Wickham, 1993, *Cell* *73*:309; Bai, 1994, *J. Virol* *68*:5925; Goldman, 1995 *J. Virol.* *69*:5951; Huang, 1996, *J. Virol.* *70*:4502). The viral DNA is replicated in the cell extrachromosomally (Horwitz, M.S., 1996 "Adenoviruses," in *Fields Virology*, Third Edition edited by B.N. Fields, D.M. Knipe, P.M. Howley *et al.*, Lippincott-Raven Publishers, Philadelphia, PA).

Recombinant adenoviral vectors are generated by a variety of techniques that include introducing the desired gene of interest into a bacterial plasmid at a site flanked by adenovirus sequences. These sequences provide control elements for gene expression and serve as sites for recombination with a compatible adenoviral genome when cotransfected together into an appropriate mammalian cell line (Horwitz, M.S., 1996 "Adenoviruses," in *Fields Virology*, Third Edition edited by B.N. Fields, D.M. Knipe, P.M. Howley *et al.*, Lippincott-Raven Publishers, Philadelphia, PA).

Adeno-associated viruses (AAV) have a linear single-stranded DNA genome and their receptor has not yet been described. These viruses only undergo productive infection if the infected cells are coinfected with a helper virus (e.g., adeno- or herpesvirus) otherwise the genome becomes integrated in a latent state at a specific site on a human chromosome (Linden, 1996, *Proc. Natl. Acad. Sci. USA* *93*:11288-

11294; Berns, K.J., "Parvoviridae: The viruses and their replication" in *Fields Virology*, Third Edition edited by B.N. Fields, D.M. Knipe, P.M. Howley *et al.*, Lippincott-Raven Publishers, Philadelphia, PA). Adeno-associated viral vectors are typically made by replacing viral genes with desired genes of interest or instead by simply adding the terminal AAV DNA sequences (ITRs) to these genes.

The negative strand RNA viruses infect cells by a variety of different mechanisms. For example, Influenza A viruses which have a segmented RNA genome, contain a surface hemagglutinin protein which binds to cell surface sialic acid receptors and mediates viral entry in a low pH endosome following receptor-mediated endocytosis (Lamb, R.A. and Krug, R.M., 1996, "Orthomyxoviridae: The viruses and their replication" *Fields Virology*, Third Edition edited by B.N. Fields, D.M. Knipe, P.M. Howley *et al.*, Lippincott-Raven Publishers, Philadelphia, PA).

Paramyxoviruses which have a non-segmented RNA genome have two surface viral proteins, the hemagglutinin (HN) and fusion protein (F), required for viral entry which occurs at neutral pH. These viruses can utilize sialic acid receptors, or protein receptors (e.g., CD46 used by measles virus), for viral entry (Lamb, R.A. and Kolakofsky, D., 1996, "Paramyxoviridae: The viruses and their replication" *Fields Virology*, Third Edition edited by B.N. Fields, D.M. Knipe, P.M. Howley *et al.*, Lippincott-Raven Publishers, Philadelphia, PA).

Rhabdoviruses (e.g., VSV) which have a non-segmented RNA genome, contain a surface protein (G)

which binds to specific cell surface receptors and mediates viral entry in a low pH endosome. A specific phospholipid appears to be one of the receptors for VSV (Wagner, R.R. and Rose, J.K., 1996, in *Fields Virology*, Third Edition edited by B.N. Fields, D.M. Knipe, P.M. Howley *et al.*, Lippincott-Raven Publishers, Philadelphia, PA).

A number of strategies are currently available for engineering negative strand viral RNA genomes to express heterologous genes (reviewed by Palese, P., 1996, *Proc. Natl. Acad. Sci. USA 93*:11354-11358; Schnell, M.J., 1996, *Proc. Natl. Acad. Sci. USA 93*:11359-11365).

The positive strand RNA viruses also infect cells by a variety of different mechanisms. For example, among the picornaviruses, different members of the immunoglobulin protein superfamily are used as cellular receptors by poliovirus, by the major subgroups of rhinoviruses, and by coxsackie B viruses, whereas an integrin protein is used by some types of ecoviruses and a low density lipoprotein receptor is used by minor subgroups of rhinoviruses (Bergelson, 1996, *Science 275*:1320-1323; Rueckert, R.R., 1996, "Picornaviridae: The viruses and their replication" *Fields Virology*, Third Edition edited by B.N. Fields, D.M. Knipe, P.M. Howley *et al.*, Lippincott-Raven Publishers, Philadelphia, PA). Following receptor-binding, it is not yet known precisely what role receptor-mediated endocytosis plays for picornaviral entry, if indeed it is required.

Because the picornaviruses lack a surface lipid bilayer, their entry pathway does not involve fusion of a viral membrane with a host cell membrane. In

contrast, the alphaviruses (e.g., Sindbis virus and Semliki virus) do contain a surface lipid bilayer. These viruses contain two (E1 and E2) surface proteins, and in some cases a third (E3) surface protein important for viral entry. These viruses use various cell surface receptors. For example, Sindbis virus can use a laminin receptor or other receptors and generally enter cells by a pH-dependent mechanism, following receptor-mediated endocytosis (Frolov, 1996, *Proc. Natl. Acad. Sci. USA* *93*:11371-11377; Schlesinger, S. and Schlesinger, M.J., 1996, "Togaviridae: The viruses and their replication," in *Fields Virology*, Third Edition edited by B.N. Fields, D.M. Knipe, P.M. Howley *et al.*, Lippincott-Raven Publishers, Philadelphia, PA).

The herpesviruses which have large double-stranded DNA genomes, contain a number of surface glycoproteins involved in viral entry and utilize various cell surface receptors. For example, herpes simplex virus and cytomegalovirus entry involves binding to a heparin sulfate cell surface receptor and herpes simplex viruses use other proteins (e.g., HVEM) for viral entry (Montgomery, R., 1996, *Cell* *87*:427-436). In contrast, Epstein-Barr virus entry is initiated by binding to a completely distinct cell surface receptor, CR2 (Wolf, 1993, *Intervirology* *35*:26-39). Strategies have been described that allow one to engineer herpes simplex viruses, cytomegaloviruses and Epstein-Barr viruses as vectors for heterologous gene expression (Roizman, 1996, *Proc. Natl. Acad. Sci. USA* *93*:11307-11312; Andreansky, 1996, *Proc. Natl. Acad. Sci. USA* *93*:11313-11318; Marconi, 1996, *Proc. Natl. Acad. Sci. USA* *93*:11319-11320; Mocarski, 1996, *Proc. Natl. Acad. Sci. USA*

93:11321-11326; Robertson, 1996, *Proc. Natl. Acad. Sci. USA 93*:11334-11340; Duboise, 1996, *Proc. Natl. Acad. Sci. USA 93*:11389-11394).

Poxviruses have large double stranded DNA genomes and enter cells by a pH-independent mechanism via receptors that remain to be defined (Moss, B., 1996: "Poxviridae: The viruses and their replication," in *Fields Virology*, Third Edition edited by B.N. Fields, D.M. Knipe, P.M. Howley *et al.*, Lippincott-Raven Publishers, Philadelphia, PA). Poxvirus vectors have been used extensively for the expression of heterologous recombinant genes and as vaccines (Moss, B., 1996, *Proc. Natl. Acad. Sci. USA 93*:11341-11348; Paoletti, 1996, *Proc. Natl. Acad. Sci. USA 93*:11349-11353).

Viral vectors which have been "pseudotyped" incorporate the env protein from a retrovirus of a desired specificity into the vector. Thus, a pseudotyped virus has the env protein from a first retrovirus and core or structural proteins from a second virus (e.g. a second retrovirus, an orthomyxovirus or a rhabdovirus). Viral pseudotypes have been described, for example, in Le Guen, 1992, *Proc. Natl. Acad. Sci. USA 89*:363-367; Rizvi, 1992, *Journal of Medical Primatology 21*:69-73; Takeuchi, 1992, *Virology 186*:792-294; Vile *et al.*, 1991, *Virology 180*:420; Miller *et al.*, 1991, *J. Virol. 65*:2220; Landau, *et al.*,, 1991, *J. Virol. 65*:162; Emi, *et al.*, 1991, *J. Virol. 65*:1207; and Dong, *et al.*, 1992, *J. Virol 66*:7374. The present invention can thus target a pseudotyped viral vector with a bifunctional molecule which binds to the target cell surface protein and binds to the heterologous env protein, inducing viral entry.

Typically, retroviral vectors are manufactured by "packaged cell lines" which provide the retroviral proteins necessary for infection (e.g., env, gag and pol), but are incapable of replication upon infection. See, for example, Miller, AD, 1992, Current Topics in Microbiology and Immunology, Vol. 158, pages 1-24.

The viral vectors employed in the present invention can be used for polynucleotide or gene delivery to a cell or animal. The polynucleotide to be delivered to the cell or animal can include a polynucleotide native to the viral vector or heterologous to the vector. In a preferred embodiment, the viral vector has been engineered to contain a polynucleotide which is itself therapeutic or encodes a therapeutic protein, which will be discussed in more detail below.

The invention, as provided for herein, is a mechanism by which the viral vector can more efficiently deliver a desired gene to a cell or animal by indirectly binding the vector to the target cell via a bifunctional molecule comprising a first and second binding moiety.

A "binding moiety," as that term is defined herein, includes a chemical entity which binds to the specified target molecule. The binding can be via a covalent bond, ionic bonding, hydrogen bonding or other mechanism. The binding moiety can be a peptide (including post-translationally modified proteins, such as amidated, demethylated, glycosylated or phosphorylated proteins), sugar, lipid, steroid, nucleic acid, small molecule, anion or cation, or combination thereof which binds the specified target molecule. Preferably, the binding will possess a high affinity. Examples of

high affinity, as that term is used herein, have an equilibrium constant of 10^{-6} (preferably 10^{-8}) or lower.

As set forth above, the first binding moiety binds a surface protein, or target molecule, on the target cell. The "target cell" is defined as the cell which is intended to be infected by the viral vector. Typically, the target cell is of animal origin and can be a stem cell or somatic cells. Suitable animal cells for use on the claimed invention can be of, for example, mammalian and avian origin. Examples of mammalian cells include human, bovine, ovine, porcine, murine, rabbit cells. The cell may be an embryonic cell, bone marrow stem cell or other progenitor cell. Where the cell is a somatic cell, the cell can be, for example, an epithelial cell, fibroblast, smooth muscle cell, blood cell (including a hematopoietic cell, red blood cell, T-cell, B-cell, etc.), tumor cell, cardiac muscle cell, macrophage, dendritic cell, neuronal cell (e.g., a glial cell or astrocyte), or pathogen-infected cell (e.g., those infected by bacteria, viruses, virusoids, parasites, or prions).

