Membrane-Tethered Proteins for Basic Research, Imaging, and Therapy

Tian-Lu Cheng,[1] Steve Roffler[2]

[1]Faculty of Biomedical Science and Environmental Biology, Kaohsiung Medical University, Kaohsiung, Taiwan
[2]Institute of Biomedical Sciences, Academia Sinica, Taipei 11529, Taiwan

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Abstract: Secreted and intracellular proteins including antibodies, cytokines, major histocompatibility complex molecules, antigens, and enzymes can be redirected to and anchored on the surface of mammalian cells to reveal novel functions and properties such as reducing systemic toxicity, altering the *in vivo* distribution of drugs and extending the range of useful drugs, creating novel, specific signaling receptors and reshaping protein immunogenicity. The present review highlights progress in designing vectors to target and retain chimeric proteins on the surface of mammalian cells. Comparison of chimeric proteins indicates that selection of the proper cytoplasmic domain and introduction of oligiosaccharides near the cell surface can dramatically enhance surface expression, especially for single-chain antibodies. We also describe progress and limitations of employing surface-tethered proteins for preferential activation of prodrugs at cancer cells, imaging gene expression in living animals, performing high-throughput screening, selectively activating immune cells in tumors, producing new adhesion molecules, creating local immune privileged sites, limiting the distribution of soluble factors such as cytokines, and enhancing polypeptide immunogenicity. Surface-anchored chimeric proteins represent a rich source for developing new techniques and creating novel therapeutics.

Key words: surface expression; transmembrane domain; GPI-anchor; single-chain antibody; membrane protein; prodrugs; imaging; chimeric proteins

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Correspondence to: Dr. Steve Roffler, Institute of Biomedical Sciences, Academia Sinica, Academia Road, Section 2, No. 128, Taipei 11529, Taiwan. E-mail: sroff@ibms.sinica.edu.tw

1. INTRODUCTION

Chimeric proteins with novel functions can be routinely created by genetic engineering. An emerging class of genetically altered proteins includes those engineered to redirect their normal location from intracellular or extracellular compartments to the surface of mammalian cells. Relocation of intracellular or nonmammalian proteins (for example, those derived from bacteria) to the cell surface facilitates the design of new molecules with novel functions. For example, soluble proteins such as antibodies or their fragments can be anchored on cells to create artificial receptors to activate immune cells, capture imaging agents and biologically active polypeptides and generate novel signaling molecules.[1–4] Likewise, tethering secreted proteins to the cell surface can minimize systemic exposure and reduce side effects associated with many biologically active polypeptides.[5,6] Surface-anchored proteins may also elicit new or more effective biological activities. For example, membrane-anchored antibodies often induce receptor signaling in target cells more effectively than secreted antibodies.[7,8] Likewise, membrane-anchored enzymes have been designed to activate anticancer prodrugs. Surface-tethered enzymes can extend the range of drugs available for therapy and promote effective killing of nontransduced cancer cells.[9–11] Redirection of polypeptides to the cell surface can dramatically alter humoral and cellular immunogenicity, providing opportunities to manipulate immune responses.[12,13] Expression of antibodies and enzymes on cells also provides opportunities for novel cell separation and imaging technologies.[3,14–16] Membrane-bound chimeric proteins therefore represent a rich source for the development of novel research tools and therapeutics.

Here we first describe how proteins can be retargeted and tethered on the surface of mammalian cells. Special emphasis is given to describing the relative effectiveness of the elements employed to tether chimeric proteins on cells. General rules for creating chimeric proteins that are efficiently expressed on the cell surface are presented. Finally, we review applications of membrane-anchored chimeric proteins including (1) membrane-anchored enzymes for prodrug activation and noninvasive imaging, (2) membrane-anchored antibodies to create artificial receptors, activate immune cells and modulate local immune responses, (3) single-chain MHC molecules to activate specific T cells, (4) surface expression of cytokines and chemokines for immune stimulation, and (5) surface expression of antigens on mammalian cells for vaccine development.

2. EXPRESSION OF CHIMERIC PROTEINS ON MAMMALIAN CELLS

Prokaryotic proteins as well as endogenous proteins that are normally secreted or expressed intracellularly can be directed to and retained on the surface of mammalian cells as an integral membrane protein or as a glycosylphosphatidylinositol (GPI)-anchored protein. Integral membrane proteins possess at least one membrane-spanning transmembrane domain (TM), typically composed of approximately 20 hydrophobic amino acids that form an alpha helix. Type I integral membrane proteins are oriented such that their *N*-terminus is extracellular and cytoplasmic tail (CT) is located inside the cytoplasmic space. Conversely, type II TM proteins possess a short *N*-terminal cytoplasmic domain and *C*-terminal extracellular domain. Proteins possessing a GPI-anchor signal peptide are post-translationally modified by attachment of a GPI anchor at their *C*-terminus that self associates with the outer sheaf of the lipid bilayer.

Genetic engineering can be employed to create transgenes that code for chimeric membrane-tethered proteins. Type I integral and GPI-anchored proteins require a hydrophobic signal peptide at their *N*-terminus to allow interaction with the signal-recognition particle, which directs import of the nascent polypeptide through a channel (translocon) into the lumen of the endoplasmic reticulum (Fig. 1).[17] The signal peptide is cotranslationally cleaved in the lumen of the endoplasmic reticulum by signal peptidase. The translocon also facilitates integration of TM domains into the endoplasmic

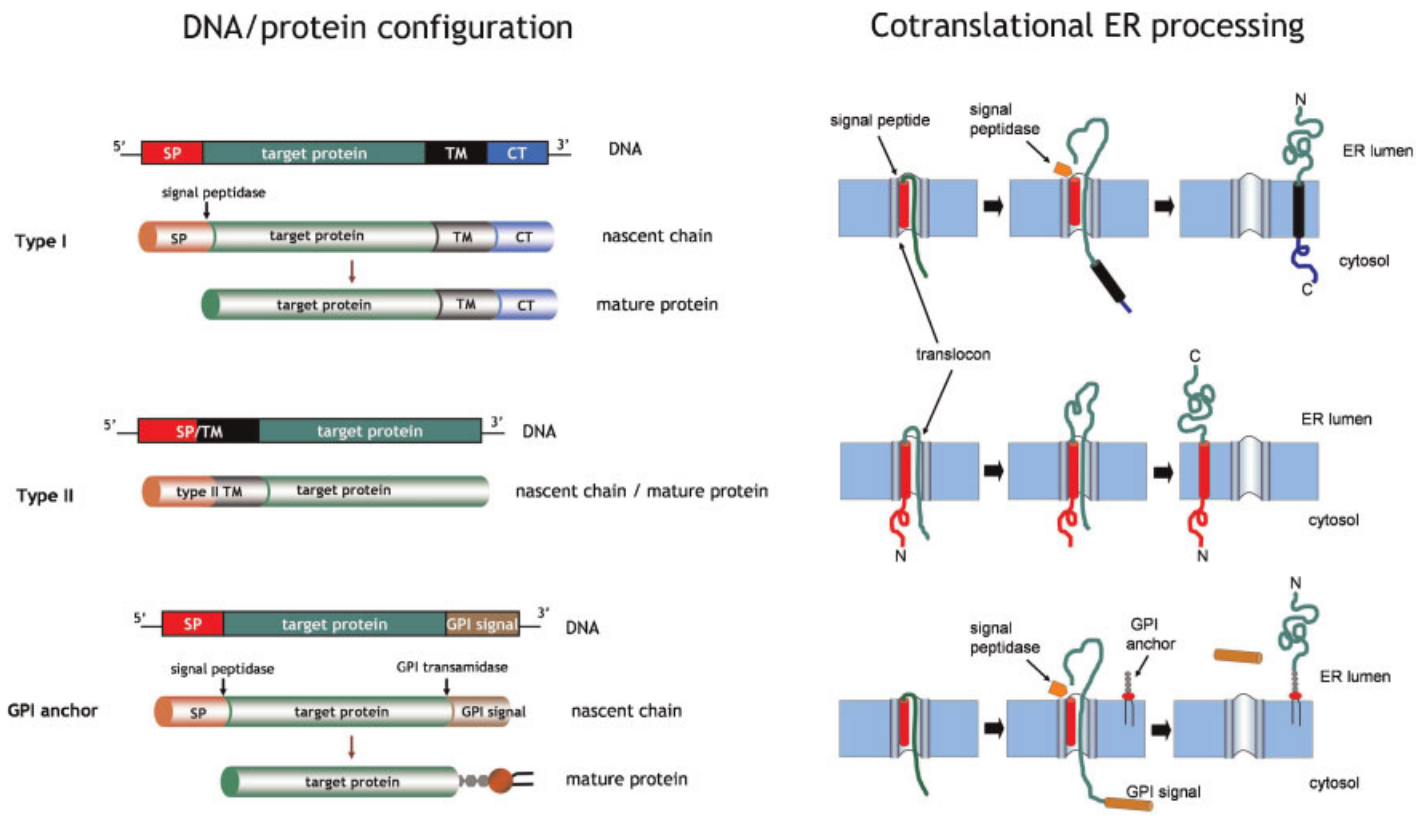

Figure 1. Expression of chimeric proteins on mammalian cells. The hydrophobic signal peptides present at the *N*-terminus of Type I integral membrane proteins and GPI-anchored membrane proteins are cotranslationally cleaved from the nascent chain in the lumen of the endoplasmic reticulum by signal peptidase. GPI transamidase in the endoplasmic reticulum cleaves the *C*-terminal GPI signal peptide and attaches the preformed GPI core structure to the newly exposed *C*-terminus of GPI-anchored proteins. GPI-anchored and type I integral membrane proteins are oriented with their *N*-terminus outside the cells. The noncleavable TM of type II integral membrane proteins acts as both signal peptide and TM to anchor the protein in the endoplasmic reticulum membrane. The *C*-terminus of type II integral membrane proteins is located outside the plasma membrane of cells. Ribosomes and signal recognition particles are omitted for clarity. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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reticulum membrane upon completion of protein translation.[18] To allow attachment of a protein to the cell surface *via* a GPI anchor, DNA coding for a GPI signal sequence can be appended to the 3′ end of a protein gene (Fig. 1). Expression of the transgene, which must contain a signal peptide at the *N*-terminus, directs the nascent polypeptide into the endoplasmic reticulum. The GPI signal sequence is cleaved in the lumen of the endoplasmic reticulum by a GPI transamidase which then attaches a preformed GPI core structure to the newly created carboxy terminus of the protein *via* an amide bond. The GPI anchor core structure consists of ethanolamine phosphate, three mannose residues, glucosamine and phophatidylinositol, which is physically inserted in the luminal side of the endoplasmic reticulum membrane.[19,20] Type I integral membrane proteins and GPI-anchored proteins are oriented with the *N*-terminus extended away from the plasma membrane. Proteins can be oriented in the reverse orientation, which may be advantageous if the *C*-terminal domain is important for biological activity, by attaching a DNA fragment encoding the *N*-terminus of a type II integral membrane protein to the 5′ end of the protein gene (Fig. 1). The *N*-terminal hydrophobic domain of type II integral membrane proteins acts as both a noncleavable signal peptide to direct the import of the protein into the lumen of the endoplasmic reticulum as well as an integral TM anchor.[21]

Once inside the lumen of the endoplasmic reticulum, membrane proteins undergo folding, assembly and post-translational modifications such as initial glycosylation and phosphorylation. Correctly folded proteins are concentrated in and exported from the endoplasmic reticulum to the Golgi complex in COPII transport vesicles (reviewed in Ref. 22). Oligosaccharide chains on proteins are further modified in the Golgi complex before membrane proteins are transported to the cell surface.[23]

3. EFFICIENT SURFACE EXPRESSION

Although a wide range of transmembrane domains (TM) and GPI anchors have been employed to anchor heterologous proteins on cells, few studies have systematically examined how to maximize the expression of chimeric proteins on mammalian cells. In an early study, Chou and colleagues[24] tested different anchoring domains to direct an alpha fetoprotein (AFP) reporter to the surface of baby hamster kidney cells. The mature alpha fetoprotein cDNA was cloned into the commercial vector pHOOK-1 (Invitrogen, Carlsbad, CA) to express a fusion protein with an immunoglobulin Vκ signal peptide at the *N*-terminus and the TM and truncated (retaining 6 amino acids) cytoplasmic tail (CT) of the human platelet-derived growth factor receptor (PDGFR) at the *C*-terminus of alpha fetoprotein (Table I). AFP-PDGFR was expressed at modest levels on transiently transfected baby hamster kidney cells as determined by flow cytometric analysis. Higher surface expression was achieved when alpha fetoprotein was fused to the TM and entire CT of mouse B7-1 (AFP-B7) or the GPI signal peptide from decay accelerating factor (AFP-DAF) (300% and 240% higher than AFP-PDGFR, respectively).[24] Fusion of the type II TM from the human asialoglycoprotein receptor to the *N*-terminus of alpha fetoprotein (ASGPR-AFP) did not enhance expression of alpha fetoprotein on cells. The half-lives of alpha fetoprotein chimeric proteins varied by more than sevenfold with AFP-B7 being most stable and AFP-PDGFR being least stable.[25] Pulse-chase experiments revealed that AFP-B7 and AFP-DAF were rapidly transported to the cell surface whereas AFP-PDGFR and ASGPR-AFP were largely retained in the endoplasmic reticulum. Even more disparate results were found with membrane-anchored single-chain antibodies (scFv). scFv are created by linking the immunoglobulin light and heavy chain variable region domain genes with a DNA fragment coding for a flexible polypeptide (Fig. 2A). Attachment of the B7-1 TM and cytoplasmic tail to a scFv (scFv-B7) allowed accumulation of approximately 5 times more antibody on baby hamster kidney cells[25] and 20 times more scFv on 3T3 fibroblasts (Fig. 2B) as compared to the PDGFR TM (scFv-PDGFR), suggesting that the presence of the B7-1 cytoplasmic tail can enhance surface expression. In agreement with this suggestion, truncation of all but five amino acids of the B7-1 cytoplasmic tail

Table I. Membrane-Anchored Chimeric Proteins Employed in Basic Transport Studies

Name	Functional extracellular domain	Juxtamembrane linker (amino acids)[a]	Glyc Sites[b]	Transmembrane domain	Cytoplasmic tail (amino acids)	Full CT?[c]	Cell target	Purpose	Surface level[d]	Ref
AFP-PDGFR	alpha-fetoprotein (AFP)	c-*myc* tag + PDGFR (20)	0	human platelet-derived growth factor receptor (PDGFR)	PDGFR (6)	no	BHK baby hamster kidney cells	reporter	++	24
AFP-B7	alpha-fetoprotein (AFP)	none	0	mouse B7-1	mouse B7-1 (38)	yes	BHK baby hamster kidney cells	reporter	+++	24
AFP-DAF	alpha-fetoprotein (AFP)	none	0	human decay-accelerating factor (DAF) GPI signal sequence	NA	NA	BHK baby hamster kidney cells	reporter	+++	24
ASGPR-AFP	alpha-fetoprotein (AFP)	ASGPR (5)	0	human asialoglycoprotein receptor (ASGPR) type II TM	ASGPR (37)	yes	BHK baby hamster kidney cells	reporter	++	24
scFv-PDGFR	scFv	c-*myc* tag + human PDGFR (20)	0	human platelet-derived growth factor receptor (PDGFR)	PDGFR (6)	no	BHK baby hamster kidney cells, 3T3 mouse fibroblasts	reporter	-	25
scFv-B7	scFv	none	0	mouse B7-1	mouse B7-1 (38)	yes	3T3 mouse fibroblasts	reporter	+	
scFv-B7t	scFv	none	0	mouse B7-1	mouse B7-1 (5)	no	3T3 mouse fibroblasts	reporter	-	
scFv-PDGFR-B7	scFv	c-*myc* tag + human PDGFR (20)	0	human platelet-derived growth factor receptor (PDGFR)	human PDGFR (6) + mouse B7-1 (33)	yes	3T3 mouse fibroblasts	reporter	+/-	
scFv-e-B7	scFv	Ig-like C type domain of mouse B7-1 (105)	3	mouse B7-1	mouse B7-1 (38)	yes	3T3 mouse fibroblasts	reporter	++++	8
scFv-γ1-B7	scFv	human IgG_1 hinge-CH_2-CH_3	1	mouse B7-1	mouse B7-1 (38)	yes	3T3 mouse fibroblasts	reporter	++	8
scFv-BGP-B7	scFv	human biliary glycoprotein-1 (BGP) (108)	3	mouse B7-1	mouse B7-1 (38)	yes	3T3 mouse fibroblasts	reporter	+++	8
scFv-BGP1-B7	scFv	mutant BGP (108)	2	mouse B7-1	mouse B7-1 (38)	yes	3T3 mouse fibroblasts	reporter	++	8
scFv-BGP2-B7	scFv	mutant BGP (108)	1	mouse B7-1	mouse B7-1 (38)	yes	3T3 mouse fibroblasts	reporter	++	8
scFv-BGP3-B7	scFv	mutant BGP (108)	0	mouse B7-1	mouse B7-1 (38)	yes	3T3 mouse fibroblasts	reporter	+/-	8
scFv-CD44-B7	scFv	human CD44E (380)	8	mouse B7-1	mouse B7-1 (38)	yes	3T3 mouse fibroblasts	reporter	++++	8

NA, not applicable.

