Expression of Chimeric Monomer and Dimer Proteins on the Plasma Membrane of Mammalian Cells

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Abstract: Targeting of proteins to the plasma membrane of cells may be useful for vaccine development, tissue engineering, genetic research, bioseparations, and disease treatment. The ability of different transmembrane domains (TM) to direct a reporter protein (human alpha-feto protein, AFP) to the surface of mammalian cells was examined. High surface expression was achieved with chimeric proteins composed of AFP and the TM and cytosolic tail of murine B7-1 (AFP-B7) as well as with AFP containing a GPI-anchor from decay-accelerating factor (AFP-DAF). Lower surface expression of AFP was observed when the TM of human platelet-derived growth factor receptor or the human asialoglycoprotein receptor H1 subunit were employed. Introduction of the hinge-CH2-CH3 region of human IgG (γ1 domain) between AFP and TM allowed efficient formation of disulfide-linked dimers. Surface expression of AFP-γ1-B7 dimers was impaired compared to AFP-B7 whereas AFP-γ1-DAF dimers were efficiently targeted to the surface. Accumulation of chimeric proteins on the cell surface did not correlate with the level of protein expression. This study demonstrates that high levels of monomeric and dimeric proteins can be targeted to the cell membrane of mammalian cells by proper selection of TM. © 1999 John Wiley & Sons, Inc. *Biotechnol Bioeng* **65:** 160–169, 1999.
Keywords: surface expression; transmembrane domain; GPI anchor; chimeric proteins; dimer; monomer.

INTRODUCTION

The display of recombinant proteins on the surface of filamentous phage (Barbas, 1993) and more recently on yeast and bacteria (Georgiou et al., 1997) has found many applications in basic research and biotechnology. Expression of homologous and heterologous proteins on the surface of mammalian cells should also be useful for a variety of bioengingeering and medical applications. Various elements can be employed to anchor proteins on the plasma membrane of cells. For example, the transmembrane domains (TM) of type-I (oriented with the N-terminus outside the cell) and type-II (oriented with the N-terminus in the cytosol) integral membrane proteins can be used to target chimeric proteins to the plasma membrane. Proteins can also be attached to the cell surface by fusion of a GPI (glycophosphatidylinositol lipid) signal to the 3′ end of genes. Cleavage of the short carboxy-terminal peptide allows attachment of a glycolipid to the newly exposed C-terminus through an amide linkage (Udenfriend and Kodukula, 1995). Although several TM have been employed to target proteins to the plasma membrane (Marais et al., 1997; Winberg et al., 1996; Moore at al., 1997; Chesnut et al., 1996), the relative efficiencies of cell surface targeting by different domains has not been investigated. A major aim of our study, therefore, was to compare the ability of different targeting domains to direct the expression of a reporter protein to the cell surface.

The cell surface targeting domains examined in this study included TM from the human platelet-derived growth factor receptor B chain (PDGFR) (Gronwald et al., 1988), the TM and cytosolic domains of murine B7-1 (B7) (Freeman et al., 1991), the type-II TM of the human asialoglycoprotein receptor H1 subunit (ASGPR) (Spiess et al., 1985), and a C-terminal GPI-anchor signal sequence from human decay accelerating factor (DAF) (Caras et al., 1987). Human α feto-protein (AFP) (Morinaga et al., 1983) was employed as a model protein for surface expression. We also investigated whether protein dimers could be expressed on the surface of cells. This was accomplished by inserting the hinge-CH2-CH3 (γ1) region of the human IgG1 heavy chain between AFP and TM to allow the formation of disulfide-linked dimers. Our results show that the choice of TM influenced the surface expression of AFP chimeric proteins. The B7 TM allowed the highest expression of AFP on the cell surface. The γ1 domain allowed efficient formation of dimers but reduced the surface expression of dimeric AFP-γ1-B7 compared to monomeric AFP-B7 whereas dimeric AFP-γ1-DAF was well expressed.