Typically, cells isolated from a specific tissue (such as epithelium, fibroblast or hematopoietic cells) are categorized as a "cell-type." The cells can be obtained commercially or from a depository or obtained directly from an animal, such as by biopsy. Alternatively, the cell need not be isolated at all from the animal where, for example, it is desirable to deliver the vector to the animal in gene therapy.

Cells are typically characterized by markers expressed at their surface that are termed "surface markers". These surface markers include surface

proteins or target molecules, such as cellular receptors, adhesion molecules, transporter proteins, components of the extracellular matrix and the like. These markers, proteins and molecules also include specific carbohydrates and/or lipid moieties, for example, conjugated to proteins. The first binding moiety binds to one or more surface proteins on the target cell. Surface proteins can be tissue- or cell-type specific (e.g. as in surface markers) or can be found on the surface of many cells. Typically, the surface marker, protein or molecule is a transmembrane protein with one or more domains which extend to the exterior of the cell (e.g. the extracellular domain). Where cell-type specific delivery is desired (as in *in vivo* delivery of a viral vector), the surface protein selected for the invention is preferably specific to the tissue. By "specific" to the tissue, it is meant that the protein be present on the targeted cell-type but not present (or present at a significantly lower concentration) on a substantial number of other cell-types. While it can be desirable, and even preferred, to select a surface protein which is unique to the target cell, it is not required for the claimed invention. It is to be appreciated, however, that specific delivery may not be required where the cell or cells are contacted with the viral vector in pure or substantially pure form, such as can be the case in an *in vitro* gene transfer. As such, the surface protein or targeted protein for the first binding moiety may be present on many different cell-types, specific or even unique to the targeted cell-type.

As set forth above, the surface protein can be a cellular receptor or other protein, preferably a cellular receptor. Examples of cellular receptors include receptors for cytokines, growth factors, and include, in particular epidermal growth factor receptors, platelet derived growth factor receptors, interferon receptors, insulin receptors, proteins with seven transmembrane domains including chemokine receptors and frizzled related proteins (Wnt receptors), immunoglobulin-related proteins including MHC proteins, CD4, CD8, ICAM-1, etc., tumor necrosis factor-related proteins including the type I and type II TNF receptors, Fas, DR3, DR4, CAR1, etc., low density lipoprotein receptor, integrins, and, in some instances, the Fc receptor.

Other examples of surface proteins which can be used in the present invention include cell-bound tumor antigens. Many of these surface proteins are commercially available and/or have been characterized in the art, including the amino acid and nucleic acid sequences, which can be obtained from, for example, GENBANK, as well as the specific binding characteristics and domains. Cytokine and chemokine receptors are reviewed for example, in Miyama, *et al. Ann. Rev. Immunol.*, *10*:295-331 (1992), Murphy, *Ann. Rev. Immunol.* *12*:593-633 (1994) and Miller *et al. Critical Reviews in Immunol.* *12*:17-46 (1992).

Typically, the first binding moiety is selected or derived from native ligands or binding partners to the surface protein of the target cell. In the case of a cellular receptor, for example, for a cytokine or growth factor, the first binding moiety can be a polypeptide

comprising at least the receptor-binding portion of the native ligand. A "native ligand" or "native binding partner" is defined herein as the molecule naturally produced by, for example, the animal or species which binds to the surface protein in nature. Preferably, the first binding moiety is a polypeptide or protein. As such, the native ligand of a cytokine receptor is the native cytokine. In another embodiment, the first binding moiety can comprise a binding fragment of an antibody, such as the variable region or a single chain antibody.

Preferably, the first binding moiety is a polypeptide ligand to a cellular receptor. Examples of preferred ligands are growth factors, epidermal growth factor, interleukins, GM-CSF, G-CSF, M-CSF, EPO, TNF, interferons, and chemokines. Generally, the cellular receptor is not presented on the cell which expresses the viral receptor (e.g., a cell receptor which is bound by the virus during infection). In one embodiment, the cellular receptor which binds to the first binding moiety is not an Fc receptor. Thus, in this embodiment, the bifunctional molecule is not an immunoadhesin.

The first binding moiety can have an amino acid sequence which is the same or substantially the same as an amino acid sequence of at least the receptor-binding portion of a native ligand for the cellular receptor. Similar to cellular receptors, many of the corresponding ligands have been identified, sequenced and characterized, including the portions thereof which bind to the receptor. The first binding moiety can, therefore, include the same or substantially the same sequence of the entire native ligand. Alternatively, first binding

moiety comprises the receptor binding portion of the native ligand, eliminating, in some cases, the effector function of the ligand.

In another embodiment, the first binding moiety is selected or derived from native ligands or binding partners to a cellular surface molecule of the target cell. A "cellular surface molecule" as defined herein can be a peptide (including post-translationally modified proteins, such as amidated, demethylated, methylated, prenylated, palmitoylated, glycosylated, myristylated, acetylated or phosphorylated proteins), sugar, lipid, steriod, anion or cation, or a combination thereof which binds the first binding moiety. Preferably, the binding of the cellular surface molecule to the binding moiety of the bifunctional molecule will be of high affinity. Examples of high affinity have an equilibrum constant of 10^{-6} (preferably 10^{-8}) or lower.

The cellular surface molecule need not be "specific" for the target cell. However, the cellular surface molecule is specific for the desired viral vector. For example, specific delivery of Influenza A viral vectors can employ sialic acid cellular surface molecules for entry into a target cell whereas targeting of VSV viral vectors can employ a phospholipid as the surface molecule. As such, the cellular surface molecule for the first binding moiety can be present on many different cell-types, specific or even unique to the target cell.

In other embodiments, the effector function can be desirable, thereby stimulating or modulating the cellular activity of the target cell which can enhance therapy. An example of where such a therapy can be

desirable is in the delivery of a negative selection marker or suicide protein to a tumor where the target cell is a lymphokine and the ligand is a cytokine. Where the lymphokine is stimulated, the cell, in addition to delivering the viral vector, can also possess therapeutic value in the recruitment of an endogenous immune response against the tumor, thereby increasing the therapeutic benefit of the therapy.

The phrase "substantially the same sequence" is intended to include sequences which bind the surface protein and possess a high percentage of (e.g., at least about 90%, preferably at least about 95%) sequence identity with the native sequence. The modifications to the sequence can be conserved or non-conserved, natural and unnatural, amino acids and are preferably outside of the binding domain. Amino acids of the native sequence for substitution, deletion, or conservation can be identified, for example, by a sequence alignment between proteins from related species or other related proteins.

Where the first binding moiety comprises a binding fragment of an antibody, many antibodies to surface proteins are known or are commercially available, as are the amino acid sequences which are responsible for binding. Alternatively, novel antibodies can be prepared by methods known in the art, such as by Harlow and Lane, "Antibodies, A Laboratory Manual," Cold Spring Harbor Laboratory (1988).

Similar to the first binding moiety, the second binding moiety is a chemical entity which binds to the specified viral target molecule on the viral vector, generally a surface protein of the viral particle. The binding can be via a covalent bond, ionic bonding,

hydrogen bonding or other mechanism. The binding moiety can be a peptide, sugar, lipid, steroid, nucleic acid, small molecule, anion or cation, or combination thereof which binds the specified viral target molecule. Generally, the second binding moiety of the bifunctional molecule is also a polypeptide. One embodiment of the second binding moiety comprises an antigen-binding fragment of an antibody which recognizes and binds to the viral vector. However, where one binding moiety comprises an antibody fragment, it is preferred that the other binding molecule does not comprise an antibody fragment.

In a more preferred embodiment, the second binding moiety is a polypeptide which has an amino acid sequence which is the same or substantially the same as an amino acid sequence of at least the viral-binding portion of a native extracellular domain of a viral cellular receptor, such as the viral cellular receptor is on a cell which is subject to infection by the viral vector. The viral surface protein is thus generally the viral protein which binds to this cell and activates or otherwise induces steps for viral entry and, thus, infection. Where the viral vector is a pseudotyped viral vector (such as where the vector is a retrovirus containing the envelope protein of another retrovirus, of an orthomyxovirus or of a rhabdovirus), as discussed above, it is understood that the viral cellular receptor generally corresponds to the env protein incorporated into the viral particle. In the case where the viral surface protein is an env protein, the second binding partner generally binds the env SU protein and, thereby, activates the env TM protein, inducing or mediating

virus-cell membrane fusion and viral entry. As such, a "viral cellular receptor" is a receptor molecule in or on a cell which binds to a natural or non-natural viral particle or protein, for example, in the course of infection. Non-natural viral particles include pseudotyped viruses, replication defective viruses, attenuated viruses, or recombinantly engineered viruses.

Examples of viral cellular receptors include the subgroup A avian leukosis virus receptor, the subgroup B and D avian leukosis virus receptor, the CD4 receptor for human and simian immunodeficiency viruses, the amphotropic and ecotropic murine leukemia virus receptors, the bovine leukemia virus receptor, the gibbon ape leukemia virus receptor, the poliovirus receptor, the major subgroup rhinovirus receptor (ICAM-1), the adenovirus and coxsackie virus receptor (CAR), the coronavirus receptors (e.g. aminopeptidase N and MHVR), and sialic acid and other carbohydrate receptors (e.g., for orthomyxoviruses and paramyxoviruses). Many viral cellular receptors are known in the art, including the sequences thereof.

Cellular receptors are generally transmembrane proteins comprising intracellular, transmembrane (characterized by highly hydrophobic regions in the sequence) and extracellular domains. It is generally preferred that the second binding moiety comprises the native extracellular domain of the receptor molecule. As above with the first binding moiety, the second binding moiety can alternatively comprise the viral binding moiety of the extracellular domain of the receptor and mutations of the extracellular domain. The viral binding moiety can be identified or confirmed by

manufacturing various fragments of the extracellular domain and screening them for their ability to bind. As above, viral-binding mutations to the extracellular domain can be manufactured as well. Mutations to the extracellular domain include conservative and non-conservative amino acid substitutions, particularly those not implicated in the binding of the viral particle. The phrase "substantially the same sequence" is intended to include sequences which bind the viral protein and possess a high percentage of (e.g., at least about 90%, preferably at least about 95%) amino acid sequence identity with the native sequence. The modifications to the sequence can be conserved or non-conserved amino acids.