[a]Number of amino acids in the juxtamembrane linker domain.

[b]Potential N-linked glycosylation sites present in the juxtamembrane linker domain.

[c]Indicates if the entire cytoplasmic tail was included in the chimeric protein.

[d]Relative surface expression level estimated from flow cytometric or fluorescence microscopy data.

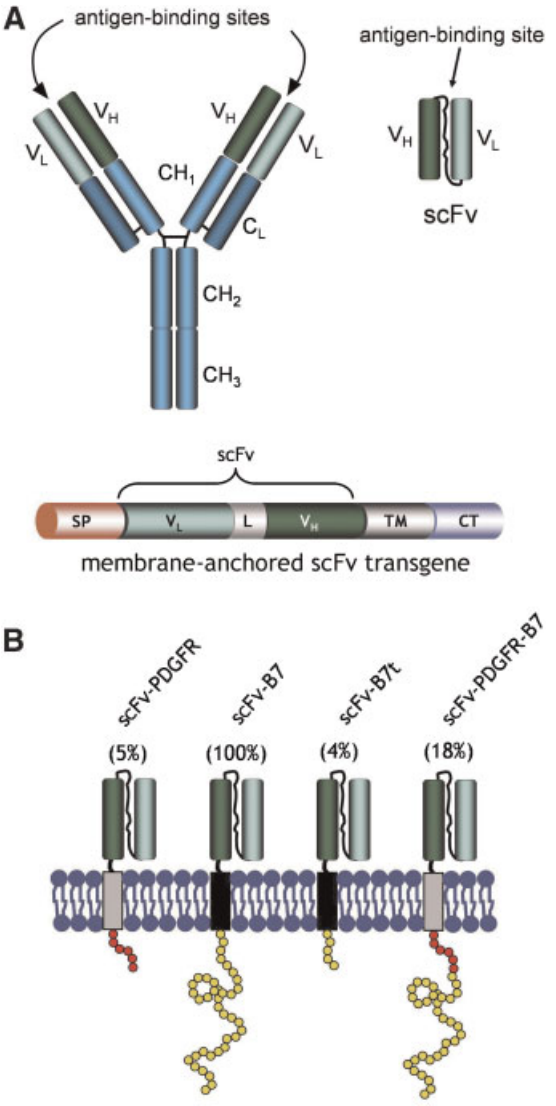


Figure 2. Membrane-anchored single-chain antibodies. **A**: A scFv is composed of the immunoglobulin heavy and light chain variable regions linked together with a flexible peptide (normally 15 amino acids in length). For expression of a membrane-anchored scFv, a signal peptide (SP) directs the nascent chain to the lumen of the endoplasmic reticulum whereas the TM anchors the antibody in the plasma membrane. **B**: A cytoplasmic tail can enhance scFv surface expression. scFv-PDGFR is composed of a scFv fused to the PDGFR TM, which includes 6 amino acids of the PDGFR CT. In scFv-B7, the same scFv was fused to the TM and complete 38 amino acid CT of murine B7-1. All but 5 amino acids of the B7-1 CT were deleted in scFv-B7t. In scFv-PDGFR-B7, the *C*-terminal 33 amino acids of the B7-1 CT were fused to scFv-PDGFR. The relative levels of scFv expressed on the surface of transiently-transfected 3T3 fibroblasts, as determined by flow cytometric analysis, are indicated in parentheses. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

caused almost complete loss of scFv from the surface of 3T3 cells (scFv-B7t in Fig. 2B). Conversely, attachment of the B7-1 cytoplasmic tail to the PDGFR TM (scFv-PDGFR-B7 in Fig. 2B) enhanced the expression of scFv on 3T3 fibroblasts. Similar results were observed when alpha fetoprotein was used as a reporter protein; AFP-B7t and AFP-PDGFR were largely retained in the endoplasmic reticulum whereas AFP-B7 and AFP-PDGFR-B7 were rapidly transported to the plasma membrane (unpublished results). Thus, the B7-1 cytoplasmic domain enhances transport from the endoplasmic

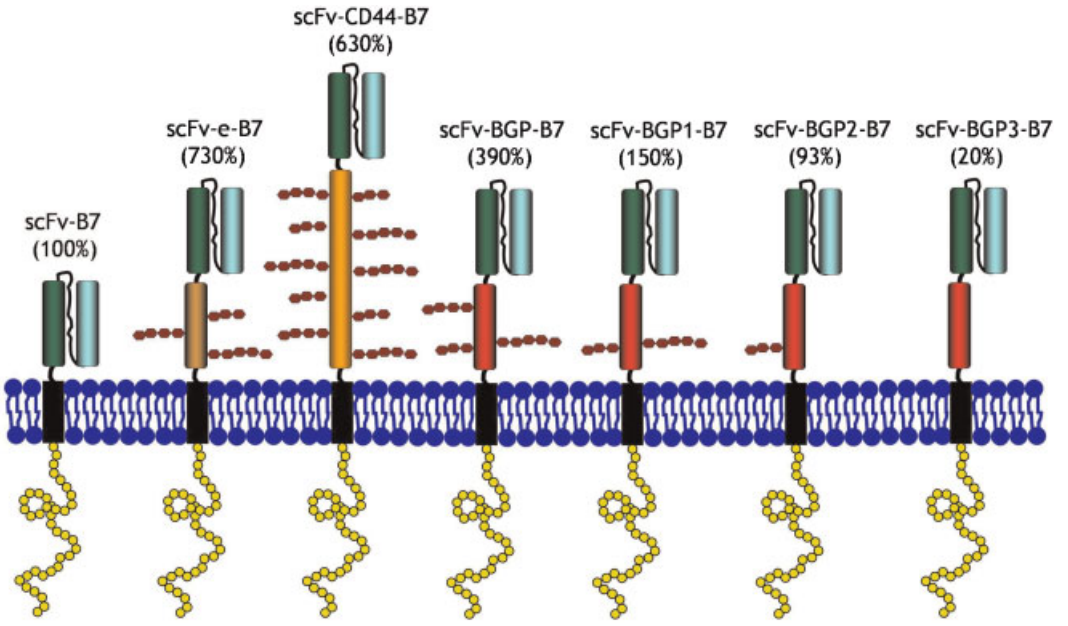


Figure 3. Spacer domains with *N*-linked or *O*-linked glycosylation sites can enhance surface expression of scFv. Spacer domains from murine B7-1 (scFv-e-B7) containing 3 *N*-linked glycosylation sites, CD44e (scFv-CD44-B7) containing multiple *N*-linked and *O*-linked glycosylation sites or biliary glycoprotein 1 (scFv-BGP-B7) containing 3 *N*-linked glycosylation sites were inserted between the scFv and B7 TM. The glycosylation sites in scFv-BGP-B7 were progressively removed in scFv-BGP1-B7 (1 site removed), scFv-BGP2-B7 (2 sites removed) and scFv-BGP3-B7 (all 3 sites removed). The relative expression levels relative to scFv-B7 are indicated in parentheses. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

reticulum and allows accumulation of chimeric proteins on the plasma membrane. These results are consistent with studies showing that signal sequences present in the cytoplasmic domain of membrane proteins may directly interact with COPII coat subunits to facilitate their recruitment into COPII vesicles and accelerate transport to the Golgi apparatus.[22]

Introduction of the hinge-CH_2–CH_3 domains of the human IgG_1 heavy chain between the scFv and B7-1 TM in scFv-B7 increased the expression of scFv on baby hamster kidney cells (~7-fold greater than scFv-PDGFR).[25] This domain contains a single *N*-linked glycosylation site. To test if the presence of oligiosaccharides in the extracellular juxtamembrane region can increase surface expression, spacer domains containing *N*-linked or *O*-linked glycosylation sites were inserted between the scFv and TM domains (Table I).[8] Compared to scFv-B7, surface expression of the scFv was increased by introduction of the Ig-like C2-type domain of murine B7-1 (~7-fold higher), the CD44E extracellular domain (~6-fold higher) and the first Ig-like V-type domain of biliary glycoprotein 1 (BGP-1) (~4-fold) (Fig. 3).[8] Removal of the three *N*-linked glycosylation sites in the BGP-1 spacer resulted in increased shedding of scFv from the cell surface and poor expression on cells (Fig. 3). Introduction of a spacer domain also enhanced the expression of an enzyme (β-glucuronidase) on cells although the enhancement was less dramatic than found for scFv.[11]

Taken together, these studies indicate that the choice of TM, CT and extracellular spacers can greatly influence the level of heterologous protein retained on mammalian cells. For a given expression level, the accumulation of proteins on the plasma membrane reflects the net rate of intracellular transport minus the rate of shedding, internalization and dilution from cellular division. Factors that increase intracellular transport (an intact cytoplasmic tail) and decrease shedding (insertion of spacers that possess glycosylation sites at the extracellular juxtamembrane position) can

increase surface expression. In general, small proteins, such as scFv, appear to be more susceptible to shedding as compared to large globular proteins such as alpha fetoprotein or the tetrameric enzyme β-glucuronidase. This may reflect relatively easier access of proteases to the juxtamembrane stalk of the small scFv molecule as compared to the bulkier alpha fetoprotein and β-glucuronidase proteins.

For a new project, it is suggested that attachment of the Ig-like *C*-type extracellular domain, TM and full cytoplasmic tail of the murine B7-1 antigen to the *C*-terminus of the target protein is a good starting point to achieve high surface expression levels. The B7-1 extracellular domain can help position the target protein above the cell surface and it also possesses three *N*-linked oligiosaccharides to reduce cleavage of chimeric proteins from the cell membrane. A sequence coding a signal peptide should be appended to the 5′ end of the target gene if it does not already possess one. For cases in which it is desirable to express dimers on cells, the B7-1 extracellular domain can be replaced with the hinge-CH_2–CH_3 domains of human or mouse IgG_1. These domains promote efficient formation of disulfide-linked dimers. Attachment of a GPI signal sequence, such as the one derived from decay-accelerating factor, to the 3′ end of a target protein sequence is a reasonable alternative to achieve good expression levels. Vectors containing these elements are available upon request from the authors.

4. MEMBRANE-ANCHORED ENZYMES

A. Enzyme-Prodrug Therapy

Several investigators have developed transgenes to express enzymes on the surface of mammalian cells (Table II), especially for cancer gene therapy. Chimeric membrane-anchored enzymes can preferentially activate prodrugs at transduced cancer cells (Fig. 4). Membrane-anchored enzymes can enhance killing of nearby nontransduced cancer cells by diffusion of activated drug.

In a pioneering study, Springer and colleagues[9] anchored carboxypeptidase G2 on breast carcinoma cells by fusion of the enzyme to a portion of the c-erbB-2 gene including a 17 amino acid juxtamembrane extracellular region, TM and 11 amino acids of the CT. Carboxypeptidase G2 (EC 3.4.17.11) derived from *Pseudomonas* RS16 is a 42-kDa homodimeric zinc-dependent exopeptidase that hydrolyzes the *C*-terminal glutamate moiety from folic acid and its analogues.[26] Removal of three cryptic *N*-linked glycosylation sites allowed active enzyme to be expressed on cells but hindered the formation of homodimers, which resulted in reduced intrinsic enzymatic activity.[27] However, even though cancer cells expressing carboxypeptidase G2 intracellularly displayed 4–5 times more total enzyme activity than cells with membrane-tethered enzyme, the later cells were approximately 10 times more sensitive to a benzoic acid mustard prodrug (Fig. 4).[9] The greater effectiveness of membrane-anchored carboxypeptidase G2 was ascribed to the poor penetration of the hydrophilic prodrug across the cell membrane. Treatment of mice bearing small (< 100 mm^3) breast cancer xenografts expressing membrane-anchored carboxypeptidase G2 with six rounds of three fractionated doses of 500 mg/kg prodrug produced substantial delay of tumor growth with one cure.[9] Prodrug treatment of mice bearing tumors containing both unmodified cancer cells and cancer cells expressing membrane-anchored carboxypeptidase G2 slightly delayed tumor growth when tumors contained 10% enzyme-positive cancer cells and produced clear growth delay and cure of 3 of 6 mice when tumors contained 50% carboxypeptidase G2-positive cancer cells, demonstrating drug-mediated bystander killing of nontransduced cells.[10]

Carboxypeptidase A1 is a zinc-dependent metalloprotease that requires trypsin-mediated processing of a 43-kDa proenzyme to generate a mature 34-kDa active enzyme.[28] Hamstra and colleagues[29] developed a mutant rat carboxypeptidase A1 (CPA_{ST3}) in which a 12 amino acid peptide was inserted between the propeptide and catalytic domains to allow trypsin-independent generation of active enzyme in cells. CPA_{ST3} was completely processed and anchored at high levels on 293 cells by attachment of the 37 amino acid GPI signal peptide of decay-accelerating factor to the *C*-terminus

of the enzyme. Surface-anchored CPA_{ST3} converted methotrexate-α-peptide prodrugs (Fig. 4) into the anticancer drug methotrexate.[29] However, good substrates for carboxypeptidase A1 tend to be unstable in serum.[30] A mutant form of human carboxypeptidase A1 (CPA T268G) can hydrolyze methotrexate analogs modified with unnatural amino acids,[30] but this enzyme is itself unstable *in vivo*.[31] It is currently unclear if continuous synthesis and replenishment of mutant carboxypeptidase A1 on the cell surface can allow accumulation of sufficient active enzyme on cancer cells for effective prodrug activation *in vivo*.