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MATERIALS AND METHODS

Cell Lines and Tissue Culture

BHK-21 (baby hamster kidney) cells were obtained from Veteran's General Hospital (Taipei, Taiwan). HepG2 human hepatoma and HeLa human cervical carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). All cells were cultured in Dulbecco's minimal essential medium (Sigma, St. Louis, MO) supplemented with 10% bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in an atmosphere of 5% CO_2.

RNA Isolation

10^7 cells were harvested and homogenized in 700 μL of solution A (0.5% SDS, 0.2 *M* sodium acetate, pH 4.2). An equal volume of water-saturated phenol was added, and the homogenate was vortexed 5 min at room temperature before extraction with 100 μL chloroform. After centrifugation at 14,000 rpm for 5 min, the aqueous supernatant was sequentially extracted with equal volumes of water-saturated phenol–chloroform and chloroform. RNA was precipitated by addition of 0.8 vol of isopropyl alcohol and centrifuged at 15,000 rpm at 4°C for 30 min. The pellet was washed with 80% ethanol and stored at −70°C.

Plasmid Construction

Human AFP cDNA was amplified by RT-PCR with primers p1 and p2 (Table I) from RNA isolated from HepG2 cells. The amplified product encompassed the first codon of mature AFP (Thr20) to the stop codon which was mutated to Val. The PCR product was cleaved with *Sfi*I and *Sal*I restriction enzymes and subcloned into pHook-1 (Invitrogen, Leek, The Netherlands) in place of the phOx scFv (single-chain antibody) gene to obtain pAFP-PDGFR.

To construct pAFP-B7, a plasmid containing the murine B7-1 gene (kindly provided by Dr. Gordon Freeman, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA) was used as template to amplify the B7-1 transmembrane domain and cytosolic tail (Pro237 to the stop codon) by PCR with the primers p3 and p4. The PCR product, after digestion with *Sal*I and *Xho*I, was ligated in the same sites of pAFP-PDGFR in place of the myc-PDGFR TM fragment.

The sequence encoding the C-terminal 37 amino acids of human decay accelerating factor (DAF) was amplified from HeLa cells by RT-PCR. In the first round of PCR, primers p5 and p6 were employed to amplify DAF cDNA from Pro345 to Thr381. cDNA encoding half of the c-myc epitope was introduced at the 5′ end and a *Not*I restriction site was added after the stop codon. A second round of PCR with primers p7 and p6 was employed to extend the c-myc epitope on the 5′ end of the cDNA and introduce a *Sal*I restriction site. The PCR product was digested with *Sal*I and *Not*I before ligation in the same sites of pAFP-PDGFR in place of the myc-PDGFR TM fragment to create pAFP-DAF.

A fragment containing the hinge, CH2 and CH3 domain (γ1) of the human IgG1 heavy chain was amplified by PCR using pUC-19-Ig1 as template (kindly provided by Dr. S. L. Hsieh, Department of Microbiology and Immunology, National Yang Ming University, Taipei, Taiwan) with the primers p8 and p9. The PCR product was digested with *Sal*I and inserted into the unique *Sal*I site in pAFP-PDGFR, pAFP-B7, and pAFP-DAF between AFP and the respective TM domains to produce pAFP-γ1-PDGFR, pAFP-γ1-B7, and pAFP-γ1-DAF.

cDNA encoding the human ASGPR H1 subunit transmembrane domain from Met1 to Gln65 was amplified by

Table I. DNA primers employed for PCR amplification.