The phrase "conservative amino acid substitutions" is intended to mean amino acids which possess similar side chains (e.g., hydrophobic, hydrophilic, aromatic, etc.), as is known in the art.

The first and second binding moieties can be directly bonded together or through a linking moiety. Where one or both of the moieties are polypeptides, a peptide bond or peptide linker is preferred, thereby obtaining a "fusion protein" of the two moieties which can be expressed by a single nucleic acid construct in series. The two moieties can alternatively be linked directly or indirectly other than via a peptide bond or peptide linker, thereby obtaining a "conjugate".

Where the moieties are directly bonded to each other, the bond can be covalent, as in a peptide bond, ionic bonding or hydrogen bonding. Where the bond is a peptide bond, the first moiety can be bonded to the N-terminus of the second moiety via the C-terminus, or

vice versa. It is acknowledged that one fusion protein may possess greater activity than a second fusion protein due to conformational or steric considerations.

Where one or more of the moieties are not polypeptides, they can be joined via chemical reaction through functional groups present on each moiety which, under the appropriate conditions, will react with each other. For example, acid groups (or activated derivatives thereof) can be reacted with amines, alcohols or thiols to form amide or ester bonds, as is known in the art.

Alternatively and advantageously, a linking moiety is employed to link the two moieties. The linker can preferably be a flexible linker and sufficient in length to separate the moieties in space, thereby not restricting the ability of the bifunctional molecule to bind independently and maintain the proper conformation. Again, where both moieties are polypeptides, the linker moiety will generally be a peptide, polypeptide, or a "pseudopeptide". A "pseudopeptide" is a bifunctional linker which contains at least one non-amino acid and reacts to form a peptide bond, or other bond, with the terminal amine or carboxyl group of the moiety. For example, a peptide characterized by substitution of the terminal amine for a carboxyl group can function to react with the amine terminus of each moiety. Such as linker is considered to be a "pseudopeptide." Similarly, a peptide characterized by substitution of the terminal carboxyl for an amine group can function to react with the carboxyl terminus of each moiety.

Generally, however, the linker will be a peptide linker which will link the amine terminus of one moiety

to the carboxyl terminus of the second moiety. One advantage to such a molecule is the ability to express the bifunctional molecule as a fusion protein in a recombinant host cell with a single nucleic acid construct.

Preferred peptide linkers can be obtained from immunoglobulin hinge regions, such as a proline rich region. Also, linkers can be characterized by little steric hindrance, thereby permitting maximal independent movement of the two moieties, such as with a polyglycine linker. Alternatively, the linker selected to be reactive to or inert to cellular proteases can be desirable. In another embodiment, the linker can be selected to avoid or minimize an immune response against the fusion protein. The length of the linker also is not particularly critical. Typically, the length of the linker will be between about 2 and about 20 amino acids. As can be seen, the selection of the particular linking group is not critical to the invention.

In yet another embodiment, the linker can be a bifunctional compound which will react with other functional groups on the two binding moieties, such as in the reaction of acids and amines or alcohols (as present in peptides, carbohydrates and lipids, for example) in the formation of amides or esters.

A preferred combination of the above first and second binding moieties includes the selection of a polypeptide ligand to a cell-type specific cellular receptor linked, via a peptide linker through a terminus of the ligand to the terminus of at least the viral-binding moiety of the extracellular domain of a viral

cellular receptor (or a mutant thereof, as defined above).

For example, the C-terminus of the first binding polypeptide is linked to the N-terminus of the second binding polypeptide via the polypeptide linker or the N-terminus of the first binding polypeptide is linked to the C-terminus of the second binding polypeptide via the polypeptide linker.

A particularly preferred combination of these elements is for targeting a retroviral vector to a cell-type comprises a receptor-binding fragment of a polypeptide ligand which binds a cellular receptor specific to the desired cell-type and a viral-binding fragment of a cellular receptor for the retroviral vector. For example, the polypeptide ligand can be human epidermal growth factor. Where the retroviral vector is avian leukosis virus, the cellular receptor can be Tva. The two polypeptides can then be linked via a hinge region, such as a proline-rich hinge region.

In another aspect of the invention, peptidomimetics (molecules which are not polypeptides, but which mimic aspects of their structures to bind to the same site), that are based upon the above-described polypeptides, can also be used as binding moieties of the bifunctional molecules. For example, polysaccharides can be prepared that have the same functional groups as the polypeptide binding moieties of the invention, and which interact with target cell and viral surface molecules in a similar manner. Peptidomimetics can be designed, for example, by establishing the three dimensional structure of the polypeptide in the environment in which it is bound or will bind to the target cell or viral vector.

The peptidomimetic comprises at least two components, a binding entity or entities and a backbone or supporting structure entity.

The binding entities of the peptidomimetic are the chemical atoms or groups which will react or complex (as in the formation of a hydrogen or covalent bond) with the target cell or viral surface molecule. In general, the binding entities in a peptidomimetic are the same as the polypeptide of the bifunctional molecule. Alternatively, the binding entities can be an atom or chemical group which will react with the target cell or viral surface molecule in the same or similar manner as the polypeptide. Examples of binding entities suitable for use in designing a peptidomimetic for a basic amino acid in a polypeptide are nitrogen containing groups, such as amines, ammoniums, guanidines and amides or phosphoniums. Examples of binding entities suitable for use in designing a peptidomimetic for an acidic amino acid in a polypeptide can be, for example, carboxyl, lower alkyl carboxylic acid ester, sulfonic acid, a lower alkyl sulfonic acid ester or a phosphorous acid or ester thereof.

The supporting structure is the chemical entity that, when bound to the binding moiety or moieties, provides the three dimensional configuration of the peptidomimetic. The supporting structure can be organic or inorganic. Examples of organic supporting structures include polysaccharides, polymers (such as, polyvinyl alcohol or polylactide). It is preferred that the supporting structure possess substantially the same size and dimensions as the polypeptide backbone or supporting structure. This can be determined by calculating or

measuring the size of the atoms and bonds of the polypeptide and peptidomimetic. For example, the nitrogen of the peptide bond can be substituted with oxygen or sulfur, thereby forming a polyester backbone. Likewise, the carbonyl of the peptide bond can be substituted with a sulfonyl group or sulfonyl group, thereby forming a polyamide. Reverse amides of the peptide can be made (e.g., substituting one or more -CONH- groups for a -NHCO- group). In addition, the peptide backbone can be substituted with a polysilane backbone.

These peptidomimetic compounds can be manufactured by art-known and art-recognized methods. For example, a polyester corresponding to the peptide RRKRK can be prepared by the substituting a hydroxyl group for each corresponding amine group on the R and K amino acids, thereby preparing a hydroxyacid and sequentially esterifying the hydroxyacids, optionally blocking the basic side chains and acids to minimize side reactions. Determining an appropriate chemical synthesis route can generally be readily identified upon determining the chemical structure using no more than routine skill.

Also encompassed by the present invention are the use of bifunctional molecules for detecting ligand and ligand-cell specific receptor binding pairs. The ligand and/or receptor can be peptides (including post-translationally modified proteins) and/or small molecules (including sugars, steroids, lipids, anions or cations). The ligands and ligand-cell specific receptors can be known or unknown. Where the ligand is known and the receptor is unknown, ligand-cell specific receptors can be identified, for example, by screening

host cells transfected with nucleotides encoding potential receptors. For example, the ligands can be secreted (such as chemokines) or non-secreted (such as the extracellular domains of chemokines receptors) proteins.

A library of host cells displaying putative ligand-cell surface receptors can be obtained by transfecting suitable host cells with nucleic acid constructs, including but not limited to cDNA or genomic libraries, under appropriate regulatory control to result in the expression of cell-surface receptors on the host cell. The bifunctional molecule with ligand and an appropriate viral vector, e.g., containing an inducible reporter gene (such as β-galactosidase or chloramphenicol acetyl transferase), is added to the population of host cells under conditions suitable for viral infection. Following infection, infection can be detected. For example, reporter gene expression can be induced under appropriate conditions and the host cells which express the ligand receptor can be identified. Experimental conditions for viral vector expression and induction and detection of reporter genes are well known in the art. A similar approach can be used to select unknown ligands in the case where the ligand-cell specific receptors are known and the ligand is unknown. In this embodiment, a library of bifunctional molecules with putative ligands (e.g., chemokines) can be obtained and contacted with one or more host cells displaying cell surface receptors and an appropriate viral vector. Infection can be detected as described above.

Thus, the invention relates to a method for detecting ligand-receptor binding pairs comprising

contacting, under conditions suitable for infection, a mixture comprising a viral vector, a bifunctional molecule and a host cell, wherein the bifunctional molecule comprises a ligand and a binding moiety which binds to a viral vector surface molecule on the viral vector and activates viral entry into a host cell expressing a receptor. Ligand binding to a receptor can be indicated by viral vector infection of the host cell by, for example, the use of reporter genes. The host cells can display receptors distinct for the known ligand or can express recombinant receptors comprising a collection of nucleic acid constructs encoding ligand-cell surface receptors.

A similar approach can be used to identify unknown ligands wherein the cell surface receptor is known, where the bifunctional molecule comprises a receptor and a binding moiety which binds a viral surface molecule. The host cell expresses a distinct ligand or a collection of recombinant ligands. Ligand-receptor binding can be detected following infection of the viral vector to the host cell.

The bifunctional molecules can be manufactured according to methods generally known in the art. For example, where one or both of the binding moieties is a nonpeptide the bifunctional molecule can be manufactured employing known organic synthesis methods useful for reacting a functional or reactive group on the moiety with a functional or reactive group on the other moiety or, preferably, a linker. In carrying out the synthesis, derivation or inactivation of the functional group(s) required for binding to the moiety's binding partner should be avoided. Appropriate syntheses are

highly dependent upon the chemical nature of the binding moiety and, generally, can be selected from an advanced organic chemistry text, such as March, *et al. Advanced Organic Chemistry*, 3rd Edition (1985) John E. Wiley & Sons, Inc., New York, NY, or other known methods.

Where the binding moieties are polypeptides, the bifunctional molecule can be a conjugate or a fusion protein and manufactured according to known methods. Where a fusion protein is desired, the molecule can be manufactured according to known methods of recombinant DNA technology. For example, the fusion protein can be expressed by a nucleic acid molecule comprising sequences which code for both moieties, such as by a fusion gene. Thus, the invention further relates to nucleic acid molecules which encode the bifunctional molecule.