β-glucuronidase has been expressed on mammalian cells to activate glucuronide prodrugs and imaging agents. This enzyme is particularly suitable for surface activation because hydrophilic glucuronide prodrugs pass very poorly through the lipid bilayer of cells.[32] Although β-glucuronidase homotetramers are normally routed to lysosomes by mannose 6-phosphate receptors in the Golgi apparatus, overexpression of β-glucuronidase results in secretion of appreciable amounts of enzyme.[33,34] Attachment of the PDGFR TM directed human β-glucuronidase to the surface of COS-7 monkey kidney, JEG-3 human choriocarcinoma and A549 human lung adenocarcinoma cells.[35] By contrast, attachment of a type II TM derived from the asialoglycoprotein receptor or respiratory syncytial virus G protein to the *N*-terminus of human β-glucuronidase resulted in low enzyme activities on cells. Treatment of mice bearing small JEG-3 tumors expressing membrane-anchored β-glucuronidase with a single injection of a glucuronide prodrug of doxorubicin (Fig. 4) resulted in tumor growth delay and cure of 2 of 6 mice.[35]

Human, murine, and *E. coli* β-glucuronidase were expressed on cells by attaching the PDGFR TM, the TM and cytoplasmic tail of murine B7-1, or the TM and cytoplasmic tail of human intercellular cell adhesion molecule-1 (ICAM-1).[11] The B7-1 TM and cytoplasmic tail directed the highest levels of human β-glucuronidase to the surface of 3T3 fibroblasts. Introduction of extracellular juxtamembrane ''spacer'' domains that contained *N*-linked or *O*-linked glycosylation sites further enhanced the expression and surface activity of β-glucuronidase on cells, presumably by reducing enzyme shedding.[8] *E. coli* β-glucuronidase was expressed at approximately 50-fold lower levels than human or mouse β-glucuronidase on human bladder cancer cells, but the high specific activity of *E. coli* β-glucuronidase (∼20,000 U/mg vs. 1,600 and 1,100 U/mg for human and mouse β-glucuronidase, respectively), largely compensated for its poor expression. Cancer cells expressing mouse β-glucuronidase were significantly more sensitive to glucuronide prodrugs of nitrogen mustard or 9-aminocamptothecin (Fig. 4). Good antitumor activity was also demonstrated against small (∼100 mm^3) tumors expressing mouse β-glucuronidase after treatment with two rounds of 150 mg/kg nitrogen mustard prodrug; tumor growth was largely suppressed and 2 of 5 mice experienced complete tumor regression.[11] CT26 mouse colon cancer tumor cells that expressed mouse β-glucuronidase on their surface displayed potent bystander effects and strong tumor suppression after treatment with a single round of 150 mg/kg nitrogen mustard prodrug.[11] These results showed that among the β-glucuronidase enzymes examined, mouse β-glucuronidase displayed the best combination of high expression and good enzyme activity when tethered to the cell surface.

A naturally occurring GPI-anchored form of rat intestinal alkaline phosphatase was expressed on gastric cancer cells to activate a phosphate prodrug of the topoisomerase II inhibitor etoposide (Fig. 4).[36] Etoposide phosphate displayed enhanced activity as compared to etoposide against tumors expressing membrane-anchored alkaline phosphatase, but tumor selectivity may be compromised by the high levels of endogenous alkaline phosphatase present in the intestinal tract.

B. Other Applications of Membrane-Anchored Enzymes

Pellegatti and colleagues[37] created a membrane ATP sensor by attaching the signal peptide and GPI signal sequence from the folate receptor to firefly luciferase. This allowed local detection of ATP levels in the proximity of the cell surface. In another study, a reporter protein was created by fusing β-lactamase (EC 3.5.2.6) from *E. coli* to the TM of human IgM.[38]

Table II. Membrane-Anchored Enzymes

Functional extracellular domain	Juxtamembrane linker (amino acids)[a]	Glyc Sites[b]	Transmembrane domain	Cytoplasmic tail (amino acids)	Full CT?[c]	Cell target	Purpose	Surface level[d]	Ref
carboxy peptidase G2	c-erbB-2 (17)	0	c-erbB-2	c-erbB-2 (11)	no	MDA-MB-361 breast carcinoma cells, COS monkey kidney cells, WiDr colon carcinoma cells, SK-OV-3 and A2780 human ovarian carcinoma cells	prodrug activation	+	9, 27, 10
carboxy peptidase A1	none	0	human decay-accelerating factor (DAF) GPI signal peptide	NA	NA	HEK-293 human embryonic kidney cells	prodrug activation	++++	29
β-glucuronidase	c-*myc* tag + PDGFR (20)	0	human platelet-derived growth factor receptor (PDGFR)	PDGFR (6)	no	COS-7 monkey kidney cells, JEG-3 human choriocarcinoma cells, A529 human lung adenocarcinoma cells	prodrug activation	++	35
β-glucuronidase	ASGPR (5)	0	human asialoglycoprotein receptor (ASGPR) (type II TM)	ASGPR (37)	yes	COS-7 monkey kidney cells, JEG-3 human choriocarcinoma cells, A529 human lung adenocarcinoma cells	prodrug activation	+/-	35
β-glucuronidase	none	0	human intercellular cell adhesion molecule-1 (ICAM-1)	none	no	3T3 mouse fibroblasts	prodrug activation	-	11
β-glucuronidase	c-*myc* tag + PDGFR (20)	0	human platelet-derived growth factor receptor (PDGFR)	PDGFR (6)	no	3T3 mouse fibroblasts	prodrug activation	+	11
β-glucuronidase	human biliary glycoprotein-1 BGP (108)	3	mouse B7-1	mouse B7-1 (38)	yes	3T3 mouse fibroblasts	prodrug activation	+++	11
β-glucuronidase	IgG_1 $H\text{-}CH_2\text{-}CH_3$ domains (232)	1	mouse B7-1	mouse B7-1 (38)	yes	3T3 mouse fibroblasts	prodrug activation	++	11
β-glucuronidase	Ig-like C type domain of mouse B7-1 (105)	3	mouse B7-1	mouse B7-1 (38)	yes	3T3 mouse fibroblasts, EJ human bladder cancer cells	prodrug activation	++++	11

β-glucuronidase	human CD44E (380)	8	mouse B7-1	mouse B7-1 (38)	yes	3T3 mouse fibroblasts	prodrug activation	++++	11
alkaline phosphatase	none	0	alkaline phosphatase GPI signal sequence	NA	NA	SNU638 human gastric cancer cells	ATP sensor	ND	36
firefly luciferase	none	0	folate receptor GPI signal sequence (28)	NA	NA	HEK-293 human embryonic kidney cells	reporter	+	37
β-lactamase	none	0	human IgM	human IgM (2)	yes	A549 human lung adenocarcinoma cells	reporter	ND	38
β-glucuronidase	c-*myc* tag + Ig-like C type domain of mouse B7-1 (105)	3	mouse B7-1	mouse B7-1 (38)	yes	CT26 mouse colon cancer cells	in vivo imaging, directed evolution	++++	15, 39
LAGILDADG homing endonuclease	c-*myc* tag + Ig-like C type domain of mouse B7-1 (105)	3	mouse B7-1	mouse B7-1 (38)	yes	DT20 chicken B cells	directed evolution	++++	16

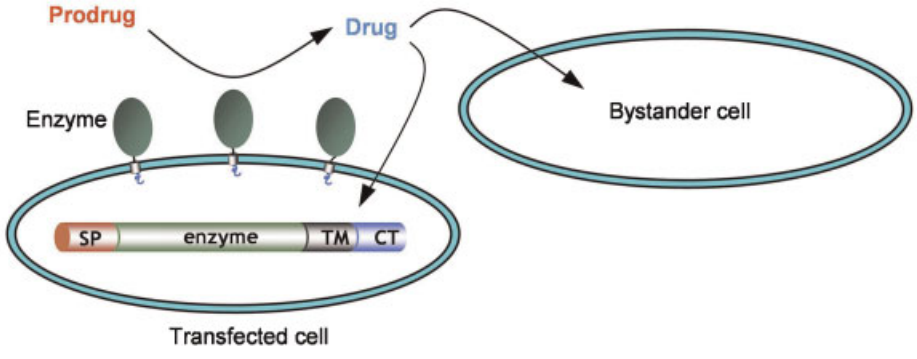
NA, not applicable; ND, not determined.

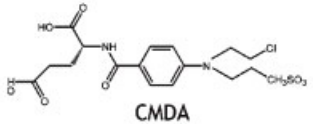
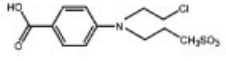
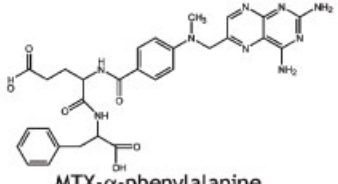
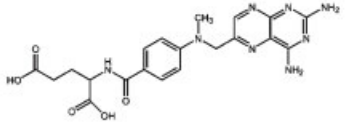
[a]Number of amino acids in the juxtamembrane linker domain.

[b]Potential N-linked glycosylation sites present in the juxtamembrane linker domain.

[c]Indicates if the entire cytoplasmic tail was included in the chimeric protein.

[d]Relative surface expression level estimated from flow cytometric or fluorescence microscopy data.



Enzyme	Prodrug	Drug	References
Carboxypeptidase G2 (CPG2)	 CMDA	 benzoic acid mustard	9, 10, 27
Carboxypeptidase A1 (CPA)	 MTX-α-phenylalanine	 methotrexate	29-31

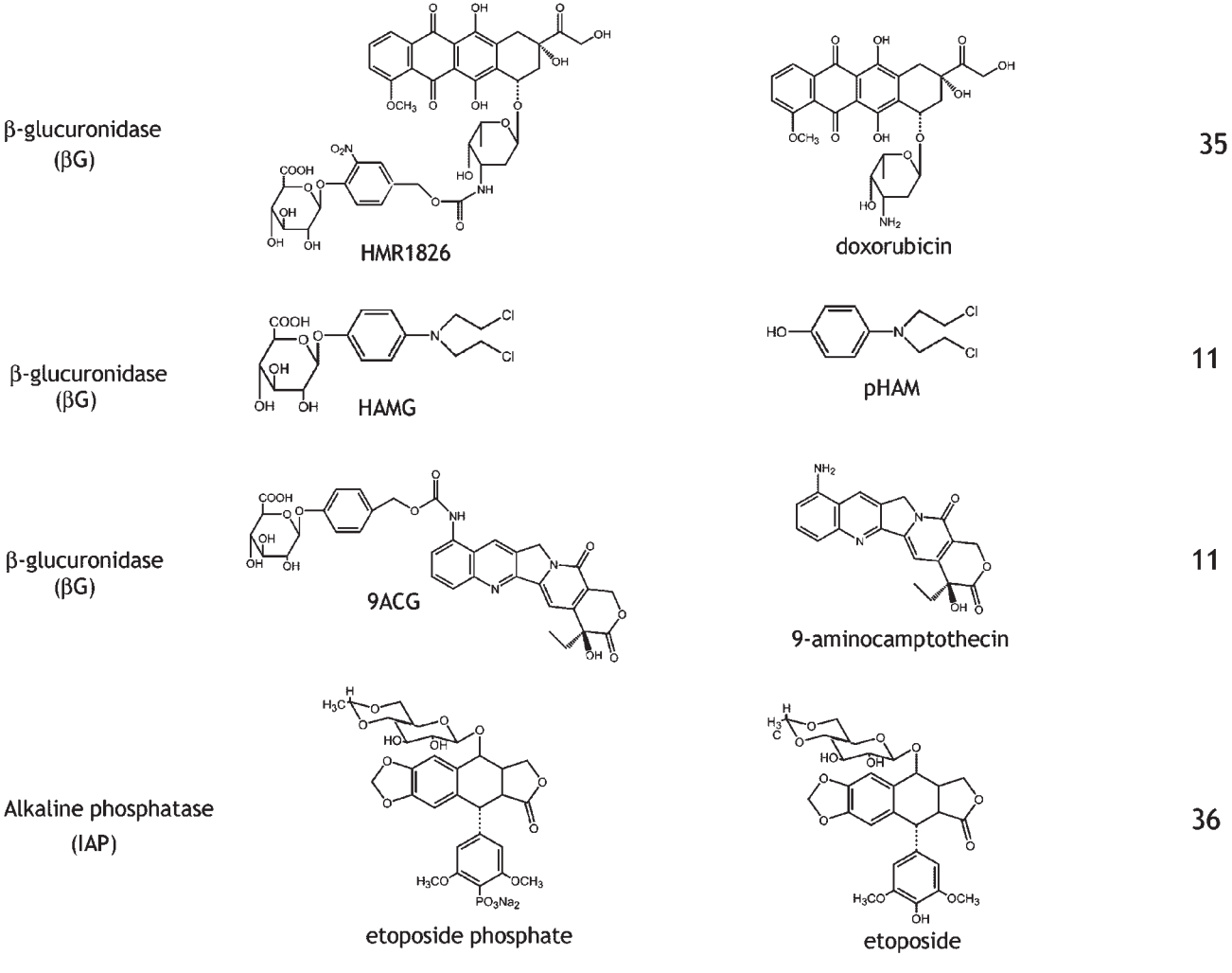


Figure 4. Prodrug activation by membrane-anchored enzymes. High densities of enzyme-TM fusion proteins accumulate on the surface of transfected cells. Administration of a relatively nontoxic prodrug promotes selective generation of cytotoxic drug outside enzyme-expressing cells, which can then enter both transfected and nontransfected bystander cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Membrane-anchored β-glucuronidase has been employed to image the location of gene expression in mice.[15] CT26 colon cancer cells expressing membrane-anchored mouse β-glucuronidase selectively hydrolyzed a nonfluorescent glucuronide probe to fluorescein, thereby allowing sensitive and specific imaging of tumors. Importantly, no immune responses were detected against murine β-glucuronidase,[15] demonstrating that this reporter offers good sensitivity with low immunogenicity. By analogy, it is anticipated that human β-glucuronidase should also display low immunogenicity in humans. Fluorescence detection is limited to relatively shallow tumors, indicating that development of alternative probes will be necessary for utilization in humans.

Human β-glucuronidase has recently been anchored on 3T3 fibroblasts to allow high-throughput screening of mutants that display altered enzymatic activity.[39] Surface display of β-glucuronidase on mammalian cells allowed glycosylation of the enzyme, which is required for proper folding of β-glucuronidase as well as allowed accurate control of the enzyme microenvironment for high-throughput isolation of mutants by flow cytometric sorting.[40] Likewise, LAGLIDADG homing endonucleases (LHEs) were expressed on the surface of B cells for high-throughput screening of enzymes with altered DNA cleavage specificities.[16] LHEs are divalent cation-dependent homodimers that bind and cleave 14–30 bp DNA sequences[41] and are of interest for generating DNA binding and cutting proteins with new specificities.[42] Fusion of a signal peptide and the B7-1 extracellular domain, TM and cytoplasmic tail allowed high expression of the normally cytosolic enzyme on B cells with retention of DNA binding specificity and enzyme activity.[16] Highly selective enrichment of minor cell populations expressing LHEs displaying defined DNA binding and cleavage properties was achieved by fluorescence-activated cell sorting as well as by magnetic cell sorting.

C. Opportunities and Limitations of Membrane-Tethered Enzymes

In agreement with studies employing reporter proteins or single-chain antibodies, high levels of β-glucuronidase could be expressed on cells by including juxtamembrane spacers that can be glycosylated and by not truncating the cytoplasmic tail of the chimeric protein. The finding that β-glucuronidase activity was adversely affected by attachment of type II TM to the enzyme reinforces the need for careful design of chimeric proteins.