Name	Sequence[a]	Restriction site or (epitope)
p1	AAATTTGGCCCAGCCGGCCACACTGCATAGAAATGAATATGGA	*Sfi*I
p2	AGCTTAGTCGACAACTCCCAAAGCAGCACGAGTTTT	*Sal*I
p3	AAAAGTCGACCCCCCAGAAGACCCTCCTGATAGC	*Sal*I
p4	AACTCGAGCTAAAGGAAGACGGTCTGTTCAGC	*Xho*I
p5	**ATCTCAGAAGAGGATCTGAAT**CCAAATAAAGGAAGTGGAACCACT	(myc)
p6	ACTCGCGGCCGCCTAAGTCAGCAAGCCCATGGTTAC	*Not*I
p7	CTATGTCGAC**GAACAAAAAACTC**ATCTCAGAAGAGGATCTGAAT	*Sal*I (myc)
p8	CCGTCGACGAGCCCAAATCTTGTGACAAAACT	*Sal*I
p9	TTTTGTCGACTTTACCCGGAGACAGGGAGAGGCT	*Sal*I
p10	TTGGGGATATCCACCATGACCAAGGAGTATCAAGACCTTCAG	*Eco*RV
p11	**ATCTGGAACATCATATGGATA**CTGGGAGTTTTGGGATCCGATCAC	(HA)
p12	CATGGCCGGCTGGGCCCC**AGCATA**ATCTGGAACATCATATGGATA	*Sfi*I (HA)
p13	CCCTGAGTCGACTTAAACTCCCAAAGCAGCACGAGT	*Sal*I
p14	TTGGGGATATCCACCATGACCAAGGAGGCTCAAGACCTTCAG	*Eco*RV

[a]All sequences are arranged from 5′ to 3′. Restriction sites are underlined. Sequences encoding partial myc or HA epitopes are shown in bold.

RT-PCR from HepG2 cells. In the first round of PCR, primers p10 and p11 were employed to introduce an *Eco*RV site at the 5′ end of the ASGPR TM sequence and append half of the HA epitope sequence to the 3′ end. The remaining portion of the HA epitope sequence and a *Sfi*I restriction site were introduced at the 3′ end of the ASGPR TM domain in a second round of PCR with primers p10 and p12. The PCR product, after digestion with *Eco*RV and *Sfi*I, was subcloned into pHook-1 in place of the sequence encoding the signal peptide-HA epitope to create pASGPR-Hook. The human AFP gene was PCR amplified from pAFP-PDGFR with primers p1 and p13 to introduce a stop codon before the *Sal*I site at the 3′ end of AFP. The PCR product was digested with *Sfi*I and *Sal*I before ligation into the same sites of pASGPR-Hook in place of the phOx scFv gene to create pASGPR(Y)-AFP. This plasmid encodes for a transgene composed of the leader/TM domain of ASGPR followed by a HA epitope and mature AFP. pASGPR(A)-AFP in which the codon for Tyr5 was mutated to Ala5, was constructed in an analogous fashion by PCR amplification of the ASGPR-TM cDNA with primers p14 and p11 in the first round of amplification, and p14 and p12 in the second round of PCR.

pNeo-Gus was constructed by excising the full length *Escherichia coli* β-glucuronidase (βG) gene from pGusN358→S (Clontech, Palo Alto, CA) by digestion with *Hin*dIII and *Eco*RI and ligating the fragment into pcDNA3 (Invitrogen). All DNA sequences were verified.

Antibodies

Female BALB/c mice (4–6 weeks old) were i.p. injected with 10 μg of AFP in complete Freund's adjuvant followed by two weekly i.p. injections of 10 μg of AFP in incomplete Freund's adjuvant and three weekly i.p. injections of 10 μg of AFP in PBS. Four days after the last immunization, spleens were removed and fused with NS1 myeloma cells as described (Yeh et al., 1979). Two hybridomas secreting IgG2a antibodies were selected based on their ELISA titers against human AFP. Competitive ELISA results demonstrated that mAb 3.3 and 36.2 bound to nonoverlapping epitopes on human AFP (data not shown). mAb 5A8 against 38C13 mouse B cell lymphoma cells was employed as a negative control (Maloney et al., 1985). Antibodies were purified from ascites produced in pristane-primed BALB/c mice on protein A-Sepharose CL-4B.

Affinity-purified human AFP was employed for rabbit immunizations. mAb 3.3 (13 mg/mL gel) was coupled to glutaraldehyde-activated AcA 22 gel. Human fetal cord serum or supernatant from HepG2 cells cultured in an Opticell 5200R bioreactor (Charles River Biotechnical Services, Wilmington, MA) was passed through the column which was then extensively washed with PBS and eluted with 0.1 *M* glycine, pH 2.5. Purified AFP was dialyzed against PBS and assayed for purity by SDS–PAGE. Rabbits were immunized with 50 μg of AFP in complete Freund's adjuvant and boosted with 25–50 μg of AFP in incomplete adjuvant at 4–6 week intervals before rabbits were bled. Polyclonal anti-AFP antibodies were purified on protein A-Sepharose CL-4B. Polyclonal anti-βG serum was prepared as described (Cheng et al., 1997).