The nucleic acid molecule can be double stranded or single stranded and can be a DNA molecule, such as cDNA or genomic DNA, or an RNA molecule. As such, the nucleic acid molecule can include one or more exons, with or without, as appropriate, introns. In one example, the nucleic acid molecule contains a single open reading frame which encodes both the first binding moiety, second binding moiety and optionally a signal sequence and/or a polypeptide linker, when present. By way of example in a multi-exon construct, the nucleic acid molecule contains a first exon which begins with an ATG, encodes one of the binding partners, and optionally the polypeptide linker, and ends with a splice donor site. The construct further would contain an intron followed by a second exon which begins with a splice acceptor site and, optionally a polypeptide linker,

coding sequences for the other binding partner and ending with a stop codon. Of course, alternative combinations of these elements would be apparent to the person of skill in the art.

As such, the nucleic acid molecule can include sequences which encode the first and second binding partners, as well as one or more of the following optional sequences, in a functional relationship, regulatory sequences (as will be discussed in more detail below) a start codon, a signal or leader sequence, splice donor sites, splice acceptor sites, introns, a stop codon, transcription termination sequences, 5' and 3' untranslated regions, polyadenylation sequences, negative and/or positive selective markers, and replication sequences.

The coding regions of the nucleic acid molecule code for the first and second binding moieties of the bifunctional molecule and any polypeptide linkers present. Where the binding moiety is a native ligand or cellular surface protein (e.g. a cellular receptor), or a binding fragment thereof, the nucleic acid molecule coding regions can correspond to the native sequences which encode the binding moiety. Because many amino acids are encoded by a plurality of codons, the coding sequence can be mutated to result in the same amino acid sequence. This may be advantageous where a codon is preferred by the selected host cell. Where the binding moiety is a mutation or variant of a native sequence, as provided above, generally, the nucleic acid sequence will be mutated correspondingly. It is preferred for ease of manufacture of the nucleic acid

sequence to maintain as much of the native sequence as possible. In one embodiment, the nucleic acid molecule shares at least about 65% sequence identity with the corresponding native sequence, preferably, at least about 75% sequence. In a more preferred embodiment, the percent sequence identity is at least about 85%, and still more preferably, at least about 95%.

Recombinant nucleic acid molecules meeting these criteria comprise nucleic acids having sequences identical to sequences of naturally occurring genes, including polymorphic or allelic variants, and portions (fragments) thereof, or variants of the naturally occurring genes. Such variants include mutants differing by the addition, deletion or substitution of one or more residues, modified nucleic acids in which one or more residues are modified (e.g., DNA or RNA analogs), and mutants comprising one or more modified residues.

Many nucleic acid molecules coding for suitable binding moieties are known in the art and can be obtained from, for example, GENBANK. Alternatively, other sequences can be employed, such as homologs of known genes.

Such homologous nucleic acids, including DNA or RNA, can be detected and/or isolated by hybridization (e.g., under high stringency conditions or moderate stringency conditions). "Stringency conditions" for hybridization is a term of art which refers to the conditions of temperature and buffer concentration which permit hybridization of a particular nucleic acid to a second nucleic acid in which the first nucleic acid may be perfectly complementary to the second, or the first

and second may share some degree of complementarity which is less than perfect. For example, certain high stringency conditions can be used which distinguish perfectly complementary nucleic acids from those of less complementarity. "High stringency conditions" and "moderate stringency conditions" for nucleic acid hybridizations are explained on pages 2.10.1-2.10.16 (see particularly 2.10.8-11) and pages 6.3.1-6 in *Current Protocols in Molecular Biology* (Ausubel, F.M. *et al.*, eds., Vol. 1, containing supplements up through Supplement 29, 1995), the teachings of which are hereby incorporated by reference. The exact conditions which determine the stringency of hybridization depend not only on ionic strength, temperature and the concentration of destabilizing agents such as formamide, but also on factors such as the length of the nucleic acid sequence, base composition, percent mismatch between hybridizing sequences and the frequency of occurrence of subsets of that sequence within other non-identical sequences. Thus, high or moderate stringency conditions can be determined empirically.

By varying hybridization conditions from a level of stringency at which no hybridization occurs to a level at which hybridization is first observed, conditions which will allow a given sequence to hybridize (e.g. selectively) with the most similar sequences in the sample can be determined.

Exemplary conditions are described in Krause, M.H. and S.A. Aaronson, *Methods in Enzymology, 200*:546-556 (1991). Also, see especially page 2.10.11 in *Current Protocols in Molecular Biology* (*supra*), which describes how to determine washing conditions for moderate or low

stringency conditions. Washing is the step in which conditions are usually set so as to determine a minimum level of complementarily of the hybrids. Generally, starting from the lowest temperature at which only homologous hybridization occurs, each °C by which the final wash temperature is reduced (holding SSC concentration constant) allows an increase by 1% in the maximum extent of mismatching among the sequences that hybridize. Generally, doubling the concentration of SSC results in an increase in T_m of ~17°C. Using these guidelines, the washing temperature can be determined empirically for high, moderate or low stringency, depending on the level of mismatch sought.

The nucleic acid molecule also preferably comprises regulatory sequences. Regulatory sequences include all cis-acting elements that control transcription and regulation such as, promoter sequences, enhancers, ribosomal binding sites, and transcription binding sites. Selection of the promoter will generally depend upon the desired route for expressing the protein. For example, where the protein will be transformed into a cell by a viral vector, preferred promoter sequences include viral, such as retroviral or adenoviral promoters. Examples of suitable promoters include the cytomegalovirus immediate-early promoter, the retroviral LTR, SV40, and TK promoter. Where the protein is to be expressed in a recombinant eukaryotic or prokaryotic cell, the selected promoter is recognized by the host cell. A suitable promoter which can be used can include the native promoter for the binding moiety which appears first in the construct.

The elements which comprise the nucleic acid molecule can be isolated from nature, modified from native sequences or manufactured *de novo*, as described, for example, in the above-referenced texts. The elements can then be isolated and fused together by methods known in the art, such as exploiting and manufacturing compatible cloning or restriction sites.

The nucleic acid molecules can be inserted into a construct which can, optionally, replicate and/or integrate into a recombinant host cell, by known methods. The host cell can be a eukaryotic or prokaryotic cell and includes, for example, baculoviruses, pichia expression systems, yeast (such as Saccharomyces), bacteria (such as, Escherichia or Bacillus), animal cells or tissue, including insect or mammalian cells (such as, somatic or embryonic human cells, Chinese hamster ovary cells, HeLa cells, human 293 cells and monkey COS-7 cells, etc.).

The nucleic acid molecule can be incorporated or inserted into the host cell, also, by known methods. Examples of suitable methods of transfecting or transforming cells include calcium phosphate precipitation, electroporation, microinjection, infection, lipofection and direct uptake. Methods for preparing such recombinant host cells are described in more detail in Sambrook *et al.*, "Molecular Cloning: A Laboratory Manual," Second Edition (1989) and Ausubel, *et al.* "Current Protocols in Molecular Biology," (1992), for example.

The host cell is then maintained under suitable conditions for expression and recovering the bifunctional molecule. Generally, the cells are

maintained in a suitable buffer and/or growth medium or nutrient source for growth of the cells and expression of the gene product(s). The growth media are not critical to the invention, are generally known in the art and include sources of carbon, nitrogen and sulfur. Examples include Dulbeccos modified eagles media (DMEM), RPMI-1640, M199 and Grace's insect media. Again, the selection of a buffer is not critical to the invention. The pH which can be selected is generally one tolerated by or optimal for growth for the host cell.

The cell is maintained under a suitable temperature and atmosphere. Anaerobic host cells are generally maintained under anaerobic conditions. Alternatively, the host cell is aerobic and the host cell is maintained under atmospheric conditions or other suitable conditions for growth. The temperature should also be selected so that the host cell tolerates the process and can be for example, between about 35° and 40°C.

The bifunctional molecule produced by the processes described herein can be isolated and purified by known means. Examples of suitable purification and isolation processes are generally known and include ammonium sulfate precipitation, dialysis, gel filtration, immuno-affinity, chromatography, electrophoresis, ultrafiltration, microfiltration or diafiltration.

The bifunctional molecule is preferably purified substantially prior to use, particularly where the protein will be employed as an *in vivo* therapeutic, although the degree of purity is not necessarily critical where the molecule is to be used *in vitro*. In one embodiment, the bifunctional molecule can be isolated to about 50% purity (by weight), more

preferably to about 80% by weight or about 95% by weight. It is most preferred to employ a molecule which is essentially pure (e.g., about 99% by weight or to homogeneity).

Bifunctional molecules which are prepared according to the above method can be used directly in the disclosed method or can be screened for activity prior to use. To screen the bifunctional molecule for activity, for example, *in vitro*, the bifunctional molecule (or mixtures of bifunctional molecules) can be contacted with, for example, the target cell or the targeted surface protein under conditions suitable for binding and then assayed for binding. Similarly the bifunctional molecule can be screened for the ability to bind the viral vector, or the surface protein of the viral vector, *in vitro*, by contacting the vector and bifunctional molecule under conditions suitable for binding and detecting binding. The bifunctional molecule can be screened for the ability to induce viral entry by contacting the vector, bifunctional molecule and target cell under typical cell culture conditions and detecting infection of the cells (e.g. replication of viral sequences).

The bifunctional molecule of the invention is particularly useful in the delivery of one or more polynucleotides (e.g., genes) or products thereof to a patient. Generally, the polynucleotide is present or has been incorporated into the genome of the viral vector. The polynucleotide or the product thereof can be a therapeutic agent. An example of a therapeutic polynucleotide includes RNA (e.g., ribozymes) and antisense DNA that prevents or interferes with the

expression of an undesired protein in the target cell. The polynucleotide can also encode a heterologous therapeutic protein. A heterologous protein or polynucleotide is one which does not exist in the virus as it is found in nature. Examples of therapeutic proteins include antigens or immunogens such as a polyvalent vaccine, cytokines, tumor necrosis factor, interferons, interleukins, adenosine deaminase, insulin, T-cell receptors, soluble CD4, epidermal growth factor, human growth factor, blood factors, such as Factor VIII, Factor IX, cytochrome b, glucocerebrosidase, ApoE, ApoC, ApoAI, the LDL receptor, negative selection markers or "suicide proteins", such as thymidine kinase (including the HSV, CMV, VZV TK), anti-angiogenic factors, Fc receptors, plasminogen activators, such as t-PA, u-PA and streptokinase, dopamine, MHC, tumor suppressor genes such as p53 and Rb, monoclonal antibodies or antigen binding fragments thereof, drug resistance genes, ion channels, such as a calcium channel or a potassium channel, and adrenergic receptors, etc.