Membrane-anchored enzymes have clear advantages for tumor-selective prodrug activation including enhanced bystander killing[9–11] and compatibility with lipid impermeable and polymeric drugs.[43] In addition to prodrug activation, surface display may be generally useful for the directed molecular evolution of enzymes, especially those that require post-translational modifications, such as glycosylation or phosphorylation, for activity. Mammalian cell surface display allows precise control of the enzyme micro environment, such as pH and ionic strength. In contrast to secreted enzymes, anchoring libraries of enzymes on the cell surface directly links phenotype and genotype for development of high-throughput screening strategies, such as, flow cytometric sorting or magnetic-bead separation of cells.[16,39] An additional advantage of mammalian surface expression is that proteins reaching the surface have passed through rigorous quality control in the endoplasmic reticulum, and therefore tend to be highly stable.[44] Finally, expression of cellular enzymes on cells may alleviate problems of drug uptake during early rounds of screening for new drug candidates.

Membrane-anchored enzymes possess great potential for developing new imaging systems. Enzymatic generation allows accumulation of high concentrations of imaging probes at sites of gene expression, which should increase detection sensitivity. In common with prodrugs, the range of potential imaging agents is extended to polymeric and hydrophilic agents due to the accessible nature of membrane-tethered enzymes. A potential limitation of membrane-anchored enzyme imaging systems is the apparent conflict between specificity and immunogenicity. Enzymes without human counterparts can be highly specific but may induce strong immune responses that damage transfected tissue and prevent persistent gene expression,[45] whereas endogenous enzymes should be less immunogenic but may lack specificity.[46] However, membrane-anchored peptides may largely bypass

the MHC class I presentation pathway, thereby minimizing cellular immune responses and promoting long-term expression of reporter proteins *in vivo*.[13]

5. MEMBRANE-ANCHORED ANTIBODIES

A. Artificial Antibody Binding Receptors

Chimeric scFv receptors have been expressed on T cells[47–50] and monocytes[51] to redirect their effector functions to tumor cells and have recently been reviewed.[52,53] Here, we review other applications of membrane-tethered antibodies.

Membrane-anchored antibodies have been developed to capture appropriately labeled haptens and proteins. In a pioneering work, a scFv with specificity for the chemical hapten phenyl-2-oxazolin-5-one (phOx) (Fig. 5) was fused to 8 amino acids of the extracellular domain, the TM and the first 11 amino acids of the CT of the human IL-6 receptor (Table III).[2] The scFv, however, was expressed at quite low levels on transfected cells. A second generation construct fused the immunoglobulin κ-chain signal peptide and PDGFR TM to the phOx scFv.[14] This vector was sold by Invitrogen as pHook-1. Although expression of phOx scFv on cells was low (personal observation), sufficient antibody accumulated on cells to allow selection of transfected cells by phOx-coated magnetic beads.[54–56]

The level of phOx scFv tethered on cells was increased by fusing the antibody to a sequence coding 47 amino acids of the juxtamembrane stalk and TM of the Fc-γ receptor II.[57] The juxtamembrane stalk can be glycosylated, which may hinder shedding of the antibody from the cell surface.[8] Human melanoma cells expressing membrane-anchored phOx scFv could be coated with phOx-labeled antibodies which, in turn, stimulated proliferation of naïve T cells.[57] In another study, high levels of phOx scFv dimers were anchored on 3T3 fibroblasts and B16 melanoma cells by attaching the hinge-CH_2–CH_3 domains of human IgG_1 and the TM and complete CT of the murine B7-1 antigen to the antibody.[58] Sufficient phOx-labeled β-glucuronidase was selectively bound by melanoma cells expressing membrane-tethered phOx scFv to activate anticancer glucuronide prodrug *in vitro* and *in vivo*. The phOx hapten, however, is a poor choice for *in vivo* applications due to its chemical instability.[58] By contrast, an antibody against the more stable dansyl molecule (5-dimethylamino-1-naphthalene sulfonic acid) (Fig. 5) allowed enhanced localization of dansyl-modified agents.[3,59] Imaging agents formed by linking one or two dansyl moieties to ^{111}In-labeled diethylenetriamine petaacetic acid (DTPA) (Fig. 5) accumulated at melanoma cells expressing membrane-anchored anti-dansyl scFv.[3] Retention of the bivalent probe (>48 h) was superior to a monovalent probe (<5 h), indicating that membrane-anchored antibodies can bind multiple determinants of a molecule to enhance binding avidity. Iron oxide nanoparticles and quantum dots

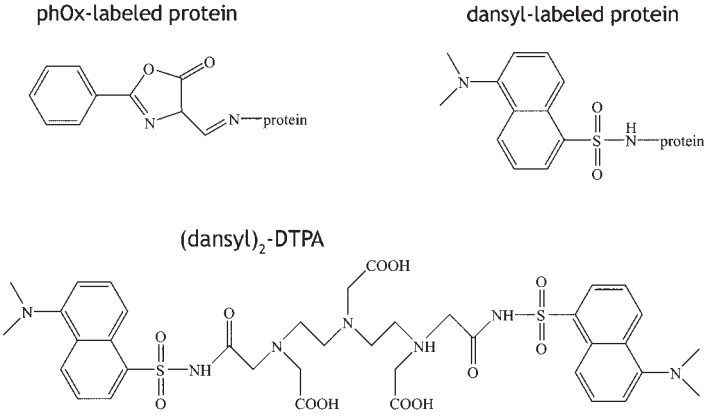


Figure 5. Haptens for targeted delivery to membrane-anchored antibodies.

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Table III. Membrane-Anchored Antibodies

Functional extracellular domain	Juxtamembrane linker (amino acids)[a]	Glyc Sites[b]	Transmembrane domain	Cytoplasmic tail (amino acids)	Full CT?[c]	Cell target	Purpose	Surface level[d]	Ref
anti-phOx scFv	c-*myc* tag + human IL-6R (8)	0	human IL-6 receptor (IL-6R)	IL-6R (11)	no	COS-7 monkey kidney cells	reporter, cell separation	+/-	2
anti-phOx scFv	2 c-*myc* tags + PDGFR (20)	0	human platelet-derived growth factor receptor (PDGFR)	PDGFR (6)	no	293 human embryonic kidney cells, HeLa human cervical carcinoma cells	reporter, cell separation	+/-	14
anti-phOx scFv	FcγRII (47)	1	human FcγRII	none	no	SK-Mel63 human melanoma cells	T cell stimulation	++	57
anti-phOx scFv	hinge-CH_2-CH_3 domains of human IgG_1	1	mouse B7-1	mouse B7-1 (38)	yes	3T3 mouse fibroblasts, B16 mouse melanoma cells	capture hapten-labeled compounds	++++	58
anti-dansyl scFv	Ig-like C type domain of mouse B7-1 (105)	3	mouse B7-1	mouse B7-1 (38)	yes	293 human embryonic kidney cells, B16 melanoma , CT26 colon cancer cells	capture hapten-labeled compounds, imaging, tumor therapy	++++	3, 59, 60
anti-*myc* scFv	human IL-6R (8)	0	human IL-6 receptor (IL-6R)	IL-6R (11)	no	SK-Mel63 human melanoma cells	capture c-*myc* tagged antibody	+/-	61
anti-foot and mouth disease virus scFv	human ICAM-1 (455)	8	human intercellular cell adhesion molecule 1 (ICAM-1)	ICAM-1 (28)	yes	CHO Chinese hamster ovary cells	pseudo virus receptor	ND	62
anti-hemagglutinin (HA) scFv	C-*myc* tag + PDGFR (20)	0	human platelet-derived growth factor receptor (PDGFR)	PDGFR (6)	no	293 human embryonic kidney cells	propagate recombinant adenovirus	+++	63
anti-polyhistidine scFv	c-*myc* tag + PDGFR (20)	0	human platelet-derived growth factor receptor (PDGFR)	PDGFR (6)	no	U118MG human glioma cells	propagate recombinant adenovirus	ND	64
anti-tobacco mosaic virus scFv	TCR β-chain (145)	1	human T cell receptor (TCR) β-chain	TCR β-chain (8)	yes	tobacco suspension culture, transgeneic plants	decoy receptor	ND	65
anti-HIV-1 gp41 scFv	human IgG_3 hinge (11)	0	human type I interferon receptor	none	no	human $CD4^+$ T cells	block HIV infection	+	66
anti-human CD28 scFv	hinge-CH_2-CH_3 domains of human IgG_1	1	human B7-1	human B7-1 (25)	yes	HeLa human cervical cancer cells, H3347 human colon cancer cells	Costimulate T cells	+++	67, 68
anti-human CD28 scFv	hinge-CH_2-CH_3 domains of human IgG_1	1	CD58 GPI signal sequence	NA	NA	HeLa human cervical carcinoma cells, H3347 human colon cancer cells	costimulate T cells	+++	67, 68
anti-human CD3 scFv and anti-human CD28 scFv	human IL-6R (8)	0	human IL6 receptor (IL-6R)	IL-6R (11)	no	SK-Mel63 human melanoma cells	activate T cells	+/-	72
anti-mouse CD3 scFv	hinge-CH_2-CH_3 domains of human IgG_1	1	mouse B7-1	mouse B7-1 (38)	yes	CT26 mouse colon cancer cells, B16 mouse melanoma cells	activate NKT and T cells	++	8, 73

anti-mouse CD3 or anti-mouse T cell receptor antibodies	none	0	rabies virus glycoprotein	rabies virus glycoprotein (47)	yes	RenCa mouse renal cell carcinoma cells, B16-F10 mouse melanoma cells	activate T cells	+++	77, 78
anti-human CD3 scFv + human CD80	c-*myc* tag + PDGFR (20)	0	human platelet-derived growth factor receptor (PDGFR)	PDGFR (6)	no	HeLa human cervical carcinoma cells	activate T cells	ND	79
anti-mouse CD137 scFv	hinge-CH_2-CH_3 domains of human IgG_1	1	human B7-1	human B7-1 (25)	yes	K1735 mouse melanoma cells, B16 mouse melanoma cells	activate T cells	+++	7, 81, 82
anti-mouse CD137 scFv	mouse B7-1 (105)	3	mouse B7-1	B7-1 (38)	yes	3T3 mouse fibroblasts, transgeneic mice	immune modulation	+++	102
anti-human CD16 (FcγRIII) scFv	c-*myc* tag + PDGFR (20)	0	human PDGFR	PDGFR (6)	no	H1299 human lung adenocarcinoma cells	activate monocytes	+++	83, 84
anti-mouse CTLA-4 and anti-mouse CD28 scFv	B7-1 (4)	0	mouse B7-1	mouse B7-1(20)	no	HEK-293 human embryonic kidney cells, mouse B cells, transgeneic mice	suppress immune responses	++	91, 93, 94
anti-mouse CTLA-4 scFv and anti-mouse CD28 scFv	c-*myc* tag + 20 amino acid synthetic spacer	0	mouse B7-1	mouse B7-1 (38)	yes	HEK-293 human embryonic kidney cells, 3T3 mouse fibroblasts	suppress immune responses	+++	92
anti-mouse CTLA-4 scFv	B7-1 (105)	3	mouse B7-1	Mouse B7-1(38)	yes	muscle fibers	enhance transgene expression	ND	95, 96
anti-mouse CTLA-4 scFv	B7-1 (105)	3	mouse B7-1	mouse B7-1(38)	yes	pancreatic islet cells	prevent diabetes	+++	97
anti-human MHC I	synthetic spacer (21)	0	CD58 GPI signal sequence	NA	NA	CHO Chinese hamster ovary cells	immune modulation	+++	99
anti-mouse C_k scFv	hinge-CH_2-CH_3 domains of rat IgG_1	1	mouse MHC (H-$2K^b$)	H-$2K^b$ (40)	yes	mouse fibroblasts, transgeneic mice	immune modulator	+++	100
anti-hen egg lysozyme (HEL) peptide V_H and V_L chains	gly-ser-gly	0	mouse erythpoietin (EPO) receptor	EPO receptor (234)	yes	Ba/F3 mouse pro-B cells	artificial cytokine-like receptor	ND	4
anti-ErbB-2 scFv	c-*myc* tag + PDGFR (20)	0	human platelet-derived growth factor receptor (PDGFR)	PDGFR (6)	no	COS-1 monkey kidney cells	tumor delivery	+/-	103
anti-fluorescein scFv	c-*myc* tag + PDGFR (20)	0	human platelet-derived growth factor receptor (PDGFR)	PDGFR (6)	no	HEK-293 human embryonic kidney cells	microarray high throughput screening	ND	104
anti-fluorogen scFv	c-*myc* tag + PDGFR (20)	0	human platelet-derived growth factor receptor (PDGFR)	PDGFR (6)	no	NIH3T3 mouse fibroblasts, M21 human melanoma cells	reporter	+	105

NA, not applicable; ND, not determined.

[a]Number of amino acids in the juxtamembrane linker domain.

[b]Potential N-linked glycosylation sites present in the juxtamembrane linker domain.

[c]Indicates if the entire cytoplasmic tail was included in the chimeric protein.

[d]Relative surface expression level estimated from flow cytometric or fluorescence microscopy data.

that were decorated with dansyl molecules also preferentially accumulated in tumors expressing membrane-tethered anti-dansyl scFv.[60] This allowed noninvasive imaging of the tumors in mice by magnetic resonance and optical imaging methods, respectively. Combination therapy of colon cancer tumors expressing membrane-bound anti-dansyl scFv was also achieved by targeting dansyl-labeled IL-2 to activate immune cells and dansyl-labeled β-glucuronidase to activate glucuronide prodrugs (Fig. 6).[59] Combination therapy produced superior tumor suppression as compared to either individual treatment. Thus, multiple therapeutic agents can be targeted to scFv receptors on cells, suggesting that multimodality therapy is feasible with this strategy.

An alternative targeting strategy involves employing an anti-myc scFv to capture proteins tagged with a c-*myc* peptide.[61] Low levels of anti-myc scFv were expressed on cells, likely due to fusion of the scFv to an IL-6 TM lacking a CT or *N*-linked glycosylation sites in the juxtamembrane region. Sufficient anti-myc antibody, however, could be expressed on melanoma cells to capture anti-CD28 antibody tagged with a myc peptide.[61]

B. Artificial Virus Receptors

Besides creating hapten-binding receptors, membrane-anchored antibodies can act as artificial receptors to promote virus entry into cells. In an innovative study, Rieder and colleagues[62] generated artificial receptors by attaching a scFv against foot-and-mouth disease virus to the *N*-terminus of human intercellular cell adhesion molecule-1. CHO cells expressing the pseudoreceptors were effectively infected by wild-type virus. In addition, noninfectious viral mutants could be propagated in CHO cells that expressed the artificial receptors.[62] Artificial virus receptors have also been developed to allow the propagation of genetically altered adenoviruses. Fusion of an anti-hemaglutinin scFv to the PDGFR TM allowed good expression of antibody on 293 cells for propagation of adenoviruses expressing an hemagglutinin tag in their fiber or penton base, which may be useful to develop adenoviruses with altered receptor binding specificities.[63] In a similar study, expression of a chimeric anti-polyhistidine scFv receptor on nonpermissive human glioma cells allowed effective propagation of adenoviruses modified to express polyhistidine-tags at the exposed *C*-terminal ends of their fibers, paving the way for development of novel viral vectors in which normal fiber binding interactions have been ablated.[64]

Membrane-anchored antibodies have also been employed as decoy receptors to block viral infectivity. Transgenic tobacco plants that expressed a scFv against tobacco mosaic virus were resistant to viral infection.[65] Interestingly, fusion of the scFv to the PDGFR TM in pHook-1 resulted in extensive shedding of scFv from cells whereas fusion to the human T-cell receptor β-chain, which contains an *N*-linked glycosylation site, largely prevented antibody shedding.[65] An non-neutralizing anti-HIV antibody also blocked HIV replication and cell–cell fusion after it was anchored on the surface of $CD4^+$ T cells.[66]

C. Activation of Immune Cells by Membrane-Anchored Antibodies

Membrane-anchored antibodies can activate immune cells (Fig. 7). In an early study, high levels of a scFv against human CD28 were anchored on cervical carcinoma or colon carcinoma cells by attachment to the hinge-CH_2–CH_3 domains of human IgG_1 followed by the TM and full CT of the human B7-1 antigen or the GPI signal sequence from human CD58.[67,68] Ligation of CD28 on T cells provides an important costimulatory signal for T cell activation.[69,70] B7-1 and B7-2, the natural ligands for CD28, however, also bind to cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, CD152) to down-regulate T cell responses.[71] It was reasoned that expression of anti-CD28 scFv on tumor cells could provide stronger amplification of T cell immune responses by avoiding CTLA-4 ligation. Although membrane-anchored anti-CD28 scFv provided similar costimulatory activity as the natural B7-1 antigen,[67,68] membrane-tethered antibodies are well suited to develop specific receptors for many applications.