Transfection of BHK Cells

2.5×10^5 BHK cells per well were cultured in 6-well dishes for 24 h. Transfection was performed by mixing 3 μg of plasmid DNA with 10 μL lipofectamine (Gibco) according to the manufacturer's instructions. For co-transfection, 1.5 μg of each AFP-TM plasmid was mixed with 1.5 μg of pNeo-Gus before addition of lipofectamine. Stable transfectants were obtained by culturing cells in G418 for 2 weeks. Stable transfectants were cloned by limiting dilution in 96-well culture plates. Clones were expanded and screened by flow cytometer analysis of surface AFP expression.

Immunoblotting of Chimeric Proteins

BHK cells were harvested 48 h after co-transfection with AFP-TM transgenes and pNeo-Gus. The cells were washed with PBS, and 5×10^4 cells were boiled for 10 min in SDS–PAGE sample buffer with or without 2-mercaptoethanol. Proteins were electrophoresed on an 8% SDS–PAGE and transferred to two sheets of nitrocellulose paper (Gelman Sciences, Ann Arbor, MI) in transfer buffer (10 m*M* Tris-HCl, pH 7.5, 50 m*M* NaCl, 2 m*M* EDTA, 0.5 m*M* 2-mercaptoethanol) by capillary diffusion. Blots were blocked with 5% skim milk and incubated with 1 μg/mL purified rabbit anti-AFP antibody or rabbit anti-βG serum diluted 1:1000 in 0.1% Tween-20/PBS for 60 min. Blots were extensively washed in the same buffer, incubated with horse radish peroxidase-conjugated goat anti-rabbit IgG diluted 1:4000, washed, and visualized by ECL detection according to the manufacturer's instructions (Pierce, Rockford, IL). X-ray films were scanned on a computing densitometer (Molecular Dynamics, Mountain View, CA), and the band intensities were quantified with Image Quant software.

Surface Immunofluorescence

BHK cells grown on glass slides in a 6-well dish were transfected with AFP-TM transgenes. The cells were fixed on the glass slides 48 h later with 2% formaldehyde. The slides were incubated with rabbit anti-AFP serum (1:500) and FITC-conjugated goat anti-rabbit IgG (1:400) before being mounted and viewed with a Leitz microscope under both visible and fluorescence illumination.

Flow Cytometer Analysis of Surface Expression

BHK cells harvested 48 h after transfection with AFP-TM transgenes were washed and suspended in DMEM containing 0.5% bovine serum at 4°C. Cells were co-transfected with pNeo-Gus in some experiments to control for transfection efficiency. 5×10^5 cells were incubated with 5 μg/mL

mAb 36.2 or mAb 5A8 for 60 min followed by FITC-conjugated goat anti-mouse antibody (1:400) for 60 min on ice. The surface immunofluorescence of 10^4 viable cells was measured with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA) after dead cells stained by addition of 5 μg/mL propidium iodide were gated out. Fluorescence intensities were measured with Cell Quest Software (Becton Dickinson).

PI-PLC Digestion of Fusion Proteins

BHK cells transfected with pAFP-PDGFR, pAFP-B7, or pAFP-DAF were harvested 48 h later and washed with PBS. 10^5 cells were incubated with 50 mU/mL phosphatidylinositol phospholipase C (PI-PLC) at 37°C in PBS for 60 min. Cells were stained for AFP surface expression and analyzed by flow cytometry as described above.