The invention is particularly useful for vaccine delivery. In this embodiment, the antigen or immunogen can be expressed heterologously (e.g., by recombinant insertion of a nucleic acid sequence which encodes the antigen or immunogen (including antigenic or immunogenic fragments) in a viral vector). Alternatively, the antigen or immunogen can be expressed in a live attenuated, pseudotyped virus vaccine, for example. Generally, the methods can be used to generate humoral and cellular immune responses, e.g. via expression of heterologous pathogen-derived proteins or fragments thereof in specific target cells.

Generally, viral vectors which contain therapeutic polynucleotide are known in the art. Examples include the vectors described in Anderson, *et al.* (United States Patent No.5,399,346), Sambrook, *et al.*, *supra*, Ausubel, *et al.*, *supra*, and Weiss, *et al.* (1985) "RNA Tumor Viruses", Cold Spring Harbor, NY, the contents of which are incorporated herein by reference.

The bifunctional molecule of the claimed invention can be employed in a method for delivering a viral vector to a cell comprising contacting the cell with the bifunctional molecule. The cell can be contacted with the viral vector and the bifunctional molecule *in vitro* or *in vivo*. Where the cell is contacted *in vitro*, the target cell containing the viral vector can then be implanted into a patient for delivery of the polynucleotide or product thereof. The target cell can be migratory, such as a hematopoietic cell, or non-migratory, such as a solid tumor cell or fibroblast. Frequently, the target cell is targeted from the patient and returned to the patient once contacted with the bifunctional molecule and viral vector. *Ex vivo* gene therapy has been described, for example, in Kasid, *et al. PNAS USA 87*:473 (1990), Rosenberg *et al. N. Engl. J. Med., 323:* 570 (1990), Williams, *et al., Nature 310*:476 (1984), Dick, *et al., Cell 42*:71 (1985), Keller, *et al. Nature 318*:149 (1985) and Anderson, *et al.* United States Patent No. 5,399,346.

Alternatively, the bifunctional molecule can be coadministered to the patient with the viral vector. Coadministration is meant to include simultaneous or sequential administration of the two agents, individually or in combination. Where the agents are

administered individually it is preferred that the mode of administration is the same and the site of administration is the same to improve efficiency. It is also preferred that the administrations are conducted sufficiently close in time so that the active ingredients are in the presence of the cells simultaneously. In one embodiment, the bifunctional molecule is administered prior to the viral vector so that the molecule can bind to the target cell initially. In another embodiment, the bifunctional molecule is contacted with the viral vector prior to administration so that the vector and molecule can bind prior to contact with the target cell.

The mode of administration is preferably at the location of the target cells. As such, the administration can be nasally (as in administering a vector expressing ADA) or by injection (as in administering a vector expressing a suicide gene to a tumor). Other modes of administration (parenteral, mucosal, systemic, implant, intraperitoneal, etc.) are generally known in the art. The agents can, preferably, be administered in a pharmaceutically acceptable carrier, such as saline, sterile water, Ringer's solution, and isotonic sodium chloride solution.

The examples presented below are provided as further guidance and are not to be construed as limiting the invention in any way.

Exemplification

EXAMPLE 1

A modified form of a human EGF minigene was obtained by PCR amplification of AX proviral DNA using the oSS1 and oSS2 primers described below. The modified form of the human EGF minigene encoded human EGF (Stern, 1987, *Science 235*:322) with a polyproline linker region (PPPELLGGP) derived from a rabbit Fc chain, added at the N-terminal end. The PCR product contained an EagI site introduced at the 5' end, and KpnI and EagI sites following the human EGF stop codon. The PCR product was digested with the EagI restriction enzyme and introduced into the EagI site of a synthetic Tva gene (Belanger, 1996, *J. Virol. 69*:1019-1024) contained within the pCI mammalian expression vector (Promega).

oSS1: gcgcggccgcccacccccctgaactcctgggggggaccggaggttcag aactctgattccgaatgc (SEQ ID NO:1)

oSS2: gcgcggccgggtaccttatcgcagttccaatttcaggtcgcg (SEQ ID NO:2)

Human 293 cells grown under standard conditions (Zingler, *J. Virol. 70*:7510-7516 (1996)) were plated at 20% confluence on 100 mm tissue culture plates. The cells were transfected with 15μg of the plasmid expression vector encoding TVA-EGF (described above) by the calcium phosphate transfection procedure (Wigler *Cell 11*:223-232 (1977)). Radioactively-labeled TVA-EGF was prepared by incubating transfected 293 cells with ^{35}S cysteine: forty eight hours after transfection, the transfected cells (or for control purposes,

untransfected cells) were incubated with cysteine/ methionine-free DME (1% dialyzed calf serum) for 1 hour and then with the same media supplemented with 100μCi/ml 35S-cysteine for 2 hours. The cells were then washed with PBS and incubated with DMEM for 4 hours and the extracellular supernatants were harvested and stored at -80°C before analysis. Aliquots of 500μl labeled cell culture supernatants were incubated with either 12μg of a subgroup A SU-immunoadhesin (SUA-rIgG)(Zingler, *J. Virol.* 70:7510-7516 (1996)) or with 26 μg of an antibody specific for human EGF for 1 hour at 4°C. These samples were then incubated with protein A-Sepharose, or protein G-Sepharose, respectively for 1 hour at 4°C. The precipitated proteins were then collected by centrifugation in a microfuge, the sepharose beads were washed 4 times each with 1 ml of ice cold PBS and the samples boiled in a non-reducing protein sample buffer for 3 minutes. The proteins were then subjected to electrophoresis on a 10% polyacrylamide gel containing SDS, the gel was then dried and exposed to Kodak XAR-5 film at room temperature. Alternatively, extracellular supernatants were collected 72 hours after transfection and 45 μl aliquots subjected to electrophoresis on 10% polyacrylamide gels containing SDS under non-reducing conditions. Electrophoretically separated proteins were then transferred to nitrocellulose and the TVA-EGF detected by immunoblotting with SUA-rIgG followed by horseradish peroxidase (HRP)-conjugated secondary antibody specific for rabbit immunoglobulins and enhanced chemiluminescence (Zingler, *et al., J. Virol.* 70:7510 (1996)). Transiently transfected human 293 cells produced bifunctional molecules containing TVA and

EGF (TVA-EGF) in the extracellular supernatant whereas supernatants from untransfected control cells did not produce TVA-EGF.

EXAMPLE 2

Unlabeled TVA-EGF was collected in extracellular supernatants from 293 cells transfected essentially as described above except that the supernatants were harvested 72 hours post-transfection. These supernatants were filtered through a 0.45 μm filter and stored at -20°C before use. Confluent plates of cells were washed with Ca^{2+}/Mg^{2+} free PBS and the cells were removed from the plates with 5 ml of Ca^{2+}/Mg^{2+} free PBS containing 1 mM EDTA and put on ice. Mouse L cells lacking EGF receptors (B82), expressing wild type EGF receptors (T23) or expressing EGF receptors lacking in kinase activity (M5) (Chen, 1987, *Nature 328*:820) (4 x 10^3 cells) were incubated either with unlabeled TVA-EGF supernatant (0.1, 0.5, 2.5, 12.5, 25, 250 or 500 μl) or with untransfected 293 cell supernatant for 30 minutes on ice. The cells were spun in a microfuge and washed with ice cold PBS and incubated with 1 μg of purified SUA-rIgG or in 1 ml of PBS for 30 minutes on ice. After washing the cells once with ice cold PBS, the cells were incubated with 5 μl of FITC-conjugated swine anti-rabbit antibody (DAKO) for 30 minutes on ice. Finally, the cells were washed once with ice cold PBS and resuspended in 0.5 ml of PBS containing 1% formaldehyde. The cells were kept in the dark at 4°C before analysis. Bound proteins were detected by flow cytometry using a Becton Dickinson FACScan. The TVA-EGF bound subgroups A SU-immunoadhesin to the M5 and T23 cells that express

surface EGF receptors (M5 and T23 cells), but not to the B82 cells which lack the EGF receptor (Figure 2). The amount of TVA-EGF bound to cells, as measured by mean fluorescence, increased with increasing amounts of TVA-EGF up to concentrations of 12.5 μg. Amounts of TVA-EGF higher than 12.5 μg did not result in a significant increase in the amount of TVA-EGF bound to cells. Thus, the TVA-EGF bifunctional molecule is capable of dual binding as indicated by binding to both an EGF receptor on the surface of M5 and T23 target cells and the SUA-rIgG antibody-like protein.

EXAMPLE 3

The specificity of TVA-EGF binding was evaluated by competition experiments using recombinant human EGF (Upstate Biotechnology).

Bifunctional molecules containing TVA and EGF (TVA-EGF) produced in the extracellular supernatant from human 293 cells were collected 72 hours post-transfection as described in Examples 1 and 2. M5 cells were plated and incubated at 4°C for 1 hour with 200 μl of extracellular supernatants containing TVA-EGF as described in Example 2 except that varying amounts of recombinant human EGF (0-5 μg) were added to the incubation media. The amount of TVA-EGF bound was determined by flow cytometry detection using SUA-rIgG as described in Example 2. The amount of TVA-EGF bound to M5 cells, as measured by mean fluorescence, decreased with increasing amounts of recombinant EGF (Figure 3). These data show that the binding of TVA-EGF to EGF receptors was specific for the EGF moiety of the TVA-EGF bifunctional molecule. As a control, the extracellular

supernatants of T23TVA cells, which stably express a transmembrane form of TVA, were also incubated with increasing amounts of recombinant EGF. T23TVA cells were generated using previously described procedures (Zingler *et al., J. Virol. 69*:4261 (1995)). EGF did not inhibit SUArIgG-TVA binding to T23TVA cells.

EXAMPLE 4

As further evidence that the TVA-EGF bifunctional molecule binds to the surface of target cells through EGF receptors, the retention of TVA-EGF proteins on the surface of M5 and T23 cells was determined.