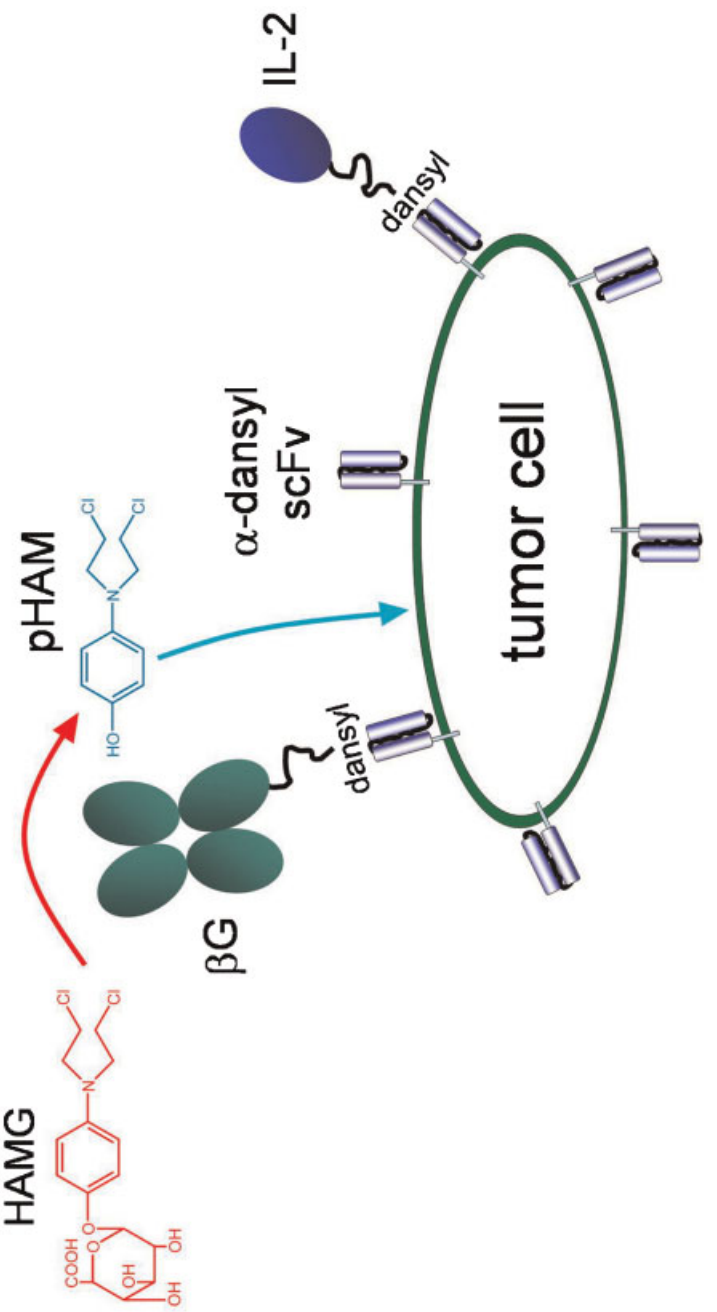
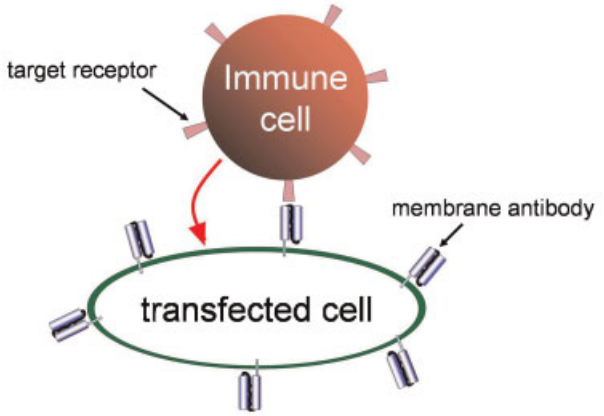


Figure 6. Targeting multiple agents to cells. Tumor cells that express membrane-anchored antibodies with specificity for the hapten dansyl bound β-glucuronidase or interleukin-2 (IL-2) proteins with dansyl moieties covalently attached to their surface *via* flexible polyethylene glycol linkers. Captured β-glucuronidase preferentially converted a glucuronide prodrug (HAMG) to a cytotoxic agent (pHAM) and captured interleukin-2 could enhance T cell activity.[59] [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]



Target receptor	Target cell	Functional outcome	References
CD28	T cells	T cell activation	67, 68, 91-94
CD3	T cells, NKT cells	proliferation, cytokine secretion, CTL, tumor rejection protective immunity	8, 72, 73, 77-80
TCR	T cells	tumor rejection protective immunity	77, 78
CD137	T cells, NK cells	cytokine secretion tumor rejection	7, 81, 82, 102
CD16	NK, neutrophils macrophages	cytokine secretion phagocytosis, tumor inhibition	83, 84
CTLA-4	T cells	suppress T cell activation delay autoimmune diabetes local immune suppression	91-97
MHC I	T cells, B cells	immune modulation	99
Ig κ-chain	B cells	immune modulation	100

Figure 7. Modulation of immune responses by membrane-anchored antibodies. Antibodies anchored on the surface of transfected cells can bind to specific receptors on immune cells to enhance or down-regulate immune responses. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Anti-CD3 antibodies have been expressed on cells to stimulate T cell proliferation and cytotoxicity. Binding of immobilized antibodies to the CD3 complex, which associates with the T cell receptor, can directly activate T cells in a MHC-unrestricted fashion. Anti-CD3 and anti-CD28 scFv fused to the human IL-6 receptor TM were individually expressed on human melanoma cells.[72] Human peripheral blood lymphocytes coincubated with melanoma cells expressing anti-CD3 or anti-CD28 scFv proliferated and exhibited cytotoxicity against tumor cells.

In another study, an anti-CD3 scFv was expressed on cells in dimeric form by attaching the scFv to the hinge-CH_2–CH_3 domains of human IgG_1 followed by the murine B7-1 TM and CT.[8,73] Incubation of naïve splenocytes with CT-26 cells expressing membrane-tethered anti-CD3 scFv induced strong cytolytic activity. Importantly, expression of anti-CD3 scFv on CT-26 cells resulted in complete rejection of the tumors in syngeneic mice,[73] demonstrating for the first time that membrane-anchored anti-CD3 scFv can produce strong anti-tumor activity *in vivo*. Coexpression on cells of anti-

CD3 scFv with CD80 or CD86, the natural ligands for the CD28 costimulatory molecule on T cells, greatly augmented the proliferation of both $CD4^+$ and $CD8^+$ T cells, stimulated cytokine production and enhanced T cell cytotoxicity.[8] The growth of poorly immunogenic B16 melanoma cells engineered to express membrane-anchored anti-CD3 scFv was also significantly delayed in comparison to unmodified B16 tumors with 50% of the mice successfully rejecting the modified tumor cells.[8] These long-term survivors were partially protected against a rechallenge with parental B16 cancer cells, indicating that specific memory T cell responses were generated. An interesting finding of this study was that extension of the anti-CD3 scFv from the plasma membrane by introduction of a large juxtamembrane ectodomain between the scFv and TM caused loss of T cell activation function even though the antibody still bound to CD3 on T cells.[8] This result is consistent with proposed models of T cell activation in which maintaining close contact between antigen-presenting cells and T cells is important for initiating signaling through the T cell receptor complex.[74,75] More recent work has revealed that anti-CD3 scFv anchored on B16 melanoma cells also activated natural killer T (NKT) cells *in vivo*, demonstrating that anti-CD3 scFv can stimulate both innate and adoptive immune responses.[76]

In an important study, modified vaccinia Ankara viruses were created to deliver membrane-anchored forms of intact antibodies against CD3 or the T cell receptor.[77] Whole antibodies were expressed on cells by attaching the TM and CT of the rabies glycoprotein to the *C*-terminus of the antibody heavy chains. Direct injection of recombinant viral particles into established subcutaneous RenCa renal cell carcinoma or B16-F10 melanoma tumors in syngeneic mice produced strong antitumor activity.[77] Interestingly, anti-CD3 was more potent in the RenCa model whereas anti-T cell receptor antibodies produced stronger antitumor activity in the poorly-immunogenic B16 model. To improve therapeutic efficacy, mice bearing RenCa tumors were treated with a combination of modified Ankara virus expressing membrane-anchored anti-CD3 antibody and adenoviruses expressing chemokines or cytokines.[78] The authors found that 100% of mice treated with a combination of viruses expressing IL-12, macrophage inflammatory protein 1β and membrane-anchored anti-CD3 scFv were cured and protected from subsequent challenge with unmodified RenCa tumors. This result demonstrates that the effectiveness of membrane-anchored anti-CD3 antibodies can be improved by combination with stimulatory cytokines.

In a recent study, a fusion protein was developed to signal through both the T cell receptor complex and CD28 simultaneously. This was accomplished by fusing an anti-CD3 scFv to the *N*-terminus of CD80 *via* a flexible linker.[79] HeLa cervical cancer cells that expressed the bifunctional protein induced the proliferation and cytotoxicity of human T cells, although it is unclear if T cell stimulatory function was superior to individually expressed anti-CD3 scFv and CD80.

The use of anti-CD3 or anti-T cell receptor membrane-bound antibodies for activation of T cells has the advantage of being able to activate both $CD4^+$ and $CD8^+$ tumor-infiltrating lymphocytes as well as natural killer T cells, even in tumors that possess defects in antigen processing or presentation. On the other hand, activation of nontumor specific T cells has the potential for generating auto-immune reactions, although this has not yet been reported. Other targets have therefore also been examined for immune stimulation and cancer therapy. CD137 (4-1BB) is a costimulatory molecule that is expressed on T cells, monocytes, dendritic cells, and natural killer cells.[80] Membrane-anchored anti-CD137 scFv, expressed on poorly immunogenic K1735 melanoma cells by fusion to the hinge-CH_2–CH_3 domains of human IgG_1 followed by the human B7-1 TM and cytoplasmic tail, caused extensive rejection of tumors, which was shown to depend on the presence of natural killer cells and $CD4^+$ T cells.[7] Tumor cells expressing anti-CD137 scFv were also effective as a therapeutic vaccine for suppressing the growth of established K1735 tumors. However, B16 melanoma cells that expressed membrane-tethered anti-CD137 scFv were ineffective as a therapeutic vaccine against established B16 tumors,[81] indicating that the effectiveness of anti-CD137 scFv may be tumor dependent. Of note, anti-CD137 scFv-expressing tumor cells produced bystander killing of unmodified cancer cells, showing that this therapeutic

strategy may help treat cancer cell antigen-loss variants.[81] In contrast to K1735 tumors, both $CD4^+$ and $CD8^+$ T cells were required to reject MHC class I-positive MMC mammary carcinoma cells expressing membrane-anchored anti-CD137 scFv in a neu-transgenic mouse model.[82] Importantly, therapeutic vaccination of established MMC tumors with anti-CD137 scFv MMC expressing tumor cells was more effective and produced fewer side effects than did injection of soluble anti-CD137 antibody.

CD16 (FcγRIII) on NK cells, neutrophils, monocytes, and macrophages binds to the Fc domains of IgG_1 and IgG_3 antibodies to initiate antibody-dependent cellular cytotoxicity, endocytosis of immune complexes, cytokine secretion and phagocytosis.[83] H1299 cells that expressed a membrane-anchored anti-CD16 scFv by fusion to the human PDGFR TM induced cytokine secretion from human monocytes as well as cellular cytotoxicity of human peripheral blood mononuclear cells toward the antibody-expressing cells.[84] Phagocytosis of H1299 tumor cells expressing anti-CD16 scFv by IFN-γ activated macrophages was also enhanced. Coengraftment of human peripheral blood mononuclear cells and tumor cells expressing anti-CD16 scFv inhibited tumor outgrowth in immunodeficient mice, demonstrating that membrane-tethered anti-CD16 scFv can induce antitumor activity.

D. Modulation of Immune Responses by Membrane-Anchored Antibodies

Creation of localized immune privileged sites could have a major impact on preventing rejection of xenogeneic and allogeneic grafts. Expression of Fas ligand (FasL) on cells has been reported to cause apoptosis of Fas-expressing infiltrating immune cells, thereby providing local immune privilege.[85,86] Cleavage and release of soluble FasL from cells, however, can induce massive neutrophil infiltration, chronic inflammation and exacerbation of graft rejection.[87–89] Mutation of FasL to prevent shedding does not eliminate this problem,[90] hindering the use of FasL for creation of local immune privilege.

Membrane-anchored antibodies offer an alternative to FasL for creation of local immune-privileged sites. Cytotoxic T lymphocyte antigen 4 (CTLA-4) is an inducible surface receptor on activated T cells that plays a pivotal role in down-regulating immune responses.[71] An anti-CTLA-4 scFv antibody was fused to a 20 amino acid flexible linker followed by the TM and first 24 amino acids of the murine B7-1 CT.[91] Anti-CD3 and anti-CD28 scFv were also anchored on 293 cells using the same domains. Anti-CTLA-4 suppressed anti-CD3 mediated activation of both $CD4^+$ and $CD8^+$ T cells *in vitro*, but only when anti-CD3 scFv and anti-CTLA-4 scFv were expressed on the same cell.[91,92] Transgenic nonobese diabetic mice in which membrane-anchored anti-CTLA-4 was expressed on B cells (antigen-presenting cells) under the control of the IgM heavy-chain promoter and enhancer experienced delayed autoimmune diabetes.[93] Simultaneous engagement of the T-cell receptor and CTLA-4 on antigen-specific $CD4^+$ T cells directly inhibited their activation and differentiation into T_{H1} cells. This important study suggests that expression of membrane-anchored anti-CTLA-4 on APCs could be employed to prevent graft rejection or treat autoimmune disease. In a separate study, expression of anti-CTLA-4 scFv on allogeneic tumor cells prevented their rejection in mice, indicating that membrane-tethered anti-CTLA-4 scFv can effectively down-regulate the function of local cytotoxic T lymphocytes.[94] Likewise, intramuscular coinjection of a plasmid encoding a membrane-anchored anti-CTLA-4 scFv with a plasmid expressing an immunogenic protein resulted in increased transduction of muscle fibers in mice.[95,96] Along the same lines, approximately 60% of transgenic nonobese diabetic mice that expressed membrane-anchored anti-CTLA-4 scFv on pancreatic beta-cells remained disease free.[97] Taken together, these studies indicate that expression of membrane-anchored anti-CTLA-4 antibodies can create local ''immune-privileged'' sites, which may be useful for preventing tissue rejection or suppressing autoimmune responses at specific anatomical sites. A major potential advantage over FasL expression is that membrane-anchored anti-CTLA-4 scFv is immunosuppressive whereas soluble monomeric anti-CTLA-4 scFv did not promote T cell activation.[98]

Kulkarni and colleagues[99] tethered an anti-human MHC class I scFv on the surface of Chinese hamster kidney cells by attachment of the CD59 GPI signal sequence *via* a 21 amino acid linker to the scFv *C*-terminus. The membrane-anchored antibody induced programmed cell death in MHC class I positive T cells and B cells as well as in primary human lymphocytes. Expression of this scFv offers a possible approach to protect cells or tissues from immune-mediated damage.