RESULTS

Generation of Vectors for Surface Expression of Chimeric Proteins

Figure 1 depicts the AFP-TM transgenes that were constructed in this study. pAFP-PDGFR was constructed by replacing the single-chain antibody cDNA in pHook-1 with the gene encoding mature AFP. This construct contains a chimeric gene that includes cDNA encoding (i) a murine immunoglobulin κ chain signal peptide; (ii) a 9 amino acid HA epitope; (iii) AFP; (iv) an 11 amino acid c-myc epitope; and (v) the PDGFR TM. pAFP-B7 was constructed by replacing the PDGFR TM cDNA with the cDNA encoding the TM and cytoplasmic domain of the murine B7-1 gene, whereas pAFP-DAF was created by inserting the cDNA encoding the C-terminal extension of decay accelerating factor. PDGFR and B7-1 are type-I integral membrane proteins, oriented such that the N-terminal is extracellular and the C-terminal is located in the cytosol. The C-terminal extension of DAF in pAFP-DAF, in contrast, directs the attachment of a GPI-anchor to the C-terminal of AFP. pASGPR(Y)-AFP was constructed by ligating cDNA encoding the N-terminal hydrophobic domain and the flanking sequence of the human ASGPR H1 subunit to the 5′ end of AFP. The human ASGPR H1 subunit is a type-II integral membrane protein oriented with the N-terminal in the cytosol and the C-terminus outside the plasma membrane. The ASGPR H1 subunit contains a tyrosine residue (Tyr5) which is believed to promote internalization of the receptor from the cell surface (Fuhrer et al., 1991). The codon encoding Tyr5 was therefore mutated to alanine in