M5 and T23 cells were prepared and incubated with 200 μl of extracellular supernatant containing TVA-EGF (4°C, 1 hour) as described in Example 2. Cells were then washed with ice cold PBS and either left on ice (zero time point) or incubated with 500 μl of Dulbecco's Modified Eagles Medium (DMEM) at 37°C conditions for 30, 60 or 120 minutes. Following incubation in DMEM, cells were placed on ice and the presence of the TVA-EGF bifunctional molecule on the cell surface of M5 and T23 cells detected by flow cytometry as described in Example 2. TVA-EGF remained present at the cell surface of M5 cells, which express EGF receptors lacking kinase activity for a longer period of time than to T23 cells, which express wildtype EGF receptors (Figure 4). The majority of TVA-EGF present at the cell surface of T23 cells was cleared from the surface of cells within 30 minutes whereas a substantial amount of TVA-EGF remained on the surface of M5 cells after 120 minutes. These data and the data presented in Figure 3 indicate that binding of TVA-EGF to target cells occurs by the EGF

receptor. Those cells which are deficient in kinase activity, the M5 cells, retain bound TVA-EGF on their surface longer than wildtype T23 cells.

EXAMPLE 5

M5 cells expressing kinase-deficient EGF receptors were plated at 10% confluence on 60 mm tissue culture plates in DME containing 10% dialyzed bovine calf serum for one day. The plates were then placed on ice, washed with ice cold PBS, and incubated with 1 ml of either ice cold untransfected 293 cell culture supernatant or with 1 ml ice cold supernatant containing TVA-EGF for 1 hour at 4°C. The plates were put on ice and washed with ice cold PBS and then incubated with 1 ml of untransfected 293 cell culture supernatant containing or lacking 300 μl of RCASH-A (a subgroup A-specific virus) containing the hygromycin B-phosphotransferase gene (Young, 1993, *J. Virol.* *67*:1811) (titer 8 x 10^3 IU/ml). After 1 hour of incubation at 4°C, the plates were placed in a 37°C incubator and 24 hours later they were selected in medium containing sufficient amounts of hygromycin B to select against growth of any uninfected cells (700 μg for M5 cells and 900 μg for B82 cells). After two weeks of selection, the cells were fixed in the solution containing 1% (w/v) methylene blue, 20% (v/v) iso-propanol and 5% (v/v) acetic acid and the colonies were counted. Cells incubated with TVA-EGF became susceptible to subgroup A viral infection (Figures 5 and 6). Only M5 cells incubated with supernatants containing TVA-EGF were infected (Figures 5 and 6). TVA-EGF rendered M5 cells highly susceptible to viral infection. The average number of M5 colonies

infected with RCASH-A virus was approximately 23% of that observed after viral infection of T23TVA cells which express a fully functional epitope-tagged TVA protein (Figure 6). B82 cells which do not express EGF receptors were not infected following the addition of TVA-EGF.

EXAMPLE 6

T23 cells, which express the wildtype EGF; M5 cells, which express an EGF receptor lacking kinase activity; or B82 cells, which lack the EGF receptor, were plated and incubated with 400 μl of subgroup A ALV virus containing a neomycin phosphotransferase gene (RCASA-Neo) at 37°C for 28 hours as described in Example 5 except that varying amounts of extracellular supernatant containing TVA-EGF were added (0, 0.5, 2.5, 12.5 or 200 μl). T23TVA and B82 cells were run in parallel in infectivity experiments as a positive and negative controls, respectively. T23TVA cells (positive control) were plated at 4°C for 1 hour, incubated with 1.5 ml of DMEM with or without 50 μl of the RCASA-Neo virus followed by incubation at 37°C for 28 hours before selection in medium containing 200 μg/ml Geneticin (G418). After two weeks of selection, infected cells (G418- resistant colonies) were determined as described in Example 5. Infectivity increased in M5 and T23 cells with increasing amounts of TVA-EGF in the incubation media (Figure 7). The average number of infected cells observed when M5 cells were incubated with an excess amount of TVA-EGF (200 μl) was approximately 10% of that obtained with T23TVA cells. The level of infectivity

obtained with T23 cells was lower than that observed with M5 cells (Figure 7).

EXAMPLE 7

Infectivity of M5 cells was diminished by the addition of recombinant human EGF (0.5 and 5 μg) (Figure 8). In these experiments M5 cells (1 X 10^5) were plated on 60 mm plates and incubated with 1 ml ice cold DMEM containing 200 μl of TVA-EGF extracellular supernatant and either 0, 0.5 or 5 μg human recombinant EGF. T23TVA cells were incubated with 1 ml DMEM and either 0 or 5 μg EGF. After washing in ice cold PBS the cells, 1.5 ml of DMEM containing 150 μl of RCASA-Neo was added and the cells were incubated at 37°C for 28 hours. The number of infected G418 resistant colonies was determined as described in Example 6. Thus, TVA-EGF mediated viral infection of target cells expressing EGF occurs by binding of the EGF moiety to EGF cell surface receptors (Figure 9).

EQUIVALENTS

Those skilled in the art will know, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.

CLAIMS

What is claimed is:

1. A bifunctional molecule comprising:
   (a) a first binding moiety which binds to a first cellular surface molecule on a target cell; and
   (b) a second binding moiety which binds to a viral surface molecule on a viral vector and activates viral entry, wherein said second binding moiety is an extracellular domain of a viral cellular receptor or a derivative thereof and said bifunctional molecule is not an immunoadhesin.

2. The bifunctional molecule of Claim 1 wherein the first cell surface molecule is selected from a group consisting of carbohydrates, lipids, polypeptides, and glycosylated polypeptides.

3. The bifunctional molecule of Claim 1 wherein the first binding moiety is a ligand to a cellular receptor.

4. The bifunctional molecule of Claim 3 wherein the cellular receptor is cell-type specific.

5. The bifunctional molecule of Claim 4 wherein the first binding moiety is a polypeptide.

6. The bifunctional molecule of Claim 5 wherein the ligand has an amino acid sequence which is the same or substantially the same as an amino acid sequence of at least the receptor-binding portion of a native ligand for the cellular receptor.

7. The bifunctional molecule of Claim 5 wherein the ligand has an amino acid sequence which is the same as an amino acid sequence of at least the receptor-binding portion of a native ligand for the cellular receptor.

8. The bifunctional molecule of Claim 5 wherein the target cell is selected from the group consisting of epithelial cells, fibroblasts, smooth muscle cells, blood cells, tumor cells, cardiac muscle cells, macrophages, dendritic cells, neuronal cells, and pathogen-infected cells.

9. The bifunctional molecule of Claim 5 wherein the cellular receptor is selected from the group consisting of the subgroup A avian leukosis virus receptor, the subgroup B and D avian leukosis virus receptor, the CD4 receptor for human and simian immunodeficiency viruses, the amphotropic and ecotropic murine leukemia virus receptors, the bovine leukemia virus receptor, the gibbon ape leukemia virus receptor, the poliovirus receptor, the major subgroup rhinovirus receptor, the adenovirus and coxsackie virus receptor, the coronavirus receptors, and sialic acid and other carbohydrate receptors.

10. The bifunctional molecule of Claim 5 wherein the ligand is selected from the group consisting of growth factors, epidermal growth factor, interleukins, GM-CSF, G-CSF, M-CSF, EPO, TNF, interferons, and chemokines.

11. The bifunctional molecule of Claim 1 wherein the first binding moiety is a single chain antibody which binds to a cellular receptor.

12. The bifunctional molecule of Claim 11 wherein the cellular receptor is cell-type specific.

13. The bifunctional molecule of Claim 1 wherein the second binding moiety is a polypeptide which has an amino acid sequence which is the same or substantially the same as an amino acid sequence of at least the viral-binding portion of a native extracellular domain of a viral cellular receptor.

14. The bifunctional molecule of Claim 13 wherein the second binding moiety has an amino acid sequence which is the same as an amino acid sequence of at least the viral-binding portion of a native extracellular domain of the viral cellular receptor.

15. The bifunctional molecule of Claim 13 wherein the native extracellular domain of the viral cellular receptor is on a cell which is subject to infection by the viral vector.

16. The bifunctional molecule of Claim 15 wherein the viral vector is selected from the group consisting of retroviral vectors, adenoviral vectors, parvovirus, coronavirus, orthomyxovirus, rhabdovirus, paramyxovirus, picornavirus, alphavirus, adenovirus, herpesvirus, poxvirus, Norwalk virus, togavirus, flavivirus, reoviruses, papovavirus, hepadnavirus, and hepatitis virus.

17. The bifunctional molecule of Claim 15 wherein the viral vector is selected from the group consisting of a retroviral vector or viral vector which comprises a retroviral envelope protein.

18. The bifunctional molecule of Claim 15 wherein the viral cellular receptor is epidermal growth factor receptor.

19. The bifunctional molecule of Claim 1 further comprising a linking moiety.

20. A bifunctional molecule comprising:

(a) a first binding polypeptide which binds to a surface molecule on a target cell, wherein said first binding polypeptide is a ligand to a cell-type specific cellular receptor;

(b) a second binding polypeptide which binds to a surface molecule on a viral vector, wherein the second binding polypeptide is a polypeptide which has an amino acid sequence which is the same or substantially the same as an amino acid sequence of at least the viral-

binding portion of a native extracellular domain of a viral cellular receptor.

21. The bifunctional molecule of Claim 20 wherein the first cell surface molecule is selected from a group consisting of carbohydrates, lipids, polypeptides, and glycosylated polypeptides.

22. The bifunctional molecule of Claim 20 wherein the first binding polypeptide and second binding polypeptide are linked via a peptide bond.

23. The bifunctional molecule of Claim 22 wherein the C-terminus of the first binding polypeptide is linked to the N-terminus of the second binding polypeptide via a peptide bond.

24. The bifunctional molecule of Claim 22 wherein the N-terminus of the first binding polypeptide is linked to the C-terminus of the second binding polypeptide via a peptide bond.

25. The bifunctional molecule of Claim 20 further comprising a linking moiety.

26. The bifunctional molecule of Claim 25 wherein the linking moiety is a polypeptide linker.

27. The bifunctional molecule of Claim 26 wherein the C-terminus of the first binding polypeptide is linked to the N-terminus of the second binding polypeptide via the polypeptide linker.

28. The bifunctional molecule of Claim 26 wherein the N-terminus of the first binding polypeptide is linked to the C-terminus of the second binding polypeptide via the polypeptide linker.

29. The bifunctional molecule of Claim 26 wherein the polypeptide linker is an immunoglobulin hinge region.

30. The bifunctional molecule of Claim 28 wherein the molecule is expressed from a single nucleic acid molecule.

31. A bifunctional molecule for targeting a retroviral vector to a cell-type comprising:
(a) a receptor-binding fragment of a polypeptide ligand which binds a cellular receptor specific to the cell-type;
(b) a viral-binding fragment of a cellular receptor for the retroviral vector.