A scFv reactive with the constant region of the mouse immunoglobulin kappa light chain was expressed on cells as a disulfide-linked dimer by fusion to the hinge-CH_2–CH_3 domains of rat IgG_1 followed by the TM and CT of the H-$2K^b$ molecule.[100] This membrane-anchored scFv was ubiquitously expressed in transgenic mice to investigate the mechanism of immune tolerance in a normal immune system.

Systemic administration of agonist antibodies against CD137 can ameliorate autoimmune disease in several experimental models.[101] Expression of anti-CD137 scFv on pancreatic islet cells in nonobese diabetic mice, however, caused earlier onset and more severe diabetes as compared to nontransgenic littermates,[102] indicating caution should be exercised on choosing the proper immunomodulatory target.

E. Other Applications of Membrane-Anchored Antibodies

An artificial receptor was created by separately fusing the V_H and V_L domains of an anti-hen egg lysozyme antibody to the TM and cytoplasmic tail of the erythpoietin receptor.[4] Addition of hen egg lysozyme peptide brought the V_H and V_L domains into close contact and induced dimerization-dependent signaling through the cytoplasmic erythropoietin domains, promoting IL-3 independent survival of pro-B cells.

Low levels of a scFv against ErbB-2 were anchored on cells by fusion to the human PDGFR TM. These cells could bind to ErbB-2 positive human colon cancer cells and were proposed as a possible vector to deliver therapeutic agents to antigen-positive cancer cells.[103]

Membrane-anchored antibodies have also been expressed on mammalian cells to create a cell microarray.[104] The authors showed that differential binding affinities of anti-fluorescein scFv could be distinguished, suggesting that this technique could be applied to high-throughput screening of antibodies or membrane-bound receptors. Recently, Szent-Gyorgyi and colleagues[105] developed single-chain antibodies that generate strong fluorescence signals upon binding to nonfluorescent fluorogens. Expression of the scFv on mammalian cells by fusion to the human PDGFR TM allowed sensitive visualization of the cell membrane, suggesting that these novel antibodies may become powerful tools to label and visualize surface proteins.

F. Opportunities and Limitations of Membrane-Tethered Antibodies

Membrane-tethered antibodies are attractive reagents for constructing artificial receptors for several reasons. Antibodies can be generated against nearly any chemical structure, peptide, polymer or protein and are highly specific, allowing fine discrimination between closely related substances. Antibodies typically display affinity constants in the nM range, allowing prolonged binding and retention of their antigens. The modular nature of antibodies means that antigen-binding can be reconstituted in small scFv molecules, increasing the range of vectors that can be employed to express membrane-tethered antibodies. Furthermore, human antibodies can now be routinely generated,[106,107] allowing creation of less immunogenic receptors for *in vivo* applications that require prolonged expression.

Several independent studies have clearly demonstrated that membrane-anchored antibodies can act as artificial receptors to bind ligands as well as artificial ligands to activate specific receptors. Besides increased discrimination, artificial ligands based on membrane-anchored antibodies can be engineered to possess higher affinity than natural ligands. For example, the binding affinity of antibodies is typically several orders of magnitude higher than the affinity of MHC-peptide

complexes for the T cell receptor,[108,109] which can translate into enhanced signaling strength and stronger biological responses.[110] Ligands can also be designed with multiple binding sites to take advantage of avidity enhancement afforded by high antibody densities on cells.[3] Likewise, multiple ligands can be targeted to the same antibody receptor to allow development of universal targeting systems and combination therapies.[59] Local retention of antibodies in defined anatomical sites may help alleviate systemic toxicity associated with the administration of activating antibodies.[111–114] It should also be feasible to use membrane-anchored antibodies to create artificial receptors that can be activated with synthetic ligands, similar to chimeric antigen receptors expressed on T cells.[49,50] Membrane-tethered antibodies may also be useful for tissue engineering as artificial receptors to create three-dimensional organs with different cell populations.

The high specificity of antibodies may be further exploited to create additional artificial ligands for specific receptors. Many endogenous membrane-anchored ligands have multiple receptor targets, often producing different cellular outcomes. Examples include B7-1 and B7-2 interacting with CD28 and CTLA-4, the lymphotoxin β receptor interacting with lymphotoxin αβ and LIGHT (lymphotoxin, showing inducible expression, and competing with herpes simplex virus glycoprotein D for HVEM),[115] herpesvirus entry mediator interacting with LIGHT, lymphotoxin α and BTLA (B- and T-lymphocyte attenuator),[115] as well as a host of receptor-ligand interactions involved in NK cell regulation.[116] Expression of membrane-tethered antibodies with specificity for individual receptors may help unravel their functions without the confounding effects of multiple receptor activation. More precise control of *in vivo* immune reactions may also be feasible by careful design of specific membrane-bound antibody ligands.

Anchorage of antibodies on cells may also reveal unexpected new properties of the antibodies. For example, the anti-polyethylene glycol antibody AGP3 binds polyethylene glycol-modified proteins with high avidity,[117,118] but binds free polyethylene glycol poorly when coated in microtiter plates (unpublished data). Conversely, membrane-anchored AGP3 binds both polyethylene glycol-protein conjugates and free polyethylene glycol with high avidity (unpublished data). Similar findings were recently reported in which a non-neutralizing anti-HIV antibody blocked HIV replication and cell–cell fusion after it was anchored on the surface of $CD4^+$ T cells.[66]

Potential problems of membrane-anchored antibodies include their susceptibility to be shed from cells, mandating careful design of membrane-tethered chimeric antibodies. In general, high expression of antibodies on cells is facilitated by employing a linker with *N*-glycosylation sites (i.e., the hinge-CH_2–CH_3 domains of human IgG_1 or the Ig-like *C*-type domain of B7-1) as well as maintaining an intact cytoplasmic tail. Antibodies are also somewhat limited by their noncatalytic nature, which may reduce their sensitivity in comparison to enzymatic systems for imaging or targeting applications.

6. SINGLE-CHAIN MHC MOLECULES

A. MHC Class I

MHC class I is composed of a 12-kDa β2-microglobulin (β2m) light chain that is noncovalently associated with a polymorphic 44-kDa heavy chain anchored on the cell surface *via* a type I TM (Fig. 8A). Specific $CD8^+$ T cells are activated after engagement of the T cell receptor by short (~9 amino acids) antigenic peptides presented by MHC class I molecules.

A single-chain MHC, in which murine β2m was fused to the complete H-$2D^d$ heavy chain *via* a 20 amino acid linker (Fig. 8B), was moderately expressed on an assortment of cells, effectively presented exogenously added peptides and stimulated IL-2 secretion from specific T cell hybridomas.[119] Similarly, a single-chain MHC molecule, in which human β2m was fused to the complete HLA-A2 molecule *via* a 15 amino acid $(GGGGS)_3$ linker, was highly expressed on β2m-

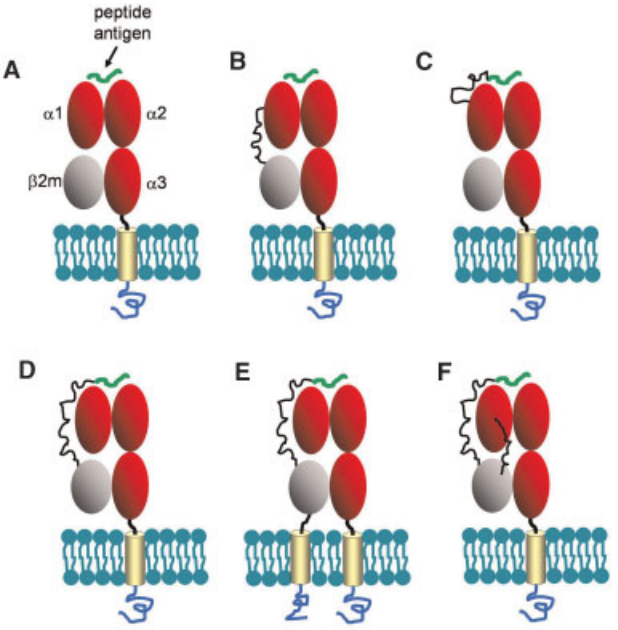


Figure 8. Membrane-anchored MHC class I molecules. **A**: MHC class I consists of a heavy chain possessing 3 extracellular domains (α1- α3), a TM and CT, and the noncovalently-associated β2m light chain. Small peptide antigens (~9 amino acids) bind to a closed cleft between the α1 and α2 domains of the heavy chain. **B**: MHC variant in which β2m is covalently linked *via* a flexible polypeptide to the α1 domain of the heavy chain. **C**: The antigenic peptide is covalently attached to the heavy chain *via* a flexible polypeptide linker. **D**: The antigenic peptide is covalently attached to the β2m light chain *via* a flexible polypeptide linker. **E**: The antigenic peptide is covalently attached to a membrane-anchored form of β2m. **F**: Single-chain trimer in which the antigenic peptide is liked to the *N*-terminus of β2m *via* a flexible polypeptide while the *C*-terminus of β2m is linked to the heavy chain *via* a second flexible polypeptide linker. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

deficient colorectal cells and effectively presented exogenously added antigenic peptide as shown by specific lysis of the transfected and peptide-pulsed cells.[120]

In another approach, T cell peptide epitopes were covalently attached to the MHC class I heavy chain (Fig. 8C). Cells expressing MART-1 or gp100 melanoma epitopes linked to the HLA-A2 heavy chain *via* a 10 amino acid flexible linker stimulated IFN-γ secretion from specific cytotoxic T lymphocytes.[121] In addition, incubation of peripheral blood mononuclear cells from melanoma patients with HMY-C1R B cells coexpressing antigen-MHC fusion proteins in combination with B7.1 or B7.2 induced melanoma-reactive cytotoxic T lymphocytes. Similarly, subdominant HIV-1 cytotoxic T lymphocyte epitopes were covalently attached to the HLA-A2 heavy chain *via* a flexible linker.[122] However, in this case, one of the peptide-MHC fusion proteins was inactive, suggesting that the linker between the antigen epitope and the MHC heavy chain can interfere with proper peptide presentation.

Antigenic peptides have also been linked to β2m to promote formation of MHC class I trimers (Fig. 8D), either by expressing the peptide-β2m fusion protein in cells or by pulsing target cells with fusion protein.[123–126] This approach can dramatically increase the antigenicity of suboptimal peptide epitopes that normally bind poorly to MHC molecules.[124] In another approach, the normally soluble human β2m light chain was anchored on cells by attachment of its *C*-terminus to 13 amino acids from the membrane-proximal extracellular portion of HLA-A2 followed by the TM and CT of the mouse CD3ζ chain (Fig. 8E).[127] Antigenic peptides derived from influenza virus that were linked to the *N*-terminus of membrane-bound β2m associated with endogenous MHC class I heavy chain on transfected cells. RMA-S cells expressing membrane-anchored peptide-β2m induced stronger CTL

responses than peptide-pulsed RMA-S cells.[128,129] Furthermore, RMA-S cells that expressed the low affinity tyrosinase-related protein 2 antigen fused to membrane-anchored human β2m protected mice from a challenge of melanoma cells significantly better than RMA-S cells saturated with tyrosinase-related protein 2 peptide. Therapeutic vaccination with RMA-S cells expressing a high affinity ovalbumin peptide antigen fused to membrane-anchored human β2m also significantly delayed the growth of ovalbumin-transfected melanoma tumors in mice.[129]

Recently, all three components of the MHC complex (antigenic peptide, β2m, and MHC heavy chain) have been covalently linked to form single-chain trimers (Fig. 8F). Yu and colleagues[130] created DNA constructs coding for the β2m signal peptide, an antigenic peptide, a flexible linker, mature murine β2m, another flexible linker and the mature MHC heavy chain. A single-chain trimer that presented an ovalbumin epitope (SIINFEKL) in the context of H-2K^b were resistant to exchange with other antigenic peptides, remained associated as trimers on the surface of transfected cells for extended periods and supported the *in vitro* generation of cytotoxic T lymphocytes. The enhanced stability of single-chain trimers appears to be due to their ability to rebind the attached peptide after it dissociates from the MHC binding groove.[131] Further engineering of the single-chain trimer by mutating tyrosine to alanine at position 84 of the H-2K^b heavy chain opened the peptide binding groove to allow better accommodation of the flexible linker between the antigen and β2m.[131] Introduction of a disulfide bond between the second amino acid of the linker and MHC class I heavy chain at position 84 further enhanced peptide binding stability, especially for low affinity peptides.[132] Further engineering of single-chain trimers may allow development of a general scaffold to bind diverse antigenic peptides without altering the original T cell specificity.