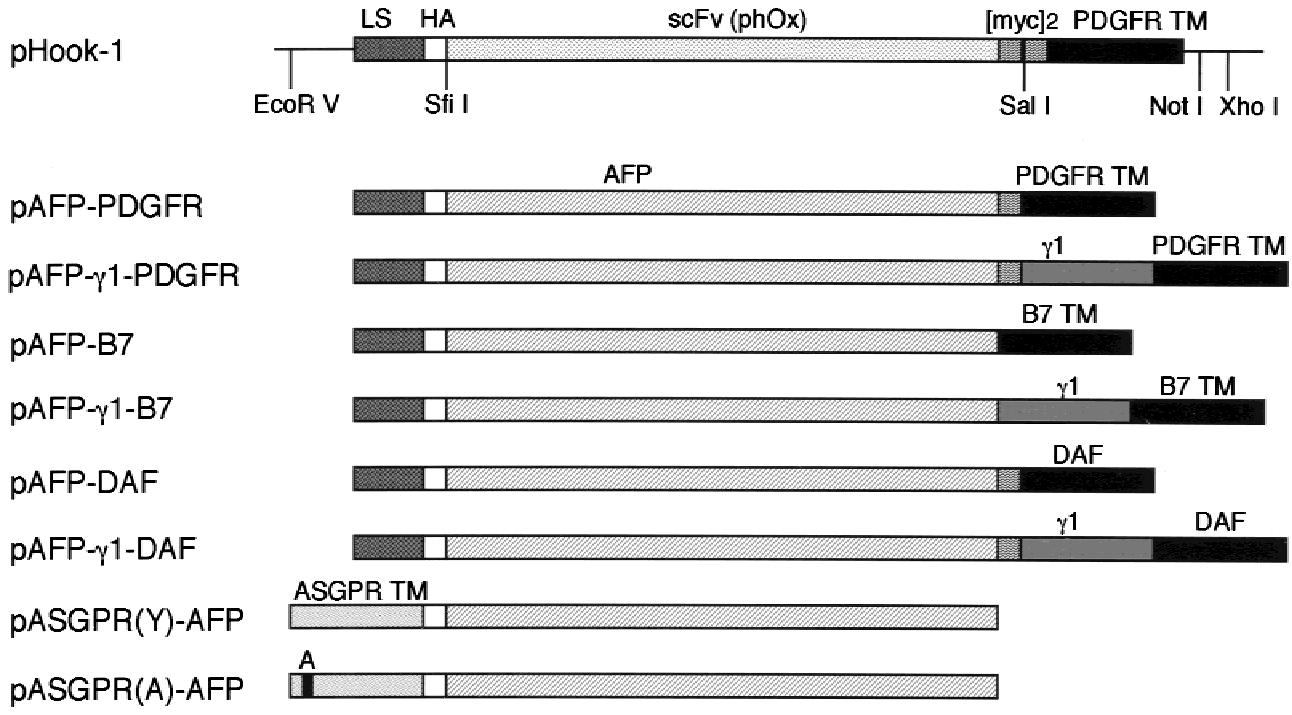

Figure 1. Diagram of AFP-TM chimeric transgenes. The AFP-TM chimeric transgenes were based on the pHook-1 vector which codes for a murine immunoglobulin κ chain signal peptide, a 9 amino acid HA epitope, the phOx scFv, two 11 amino acid myc epitopes, and the TM of PDGFR. pAFP-PDGFR contains the human AFP gene in place of the phOx scFv cDNA. The PDGFR TM cDNA was replaced with the murine B7-1 TM and cytosolic tail cDNA in pAFP-B7. The c-myc epitope was removed during the cloning procedure in this construct. pAFP-DAF contains cDNA coding for the C-terminal 37 amino acids of human DAF. The human IgG1 hinge-CH_2-CH_3 domain (γ1) cDNA was inserted between the sequences coding for AFP and the TM of pAFP-PDGFR, pAFP-B7, and pAFP-DAF to generate pAFP-γ1-PDGFR, pAFP-γ1-B7, and pAFP-γ1-DAF. The type-II TM cDNA from the human asialoglycoprotein receptor H1 subunit was ligated to the 5′ end of the AFP gene to create pASGPR(Y)-AFP. pASGPR(A)-AFP is identical to pASGPR(Y)-AFP except that tyr5 codon in the TM was mutated to alanine. All transgenes are under the control of the CMV promoter in pHook-1.

pASGPR(A)-AFP to reduce internalization. The γ1 domain (hinge-CH2-CH3) cDNA of human IgG1 was also inserted between AFP and transmembrane domain sequences (pAFP-γ1-PDGFR, pAFP-γ1-B7, and pAFP-γ1-DAF) to create chimeric genes that can potentially express proteins that form disulfide-linked dimers on the cell surface.

Chimeric Proteins Can Be Expressed as Monomers or Dimers

The expression of AFP-TM chimeric proteins was examined by immunoblotting whole cell lysates prepared from BHK cells that were co-transfected with AFP-TM transgenes and pNeo-Gus to express β-glucuronidase (βG) as an internal transfection control. Immunoblotting with polyclonal rabbit serum against AFP revealed that all the AFP-TM transgenes were expressed in BHK cells (Fig. 2A). Immunostaining for AFP was specific as shown by the lack of a signal in untransfected BHK cells (Fig. 2A, lane 1). The molecular weights of the chimeric proteins estimated by their relative positions on the gel corresponded to their predicted sizes. The molecular sizes of AFP-γ1-PDGFR, AFP-γ1-B7, and AFP-γ1-DAF were increased by approximately 28 kDa, in agreement with the predicted size of the γ1 domain. All chimeric proteins predominately migrated as monomers on a reducing SDS–PAGE. The appearance of multiple bands in several of the lanes (AFP-γ1-PDGFR, lane 3; AFP-γ1-B7, lane 5; AFP-DAF, lane 6; ASGPR(Y)-AFP, lane 8) is likely due to degradation of the chimeric proteins.

The relative expression of the fusion proteins was determined by densitometric scanning of the bands in each lane, normalized for the expression of cytosolic βG to control for differences in transfection efficiency. Figure 2B shows that AFP-PDGFR, ASGPR(Y)-AFP, and ASGPR(A)-AFP were expressed at the highest levels whereas the expression of all other chimeric proteins was significantly lower than AFP-PDGFR. Introduction of the γ1 domain decreased the expression of AFP-γ1-PDGFR by 40% compared to AFP-PDGFR. The expression AFP-γ1-DAF but not AFP-γ1-B7 was also significantly ($p \leq 0.05$) reduced compared to the same chimeric protein without a γ1 domain.