32. The bifunctional molecule of Claim 31 wherein the polypeptide ligand is human epidermal growth factor.

33. The bifunctional molecule of Claim 31 wherein the retroviral vector is avian leukosis virus and the cellular receptor for the retroviral vector is Tva.

34. The bifunctional molecule of Claim 31 further comprising a hinge region linking the receptor-binding fragment to the viral-binding fragment.

35. A nucleic acid molecule which encodes a bifunctional molecule of Claim 1.

36. A nucleic acid molecule which encodes a bifunctional molecule of Claim 20.

37. The nucleic acid molecule of Claim 36 wherein the bifunctional molecule is encoded in a single open reading frame.

38. A nucleic acid molecule which encodes a bifunctional molecule of Claim 31.

39. The nucleic acid molecule of Claim 38 wherein the bifunctional molecule is encoded in a single open reading frame.

40. A method for preparing a bifunctional molecule comprising:

(a) a first binding moiety which binds to a surface molecule on a target cell; and

(b) a second binding moiety which binds to a surface molecule on a viral vector, wherein said second binding moiety is an extracellular domain of a viral cellular receptor or a derivative thereof and said bifunctional molecule is not an immunoadhesin;

wherein the method comprises maintaining a host cell which expresses the bifunctional molecule under conditions suitable for expression and recovering the bifunctional molecule.

41. The method of Claim 40 wherein the first cell surface molecule is selected from a group consisting of carbohydrates, lipids, polypeptides, and glycosylated polypeptides.

42. A method for preparing a bifunctional molecule comprising conjugating a first binding moiety which binds to a surface protein on a target cell and a second binding moiety which binds to a surface protein on a viral vector, wherein said second binding moiety is an extracellular domain of a viral cellular receptor or a derivative thereof.

43. A method for delivering a viral vector to a cell comprising contacting the cell with the viral vector and a bifunctional molecule comprising:

(a) a first binding moiety which binds to a surface molecule of the target cell; and

(b) a second binding moiety which binds to a surface molecule on a viral vector, wherein said second binding moiety is an extracellular domain of a viral cellular receptor or a derivative thereof.

44. The method of Claim 43 wherein the first cell surface molecule is selected from a group consisting of carbohydrates, lipids, polypeptides, and glycosylated polypeptides.

45. The method according to Claim 43 wherein the cell is contacted with the viral vector *in vitro*.

46. The method according to Claim 43 wherein the cell is contacted with the viral vector *in vivo*.

47. The method according to Claim 43 wherein the viral vector encodes a heterologous protein.

48. The method according to Claim 47 wherein the heterologous protein is selected from the group consisting of immunogens, cytokines, tumor necrosis factor, interferons, interleukins, adenosine deaminase, insulin, T-cell receptors, soluble CD4, epidermal growth factor, human growth factor, blood factors, such as Factor VIII, Factor IX, cytochrome b, glucocerebrosidase, ApoE, ApoC, ApoAI, the LDL receptor, negative selection markers, anti-angiogenic factors, Fc receptors, plasminogen activators, dopamine, MHC, tumor suppressor genes, monoclonal antibodies or antigen binding fragments thereof, drug resistance genes, ion channels and adrenergic receptors.

49. A method for delivering a viral vector to a cell comprising contacting the cell with the viral vector and a bifunctional molecule comprising:

(a) a first binding polypeptide which binds to a surface molecule on a target cell, wherein said first binding polypeptide is a ligand to a cell-type specific cellular receptor;

(b) a second binding polypeptide which binds to a surface molecule on a viral vector, wherein the second binding polypeptide is a polypeptide which has an amino acid sequence

which is the same or substantially the same as an amino acid sequence of at least the viral-binding portion of a native extracellular domain of a viral cellular receptor.

50. The method of Claim 49 wherein the first cell surface molecule is selected from a group consisting of carbohydrates, lipids, polypeptides, and glycosylated polypeptides.

51. A method for delivering a retroviral vector to a cell comprising contacting the cell with the retroviral vector and a bifunctional molecule comprising:

(a) a receptor-binding fragment of a polypeptide ligand which binds a cellular receptor specific to the cell-type;

(b) a viral-binding fragment of a cellular receptor for the retroviral vector.

52. The method according to Claim 51 wherein the cell is contacted with the retroviral vector *in vitro*.

53. The method according to Claim 51 wherein the cell is contacted with the retroviral vector *in vivo*.

54. The method according to Claim 51 wherein the retroviral vector encodes a heterologous protein.

55. The method according to Claim 54 wherein the heterologous protein is selected from the group consisting of immunogens, cytokines, tumor necrosis

factor, interferons, interleukins, adenosine deaminase, insulin, T-cell receptors, soluble CD4, epidermal growth factor, human growth factor, blood factors, such as Factor VIII, Factor IX, cytochrome b, glucocerebrosidase, ApoE, ApoC, ApoAI, the LDL receptor, negative selection markers, anti-angiogenic factors, Fc receptors, plasminogen activators, dopamine, MHC, tumor suppressor genes, monoclonal antibodies or antigen binding fragments thereof, drug resistance genes, ion channels and adrenergic receptors.

56. The method according to Claim 54 wherein the polypeptide ligand is human epidermal growth factor.

57. The method according to Claim 56 wherein the retroviral vector is avian leukosis virus and the cellular receptor for the retroviral vector is Tva.

58. The method according to Claim 57 wherein the bifunctional molecule further comprising a hinge region linking the receptor-binding fragment to the viral-binding fragment.

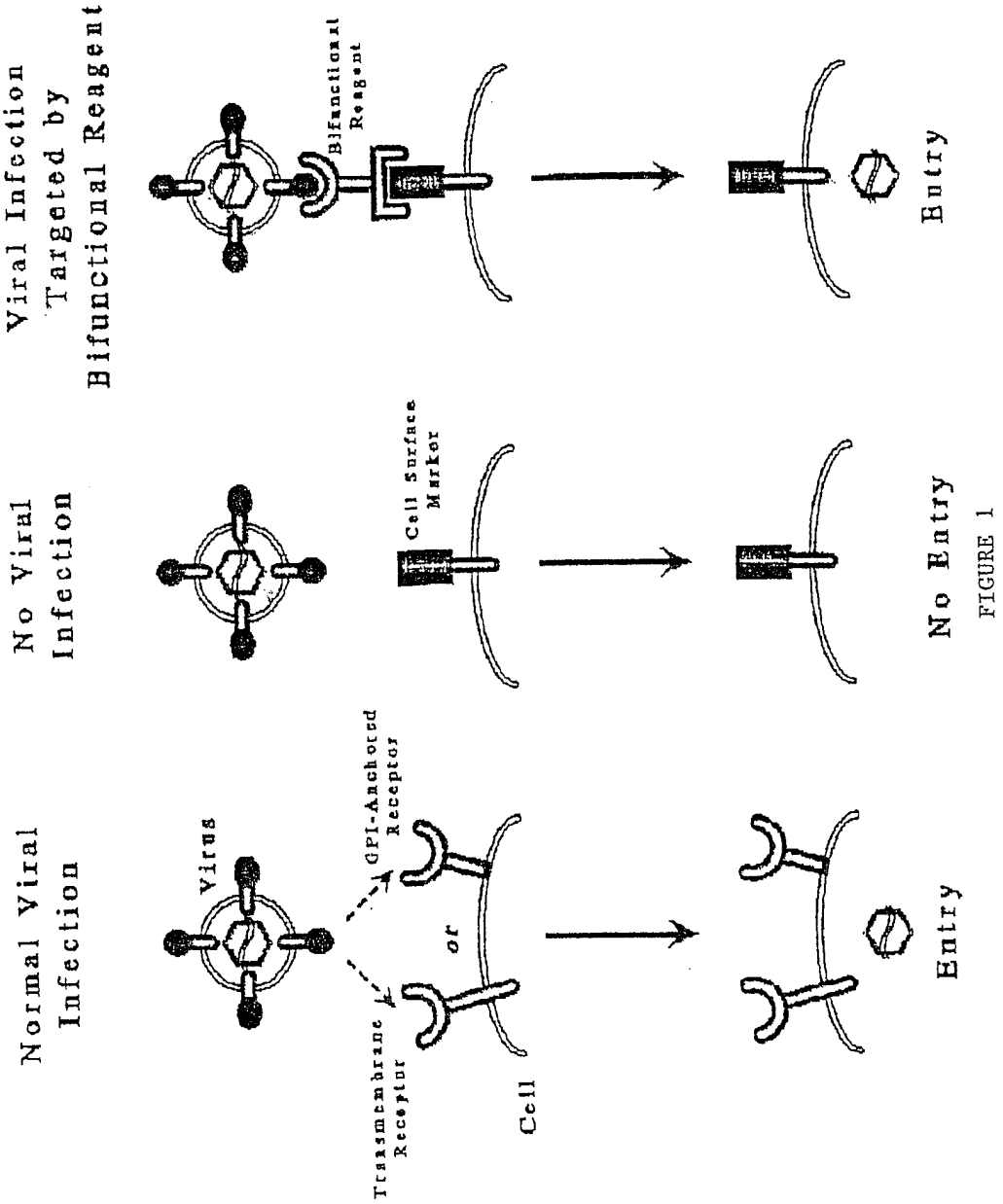
Normal Viral Infection
Virus
GPI-Anchored Receptor
or
Transmembrane Receptor
Cell
Entry
No Viral Infection
Cell Surface Marker
No Entry
Viral Infection Targeted by Bifunctional Reagent
Bifunctional Reagent
Entry

FIGURE 1

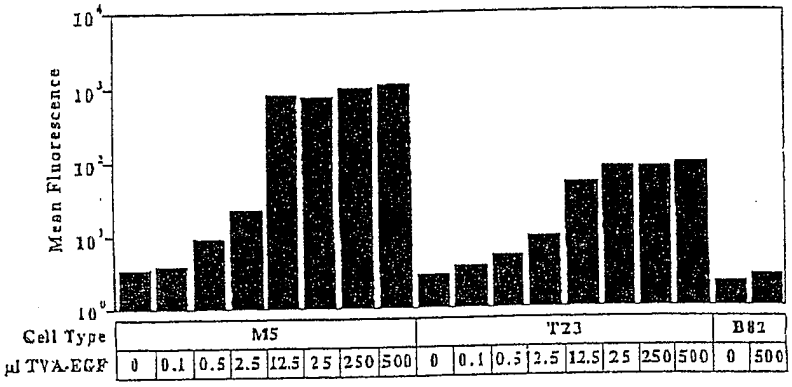
Mean Fluorescence
10^4
10^3
10^2
10^1
10^0
Cell Type
M5
T23
B82
µl TVA-EGF
0 0.1 0.5 2.5 12.5 25 250 500 0 0.1 0.5 2.5 12.5 25 250 500 0 500