DNA vaccination of HLA-A2$^+$/CD8$^+$ transgenic mice with a single-chain trimer construct presenting antigenic peptides derived from the breast cancer-associated antigen mammaglobin-A in the context of HLA-A2 induced the expansion of antigen-specific CD8$^+$ CTLs that lysed mammaglobin-A$^+$ UAC-812 breast cancer cells.[133] DNA immunization with a single-chain trimer vector in which a human mesothelin peptide antigen was presented in the context of HLA-A2 generated an antigen-specific CD8$^+$ T cell response and significantly protected HLA-A2$^+$ transgeneic mice from syngeneic tumor cells that overexpressed human mesothelin.[134]

A single-chain trimer presenting an immunodominant epitope of the human papillomavirus (HPV) E6 protein in the context of H-2K^b was tested as a DNA vaccine in C57BL/6 mice.[135] Mice receiving 4 weekly gene-gun immunizations of single-chain trimer plasmid generated much stronger CD8$^+$ T cell responses as compared to immunization with a human papillomavirus E6 expressing plasmid, which also contains the immunodominant T cell epitope. DNA immunization with the single-chain trimer plasmid protected mice from a challenge with E6-expressing cancer cells. In another application, a single-chain trimer presenting an HLA-E-binding peptide in the context of HLA-E protected porcine cells from natural killer (NK) cell cytotoxicity and cytokine secretion, which may help prevent the NK-mediated rejection of xenografts.[136]

B. MHC Class II

Single-chain MHC class II molecules have also been expressed on cells. MHC class II is composed of noncovalently associated α and β chains which fold to form a peptide-binding grove (Fig. 9A).[137,138] A single-chain MHC class II molecule was constructed in which antigenic peptides were linked *via* a 10 amino acid linker to a truncated murine I-A^d β chain (lacking the TM), which in turn was linked *via* a 24 amino acid linker to the intact I-A^d α chain (Fig. 9B).[139] Expression of single-chain MHC II molecules on NS-0 murine plasmacytoma cells effectively stimulated IL-2 production from CD4$^+$ T cells. An analogous human single-chain MHC class II molecule was also active when expressed on cells.[140]

A novel single-chain MHC class II molecule was created in which a truncated I-A^b α chain was connected *via* a 10 amino acid linker to a portion of the invariant chain (residues 58–85), which in

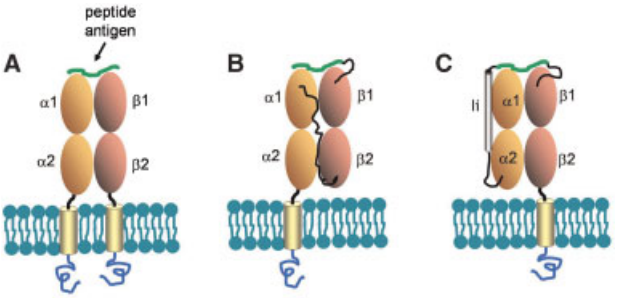


Figure 9. Membrane-anchored MHC class II molecules. **A**: MHC class II consists of alpha and beta heavy chains that are anchored to the membrane as type I integral membrane proteins. Peptide antigens bind to an open cleft formed by the α and β chains. **B**: The antigenic peptide is covalently attached *via* a flexible polypeptide linker to a truncated β heavy chain, which in turn was linked to an intact α chain. **C**: Single-chain MHC is which a truncated α chain is linked to a portion of the invariant chain (Ii), followed by the antigenic peptide which is in turn liked *via* a flexible polypeptide to the *N*-terminus of the β chain. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

turn was fused to an antigenic peptide, a 15 amino acid linker and the entire I-A^b β chain (Fig. 9C).[141] The invariant chain was included in this chimeric MHC to position the antigenic peptide correctly in the antigen binding groove.[142] COS cells expressing this single-chain MHC class II molecule effectively stimulated IL-2 secretion of antigen-specific $CD4^+$ T cells.[141]

C. Opportunities and Limitations of Membrane-Tethered Single-Chain MHC Molecules

Single-chain trimers hold promise for many emerging applications including *in vivo* expansion of adoptively transferred T cells, *in vitro* and *in vivo* induction of specific immune responses and creation of artificial antigen presenting cells with defined characteristics. single-chain trimers may also be useful for cancer gene therapy since they could help overcome defects in antigen processing and presentation commonly observed in advanced cancers.[143,144] Single-chain trimers are also proving to be very useful reagents for answering basic immunological questions, largely based on their ability to reduce artifacts associated with transfer of antigenic peptides to endogenous MHC molecules. For example, transgenic mice expressing single-chain trimers were employed to help show that natural killer cells require interactions with MHC class I molecules during their development to be ''licensed'' for functional competence.[145] Single-chain trimers have also been employed to address the mechanism by which peptide-MHC engagement of T cell antigen receptors initiates signaling in T cells.[74,75]

Currently, single-chain trimers are limited by interference of the polypeptide linker between the peptide and β2m with the proper orientation of the peptide in the MHC binding grove. Subtle changes in peptide orientation can result in loss of T cell stimulatory activity.[122] It is unclear how common this problem is but further advances in design of single-chain trimers may alleviate this limitation and expand the use of single-chain trimers.

7. MEMBRANE-ANCHORED CYTOKINES

A. Immune Stimulation

Local retention of bioactive polypeptides is an attractive method to reduce systemic toxicity. For example, murine tumor-necrosis factor (TNF-α) displays potent antitumor activity but its use is limited by severe systemic toxicity, including septic shock, and cachexia.[146] Marr and colleagues[5] compared the expression of wild-type TNF-α, which is retained on the membrane but is also extensively shed in a soluble form and a mutant form of TNF-α, which was largely retained on the membrane of transfected tumor cells (Table IV). High levels of TNF-α were detected in the serum of

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Table IV. Membrane-Anchored Cytokines

Functional extracellular domain	Juxtamembrane linker (amino acids)[a]	Glyc Sites[b]	Transmembrane domain	Cytoplasmic tail (amino acids)	Full CT?[c]	Cell target	Purpose	Surface level[d]	Ref
mouse TNF-α	none	0	mouse TNF-α type II TM	TNF-α (34)	yes	MRC5 human lung fibroblasts, MT1A2 mouse mammary adenocarcinoma cells	antitumor	+	5
mouse GM-CSF	c-*myc* tag + PDGFR (20)	0	human platelet-derived growth factor receptor (PDGFR)	PDGFR (6)	no	P815 mouse mastocytoma cells, B16-F10 mouse melanoma cells	antitumor	+++	149, 150
mouse GM-CSF	none	0	CD59 GPI signal sequence	NA	NA	CHO monkey kidney cells, K1735M2 mouse melanoma cells, B16-F10 mouse melanoma cells	antitumor	+++	152
mouse IFN-γ mouse GM-CSF	not specified	NA	FcεRI	not specified	NA	mouse Lewis lung carcinomacells	antitumor	+/-	153
human G-CSF	mSCF (44)	1	mouse stem cell factor (mSCF)	mSCF (35)	yes	NIH3T3 mouse fibroblasts	immune stimulation	+	154
human M-CSF	NA	0	human decay accelerating factor (DAF) GPI signal sequence	NA	NA	KM-102 human stromal cells	artificial adhesion molecule	+/-	155
mouse IL-4	mouse CD4 (368)	4	mouse CD4	CD4 (40)	yes	MethA mouse fibrosarcoma cells	antitumor	+	156
mouse IL-4	mouse TNF (34)	0	mouse TNF type II TM	TNF (19)	yes	MethA mouse fibrosarcoma cells	antitumor	++	156
mouse IL-12	none	0	CD59 GPI signal sequence	NA	NA	P815 mouse mastocytoma cells	antitumor	+++	6
human IL-2 mouse IL-12 p40 chain	none	0	human decay accelerating factor (DAF) GPI signal sequence	NA	NA	B16-F0 mouse melanoma cells	antitumor	+/-	162, 163
mouse IL-2	mouse TNF (34)	0	tumor-necrosis factor (TNF) GPI signal sequence	NA	NA	B16-F10 mouse melanoma cells	antitumor	+/-	164

NA, not applicable.

[a]Number of amino acids in the juxtamembrane linker domain.

[b]Potential N-linked glycosylation sites present in the juxtamembrane linker domain.

[c]Indicates if the entire cytoplasmic tail was included in the chimeric protein.

[d]Relative surface expression level estimated from flow cytometric or fluorescence microscopy data.

mice after direct tumor injection of an adenoviruses expressing native TNF-α whereas only background levels of circulating TNF-α were detected in mice injected with adenovirus expressing membrane-anchored TNF-α. In agreement with these results, injection of tumors with adenovirus expressing membrane-anchored TNF-α produced significantly less systemic toxicity as compared to mice treated with the native TNF-α adenovirus. Membrane-anchored TNF-α displayed similar antitumor activity as wild-type TNF-α, providing a strong rationale for localized expression of cytokines: retention of biological activity with reduced systemic toxicity.

Tumor cells have been engineered to secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) to stimulate the *in vivo* expansion and differentiation of dendritic cells, thereby promoting the development of strong, long-lasting antitumor immunity in several murine tumor models.[147,148] GM-CSF was anchored at good levels on immunogenic P815 mouse mastocytoma cells by fusion to the PDGFR TM.[149] Membrane-tethered GM-CSF retained biological activity and enhanced the rejection of P815 transfectants as compared to unmodified P815 tumors.[149] Likewise, the antitumor activity of poorly immunogenic B16-F10 melanoma cells expressing membrane-anchored GM-CSF produced superior activity in several *in vivo* assays as compared to B16-F10 cells that secreted GM-CSF.[150] Superiority of membrane-anchored GM-CSF, however, is obscured by the large amounts of membrane-tethered GM-CSF that were shed from cells, likely due to proteolytic cleavage at a susceptible site near the *C*-terminus of GM-CSF.[151] High levels of GM-CSF were also expressed on cells by attaching the CD59 GPI signal sequence to the *C*-terminus of GM-CSF.[152] GPI-anchored GM-CSF stimulated the proliferation of bone-marrow derived cells and promoted the *in vivo* generation of dendritic cells. However, in common with TM-anchored GM-CSF, substantial amounts of GM-CSF were shed from cells, likely due to proteolytic cleavage since shedding was reduced by addition of the metallo-protease inhibitor 1,10-phenathroline.[152] Introduction of glycosylation sites near the TM may help alleviate shedding of GM-CSF from cells. Murine interferon gamma and GM-CSF have also been anchored at low levels on Lewis lung carcinoma cells by fusing a fragment of the gamma chain of the high affinity receptor for IgE (FcεRI) to these cytokines.[153] Postsurgical immunotherapy of mice with tumor cells expressing membrane-bound cytokines provided similar protection as tumor cells expressing the secreted cytokines.

Human granulocyte colony-stimulating factor (G-CSF) was tethered on mouse fibroblasts by fusion to a 44 amino acid portion of the extracellular domain, TM and CT of mouse stem cell factor.[154] Membrane-tethered G-CSF retained the ability to stimulate the proliferation of G-CSF responsive NFS60 myelogenous leukemia cells. Macrophage colony-stimulating factor (M-CSF) has also been anchored on KM-102 stromal cells *via* the human decay-accelerating factor GPI anchor to act as an artificial adhesion molecule for cells expressing M-CSF receptors.[155]

Interleukin-4 (IL-4) was tethered on MethA fibrosarcoma cells as a type I membrane protein by fusion to the complete mature CD4 protein or as a type II membrane protein by attaching the first 74 amino acids of tumor necrosis factor to its *N*-terminus.[156] Although both forms of IL-4 were expressed at low levels on MethA cells, the type II fusion protein displayed superior *in vitro* and *in vivo* activity.

Interluekin-12 (IL-12) is a heterodimeric secreted cytokine that stimulates elaboration of interferon gamma from natural killer and T cells, and exhibits potent antitumor and antimetastatic activity.[157,158] Systemic administration of IL-12, however, can produce unacceptable toxicity.[159] To reduce systemic release of IL-12 from tumors, the CD59 GPI anchor signal sequence was fused to the *C*-termini of both the p35 and p40 subunits of IL-12.[6] Cotransfection of P815 mastocytoma cells with plasmids encoding GPI-anchored IL-12 subunits resulted in high expression of functional IL-12 as assessed in T cell proliferation and IFN-γ release assays. IL-12 appeared to be stably retained on transfected cells, resulting in undetectable IL-12 and interferon gamma levels in the circulation of mice inoculated with P815 tumor cells expressing GPI-anchored IL-12 subunits. By contrast, both IL-12 and interferon gamma were detected in the serum of mice inoculated with P815 tumors expressing secreted IL-12. The growth of a P815 tumor clone expressing GPI-anchored IL-12

subunits was suppressed to a similar degree as tumors secreting IL-12, suggesting that localization of IL-12 on the membrane of tumor cells may allow retention of substantial antitumor activity with reduced systemic toxicity.

Interleukin-2 (IL-2) is a 15.5 kDa glycoprotein produced mainly by activated T cells. Although IL-2 can enhance the proliferation of effector T cells, it is also important for generation of regulatory T cells and maintenance of peripheral T cell tolerance.[160] High doses of IL-2 are toxic, causing vascular leak syndrome.[161] Attachment of the GPI signal sequence of decay-accelerating factor to human IL-2 tethered very low levels of IL-2 on B16-F0 melanoma cells.[162] IL-2 was also released from transfected B16-F0 cells, possible with an intact GPI anchor since it could rebind to untransfected cells.[162] *In vivo* analysis showed that GPI-anchored but not secreted IL-2 accumulated in transfected tumors. Interestingly, the number of lung colonies in mice after i.v. injection of B16-F0 cells expressing GPI-anchored IL-2 was significantly reduced as compared to B16-F0 cells secreting IL-2, suggesting that local retention of IL-2 at tumors can increase IL-2 antitumor activity. In a subsequent study, in addition to IL-2, murine IL-12 was also anchored on B16-F0 cells by attaching the GPI signal sequence from decay-accelerating factor to the *C*-terminus of the IL-12 p40 chain.[163] Expression of low levels of IL-2 and IL-12 on B16-F0 melanoma cells caused tumor rejection after i.v. or s.c. inoculation in syngeneic mice. Cells expressing both GPI-anchored IL-2 and IL-12 displayed enhanced antitumor activity as compared to tumor cells expressing individual cytokines. Unfortunately, systemic effects of the therapy were not reported. In a similar study, expression of mouse IL-2 on B16-F0 melanoma cells as a type II TM fusion protein suppressed tumor growth as compared to unmodified B16-F0 cells in subcutaneous, intraperitoneal and intravenous tumor models.[164]

B. Opportunities and Limitations of Membrane-Tethered Cytokines

Localized expression of cytokines and chemokines is an attractive method to reduce systemic exposure and toxicity, as long as biological activity is retained in a membrane-bound form. It appears that most cytokines examined to date retain substantial biological activity but the benefits of localized expression have rarely been rigorously documented.[5] Since most cytokines and chemokines have short half-lives, retention on the cell surface may prevent rapid clearance of the proteins, thereby increasing their effective half-life. However, more careful comparison between membrane-tethered and secreted cytokine function and toxicity are required before the superiority of localized expression can be confirmed. GPI-anchored cytokines and chemokines may be attractive to allow limited *in vivo* spread to neighboring cells.[162] Careful design of the elements employed to attach chemokines and cytokines to cells is necessary as shown by the superior activity of a type II TM for IL-4 function and the severe shedding of GM-CSF from the surface of transfected cells.[150,152,156]

8. SURFACE EXPRESSION OF ANTIGENS ON MAMMALIAN CELLS

A. Vaccines

In an early work, Langford and colleagues[12] tethered the normally secreted, highly repetitive S-antigen of *plasmodium falciparum* to cells by fusion to a 6 amino acids juxtamembrane domain, TM and 28 amino acid CT of murine IgG_1 (Table V). Both mice and rabbits generated a robust antibody response after injection of vaccinia virus expressing membrane-anchored but not secreted S antigen. Likewise, Vijaya and colleagues[165] fused the type II TM of respiratory syncytial virus glycoprotein G to repeating epitopes of the circumsporozoite protein of *Plasmodium falciparum*. Mice and rabbits immunized with vaccinia virus expressing the membrane-anchored protein generated antibodies that reacted with sporozoites.

The 92 amino acids human chorionic gonadtropin polypeptide (hCG-α) was successfully anchored on COS-1 cells *via* the TM and CT of vesicular stomatitis virus glycoprotein.[166] A

Table V. Membrane-Anchored Antigens

Functional extracellular domain	Juxtamembrane linker (amino acids)[a]	Glyc Sites[b]	Transmembrane domain	Cytoplasmic tail (amino acids)	Full CT?[c]	Cell target	Purpose	Surface level[d]	Ref
Plasmodium falciparum S-antigen	mouse IgG_1 (6)	0	mouse IgG_1	mouse IgG_1 (28)	yes	BSC-1 monkey kidney cells, rabbits	vaccine	+	12
Plasmodium falciparum CS repeats	RSVG (117) RSVG (167)	3 3	respiratory syncytial virus glycoprotein G (RSVG) type II TM	RSVG (40)	yes	CV-1 monkey kidney cells, rabbits	vaccine	+	165
rotavirus VP7 outer capsid glycoprotein	none	0	Influenza virus hemagglutinin (HA)	influenza virus HA (10)	yes	COS monkey kidney cells, human 143B osteosarcoma cells, rabbits, CBA/H mice	vaccine	+	168 - 170
rotavirus VP6	mouse IgG_1 (6)	0	mouse IgG_1	mouse IgG_1 (28)	yes	CV-1 monkey kidney cells, CBA/H mice	vaccine	+	171
human papillomavirus (HPV) E7 antigen	vaccinia virus HA Ig-like domain (42)	0	vaccinia virus hemagglutinin (HA)	Vaccinia virus HA (13)	yes	CV-1 monkey kidney cells, C57BL/6 mice	vaccine	+/-	13
foot and mouth disease VP1 antigen	none	0	human platelet-derived growth factor receptor (PDGFR)	PDGFR (6)	no	BHK-21 baby hamster kidney cells, BALB/c mice	vaccine	+	172
β-subunit of human chorionic gonadtropin	none	0	vesicular stomatitis virus glycoprotein (VSVG)	VSVG (29)	yes	293 human embryonic kidney cells, COS-1 monkey kidney cells, Wistar rats	marker protein, anti-fertility vaccine	++	166, 167

[a]Number of amino acids in the juxtamembrane linker domain.

[b]Potential N-linked glycosylation sites present in the juxtamembrane linker domain.

[c]Indicates if the entire cytoplasmic tail was included in the chimeric protein.

[d]Relative surface expression level estimated from flow cytometric or fluorescence microscopy data.

recombinant vaccinia virus expressing membrane-anchored hCG-α generated a robust antibody response in Wistar rats whereas no response was observed in rats immunized intradermally with vaccinia virus expressing soluble hCG-α.[167] Membrane-anchored hCG-α has been proposed as a possible anti-fertility vaccine.

The rotavirus VP7 outer capsid glycoprotein, which is normally retained in the ER, was converted to a secreted form by replacement of the VP7 signal peptide with the influenza virus hemagglutinin signal peptide. Subsequent attachment of the TM and CT of influenza virus hemagglutinin anchored VP7 on cells.[168] Female mice immunized with a vaccinia virus expressing membrane-anchored VP7 produced earlier and higher antibody titers as compared to a vaccinia virus expressing wild-type VP7 and provided better protection to their offspring against rotavirus-induced diarrhea.[169,170] The rotavirus VP6 protein was also anchored on cells by attachment of the mouse IgG_1 TM and CT.[171] Interestingly, membrane-tethered VP6 formed large two-dimensional arrays on the surface of monkey kidney cells. Much stronger anti-VP6 antibody responses were generated in mice immunized with vaccinia virus expressing membrane-anchored VP6 as compared to wild-type or secreted VP6.

In a more recent study, insertion of the 98 amino acid E7 protein of human papilloma virus 16 in place of an internal portion (amino acids 64–275) of vaccinia virus hemagglutinin allowed expression of moderate levels of E7 on virus-infected monkey kidney cells.[13] Comparison of mice immunized with vaccinia virus expressing unmodified E7, membrane-anchored E7 or a form which targets E7 to lysosomes, revealed that only membrane-anchored E7 induced anti-E7 antibody responses. Conversely, viruses expressing unmodified E7 and lysosome-targeted E7, but not virus expressing membrane-anchored E7, induced $CD8^+$ cell-mediated immune responses against E7 and protection against challenge of E7-expressing TC-1 tumor cells. In a separate study, mice generated neutralizing antibodies only after electroporation of a plasmid expressing membrane-anchored but not secreted or intracellular VP1 antigen from foot and mouth disease virus,[172] again demonstrating that membrane expression of antigens can alter their immunogenicity.

B. Opportunities and Limitations of Membrane-Tethered Antigens

Based on several studies, membrane-anchored antigens appear to effectively induce potent humoral responses, but poor cellular immunity. This is likely due to segregation of antigen from the proteosome, thereby minimizing the generation of antigenic peptides for presentation by MHC class I molecules. In most cases, strong humoral immune responses were observed after antigens were tethered on the cell surface, even when the soluble antigen was unable to induce an immune response. Membrane-anchored antigens therefore appear to be a good choice for generating antibody responses against glycoproteins and poorly immunogenic polypeptides. Membrane-anchored antigens can be easily produced in large amounts, but their complex nature can make standardization difficult. Alternative technologies, such as production of purified particulate antigens, are highly competitive for *in vivo* human use.

9. OTHER APPLICATIONS OF MEMBRANE-ANCHORED CHIMERIC PROTEINS

Besides acting as molecular chaperones, heat shock proteins can also stimulate innate and adaptive immunity.[173] The resident endoplasmic reticulum heat shock protein gp96 binds to receptors present on dendritic cells to induce their maturation and enhance the transfer of gp96-associated proteins to MHC class I molecules (reviewed in Ref. 174). To enhance the immunostimulatory activity of gp96, Zheng and colleagues[175] replaced a *C*-terminal endoplasmic reticulum–retention sequence with the PDGFR TM (Table VI). Although the level of gp96 expressed on the surface of MethA fibrosarcoma cells and CT-26 colon carcinoma cells was modest, direct contact with DCs upregulated markers

Table VI. Other Membrane-Anchored Proteins

Functional extracellular domain	Juxtamembrane linker (amino acids)[a]	Glyc Sites[b]	Transmembrane domain	Cytoplasmic tail (amino acids)	Full CT?[c]	Cell target	Purpose	Surface level[d]	Ref
gp96	c-*myc* tag + PDGFR (20)	0	human platelet-derived growth factor receptor (PDGFR)	PDGFR (6)	no	MethA mouse fibrosarcoma cells, CT-26 mouse colon cancer cells, transgenic mice	antitumor	+	175-177
IgG_1 Fc	TfR (11)	0	human transferrin receptor (TfR) type II TM	TfR (63)	yes	BHK hamster kidney cells	monocyte activation	++	1
SEA superantigen	(GGGGS)3	0	human B7-1	human B7-1 (25)	yes	HepG2 human hepatoma cells, Hepa1-6 mouse hepatoma cells, NIH3T3 fibroblasts, B16 melanoma cells	lymphocyte activation	+++	179, 180
human platelet-derived growth factor receptor (PDGFR)	none	0	human platelet-derived growth factor receptor (PDGFR)	human fibroblast growth factor receptor-1 (422)	yes	rat pancreatic islets, COS monkey kidney cells	artificial receptor	ND	181
human PDGFR	none	0	human platelet-derived growth factor receptor (PDGFR)	CD4 (37)	yes	NMuMG mammary gland epithelial cells, COS-7 monkey kidney cells	artificial kinase	ND	182
mouse erythpoietin (EPO) receptor	TNF-R1a (15) TNF-R1b (24)	1	Mouse tumor necrosis factor receptor (TNF-R)	TNF-R1a (219) TNF-R1b (188)	yes	NIH 3T3 fibroblasts, COS monkey kidney cells, PAM 212 transformed mouse keratinocytes, HeLa human cervical carcinoma cells	artificial receptor	ND	183
apolipoprotein B	none	0	human decay accelerating factor (DAF) GPI signal sequence	NA	NA	CHO monkey kidney cells	artificial receptor	ND	184
CCR2 fragment	none	0	human CD8	CD8 (28)	yes	HEK-293 human embryonic kidney cells	artificial receptor	+	185
CCR2 fragment	EPO receptor (6)	0	erythropoietin (EPO) receptor	EPO receptor (234)	no	HEK-293 human embryonic kidney cells	artificial receptor	+	185
peptide libraries	none	0	mouse CD8	Mouse CD8 (27)	yes	*Xenopus* oocytes	screening of receptor agonists	ND	186, 187

NA, not applicable; ND, not determined.

[a]Number of amino acids in the juxtamembrane linker domain.

[b]Potential N-linked glycosylation sites present in the juxtamembrane linker domain.

[c]Indicates if the entire cytoplasmic tail was included in the chimeric protein.

[d]Relative surface expression level estimated from flow cytometric or fluorescence microscopy data.

associated with dendritic cell maturation. MethA tumor cells expressing gp96 were rejected in a $CD8^+$ and $CD4^+$ T cell dependent fashion.[176] More interestingly, wild-type MethA tumors injected into the contralateral flank were also rejected, indicating that systemic antitumor immunity was induced. Transgenic mice that expressed membrane-anchored gp96 in various tissues including the heart, kidney, lung, thymus, skin, gut and skeletal muscle experienced chronic activation of dendritic cells and developed autoimmune disease typified by severe glomerulonephritis.[177]

In an interesting application, Stabila and colleagues[1] fused the first 97 amino acids of the type II human transferrin receptor to the *N*-terminus of the human IgG_1 Fc domain (CH_2 and CH_3 domains) to express Fc molecules on baby hamster kidney cells in the proper orientation. Although the immunoglobulin hinge region was deleted from the construct, a cysteine residue present in the extracellular domain of the transferrin receptor allowed formation of disulfide-linked dimers. Membrane-anchored Fc molecules did not activate complement but specifically bound to Fc receptors on monocytes, leading to monocyte activation as assessed by superoxide production. Surface Fc receptors may be useful for macrophage-mediated elimination of cells leaking from encapsulated cell devices as well as for tumor therapy.

Superantigens are proteins that can bridge MHC class II molecules and specific T cell receptor β chains, thereby activating up to 20% of all T cells and producing toxic shock syndrome.[178] The superantigen staphylococcal enterotoxin A was fused to the human B7-1 TM to allow accumulation on the surface of HepG2 hepatocellular carcinoma cells.[179] A replication-defective adenovirial vector was created to express membrane-anchored staphylococcal enterotoxin A on hepatocellular carcinoma cells under the control of the alpha fetoprotein enhancer/promoter.[180] Direct injection of Hepa1-6 tumors with the adenovirus resulted in systemic antitumor immunity and increased survival time of tumor-bearing mice.

Mares and colleagues[181] created a chimeric receptor by fusing the extracellular and TM domains of the human platelet-derived growth factor receptor to the cytoplasmic tail of human fibroblast growth factor receptor-1. DNA synthesis was stimulated upon addition of soluble platelet-derived growth factor to rat pancreatic islets expressing the chimeric receptor. Along similar lines, an artificial receptor tyrosine kinase was constructed by fusing the extracellular and TM domains of the mouse platelet-derived growth factor receptor to the cytoplasmic tail of CD4, which can bind the cytoplasmic tyrosine protein kinase $p56^{lck}$. Addition of platelet-derived growth factor to mouse mammary gland epithelial cells expressing the chimeric receptor and $p56^{lck}$ resulted in increased $p56^{lck}$ protein kinase activity and the phosphorylation of several protein substrates.[182]

A chimeric receptor was created by fusing the extracellular domain of the murine erythropoietin receptor to the stem, TM and cytoplasmic tail of the 55- and 75-kDa forms of mouse tumor necrosis factor.[183] Both chimeric receptors were constitutively active, arguing that the extracellular domain of tumor necrosis factor normally prevents dimerization of the cytoplasmic tail signaling domain in the absence of ligand, whereas the chimeric receptors constitutively form dimers.

The GPI signal sequence from decay-accelerating factor was fused to the *C*-terminus of a fragment of apolipoprotein B and expressed on cells.[184] These cells specifically bound lipoprotein lipase, thus demonstrating that an *N*-terminal fragment of apolipoprotein B can function as a lipoprotein lipase binding protein.

The 35 amino acid *N*-terminal fragment of the seven-transmembrane domain G-protein-coupled monocyte chemoattractant protein 1 receptor was fused to the human CD8 TM and CT or the TM of the murine erythropoietin receptor to anchor this fragment on HEK-293T cells.[185] This allowed the authors to unambiguously demonstrate that the *N*-terminal domain of the receptor was sufficient for high affinity binding to monocyte chemoattractant protein 1.

In an interesting application, Chen and colleagues[186] anchored the *N*-terminal fragment of the thrombin receptor on *Xenopus* oocytes *via* the mouse CD8 TM and CT to demonstrate that receptor activation by protease cleavage is due to unmasking of a tethered peptide ligand that then binds intramolecularly to initiate receptor signaling. They employed the same principle to tether a random

peptide library on oocytes and showed that novel and potent receptor agonists could be isolated from the library.[187]

10. CONCLUDING REMARKS

The potential of membrane-anchored chimeric proteins is just beginning to be tapped. Any application that can benefit from cell surface-located accumulation of a biologically active protein should be amenable to using membrane-anchored chimeric proteins. Expression of membrane-anchored chimeric proteins may also compliment other methods to target proteins to cellular targets *in vivo*, such as employing antibodies or antibody conjugates. Antibody targeting requires identification of suitable specific antigens on the cell membrane, and is limited to antigens that do not undergo endocytosis if extracellular retention of the conjugate is desirable. *In vivo* expression of membrane-anchored chimeric proteins requires development of suitable nonviral or viral vectors to allow expression in defined target tissues. Regulatory elements and vector design can be employed to restrict gene expression to selected tissues.[188,189] For example, adenoviral vectors are being developed that (1) preferentially target specific cells by altering virus tropism through genetically incorporating targeting moieties in the virus capsid or pseudotyping the virus with other adenovirus serotypes, (2) expressing transgenes in selected cells by transcriptional targeting, or (3) developing adenoviruses that conditionally replicate in target cells such as tumor cells.[190] Similar strategies are being applied to selectively target lentiviral and retroviral vectors.[188] Rapid progress is also being made in developing polymeric and liposomal systems to deliver nonviral DNA vectors to specific cell targets.[191,192] New nonviral vectors are also being developed to allow long-term gene expression in mammalian cells.[193] Antibodies typically display half-lives on the order of hours to a week.[194] Gene expression, by contrast, can be extended for weeks to years.[195,196] These technologies thus possess strengths and weaknesses that compliment each other. Numerous opportunities exist to develop novel technologies and therapeutic agents based on membrane-anchored chimeric proteins alone or with other tissue targeting strategies.

11. ABBREVIATIONS

AFP	alpha fetoprotein
ASGPR	asialoglycoprotein receptor
β2m	beta-2-microglobulin
CPA	carboxypeptidase A1
CT	cytoplasmic tail
CTLA-4	cytotoxic T-lymphocyte antigen 4
DAF	decay-accelerating factor
Dansyl	5-dimethylamino-1-naphthalene sulfonic acid
DTPA	diethylenetriamine pentaacetic acid
GPI	glycosylphosphatidylinositol
MHC	major histocompatibility complex
PDGFR	platelet-derived growth factor receptor
phOx	phenyl-2-oxazoline-5-one
TM	transmembrane domain
TNF	tumor-necrosis factor

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Tian-Lu Cheng *received his Ph.D. degree from the National Defense Medical Center in Taipei, Taiwan in 1999. He has been at the Kaohsiung Medical University since 2001 where he is currently an Associate Professor in the Faculty of Biomedical Science and Environmental Biology and Division Leader in the Biomedical Innovation Incubation Center at the Kaohsiung Medical University. The recent focus of his research group includes development of membrane antibody and enzyme reporters for noninvasive imaging of gene expression in vivo*, membrane cytokines as cancer vaccines and cell surface display of antibody for affinity evolution.

Steve Roffler *received his Ph.D. degree in Chemical Engineering from the University of California at Berkeley in 1986. He has been at the Institute of Biomedical Sciences, Academia Sinica in Taipei, Taiwan since 1991 where he is currently a Full Research Fellow. The current focus of his research group includes targeted cancer therapy with glucuronide prodrugs, mammalian cell surface display for directed evolution and therapy, and elucidation of the mechanism of surface receptor signal initiation in immune cells.*