FIGURE 2

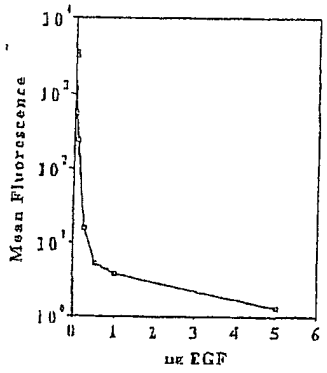

FIGURE 3

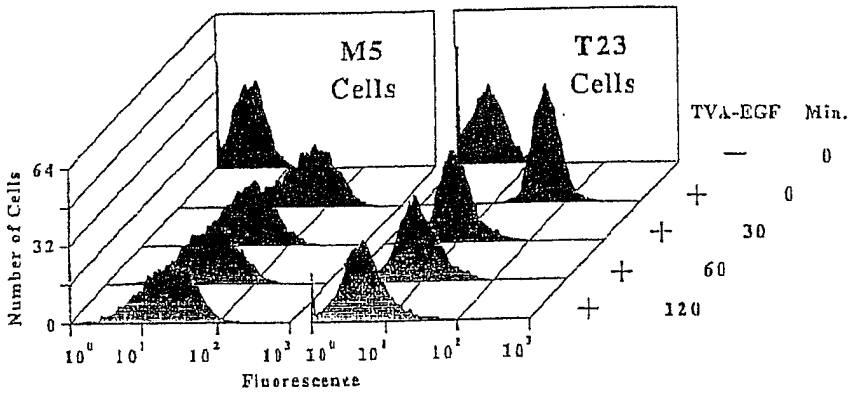
M5
Cells
T23
Cells
TVA-EGF
Min.
—
0
+
0
+
30
+
60
+
120
Number of Cells
64
32
0
10^0 10^1 10^2 10^3 10^0 10^1 10^2 10^3
Fluorescence

FIGURE 4

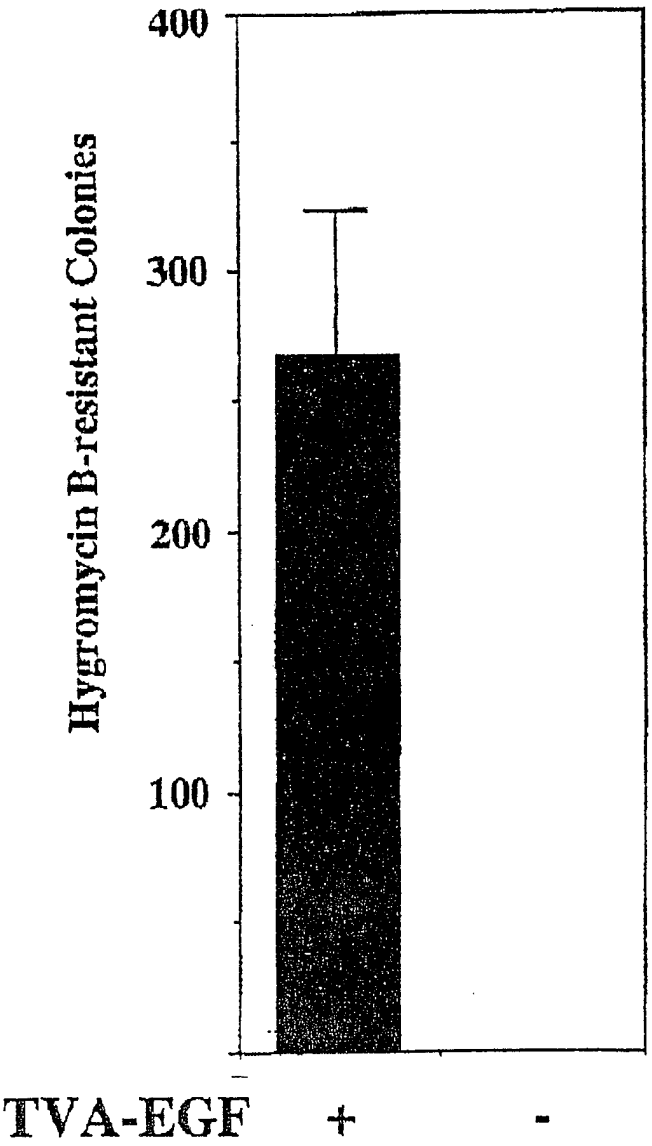

FIGURE 5

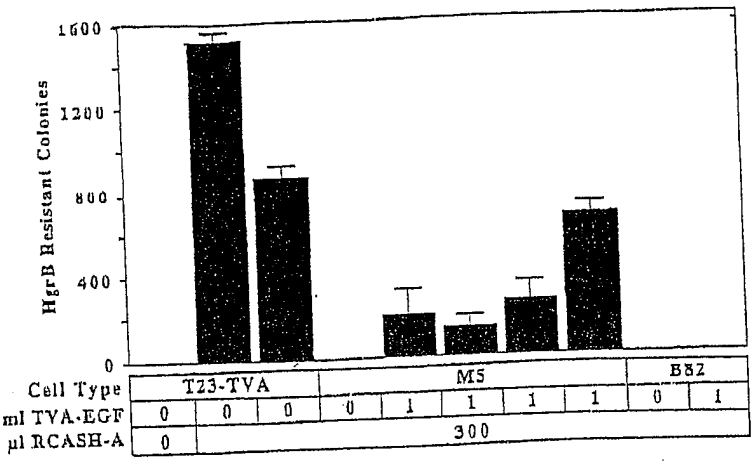
1600
1200
800
400
0
HgrB Resistant Colonies
Cell Type
T23-TVA
M5
B82
ml TVA-EGF
0 0 0 0 1 1 1 1 0 1
µl RCASH-A
0
300

FIGURE 6

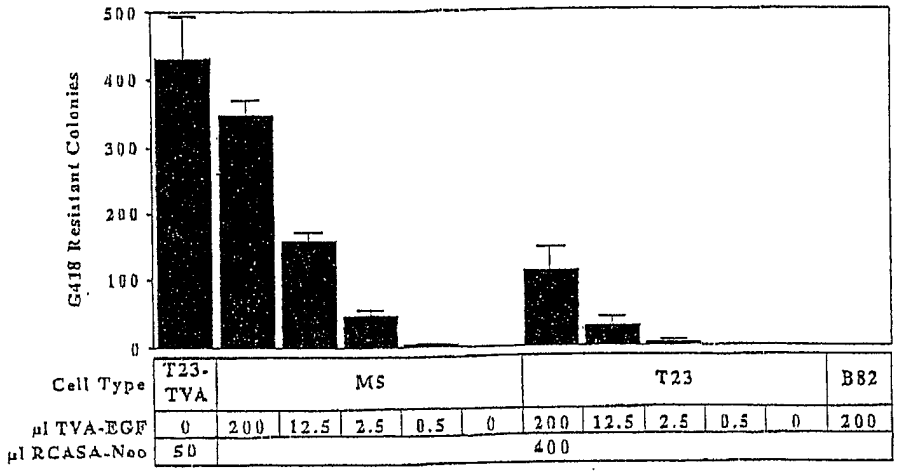
G418 Resistant Colonies
500
400
300
200
100
0
Cell Type
T23-TVA
MS
T23
B82
µl TVA-EGF
0
200
12.5
2.5
0.5
0
200
12.5
2.5
0.5
0
200
µl RCASA-Neo
50
400

FIGURE 7

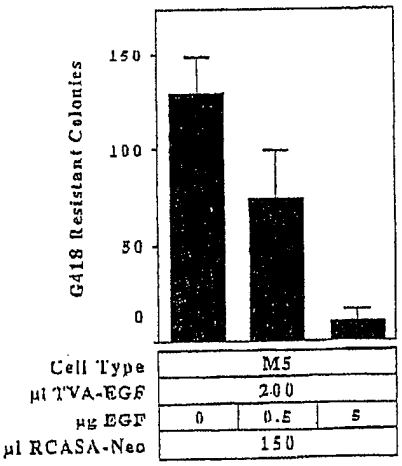
G418 Resistant Colonies
150
100
50
0
Cell Type
M5
µl TVA-EGF
200
µg EGF
0
0.5
5
µl RCASA-Neo
150

FIGURE 8

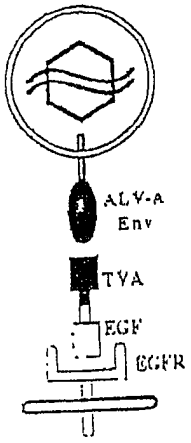

FIGURE 9

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/07720

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/00 C12N15/62

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 27643 A (TARGETED GENETICS CORP ;PAUL RALPH W (US); OVERELL ROBERT (US)) 8 December 1994 see page 7, line 22 - page 9, line 23 see page 13, line 31 - page 16, line 28 see page 38, line 1 - page 40, line 5 ---	1-58
X	FR 2 649 119 A (CENTRE NAT RECH SCIENT) 4 January 1991 see page 1, line 31 - page 2, line 25 see claims 1-15 ---	1-58
X	WO 93 25682 A (UNIV NEW YORK ;MERUELO DANIEL (US); YOSHIMOTO TAKAYUKI (JP)) 23 December 1993 see page 51, line 27 - page 52, line 15 --- -/--	1-58

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 September 1998	06/10/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Seegert, K

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

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/07720

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication.where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 09221 A (THERA GENE HB) 13 May 1993 see page 1, line 30 - page 2, line 13 ---	1-58
X	ETIENNE-JULAN M ET AL: "THE EFFICIENCY OF CELL TARGETING BY RECOMBINANT RETROVIRUSES DEPENDS ON THE NATURE OF THE RECEPTOR AND THE COMPOSITION OF THE ARTIFICIAL CELL-VIRUS LINKER" JOURNAL OF GENERAL VIROLOGY, vol. 73, December 1992, pages 3251-3255, XP000612121 see abstract -----	1-58

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
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