Cell lysates from BHK cells transfected with AFP transgenes were also electrophoresed on a nonreducing SDS–PAGE and immunoblotted with AFP polyclonal serum. Figure 3 shows that proteins containing the γ1 domain exhibited reduced migration on a nonreduced gel (Fig. 3, lanes 3, 5, 7) compared to their migration on a reduced gel (Fig. 2, lanes 3, 5, 7), demonstrating that the γ1 domain allowed formation of disulfide-linked dimers. The efficiency of dimerization was good with only small amounts of monomeric AFP-γ1-PDGFR and AFP-γ1-B7 evident (Fig. 3, lanes 3 and 5).

Monomeric and Dimeric Proteins Are Expressed on the Cell Surface

To determine whether AFP-TM chimeric proteins were expressed on the cell surface, viable transfected cells were

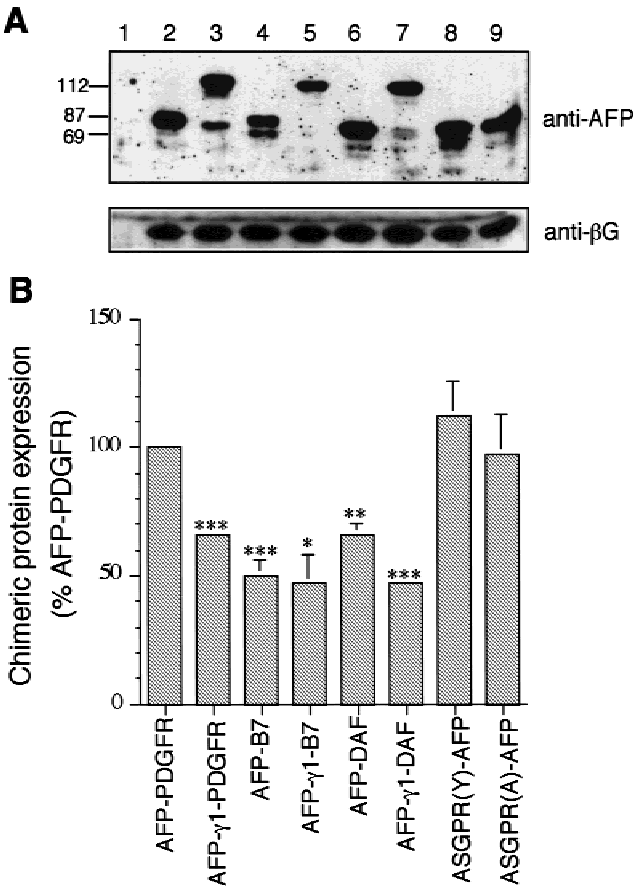

Figure 2. Immunoblot of AFP-TM chimeric proteins in transfected cells. (A) Cells harvested 48 h after transfection with AFP-TM transgenes and βG reporter plasmid were lysed and separated on a 8% reducing SDS–PAGE. Proteins transferred to nitrocellulose paper were immunoblotted with anti-AFP (top) or anti-βG (bottom) antibodies. Lane 1, untransfected BHK cells; lane 2, AFP-PDGFR; lane 3, AFP-γ1-PDGFR; lane 4, AFP-B7; lane 5, AFP-γ1-B7; lane 6, AFP-DAF; lane 7, AFP-γ1-DAF; lane 8, ASGPR(Y)-AFP; lane 9, ASGPR(A)-AFP. The molecular masses of the proteins are indicated in kDa. (B) X-ray films similar to those shown in panel A were scanned on a densitometer and quantified by Image Quant software. Relative AFP expression was calculated as $100[Da/Db]_{AFP\text{-}TM}/[Da/Db]_{AFP\text{-}PDGFR}$, where Da and Db represent the integrated band densities of the indicated chimeric protein and βG, respectively. Results represent mean values from 4 independent experiments. Significant differences relative to AFP-PDGFR are indicated (*, $p \leq 0.05$; **, $p \leq 0.005$; ***, $p \leq 0.0005$). Bars, SE.

viewed under fluorescence illumination after staining with AFP antibody and FITC-conjugated second antibody. BHK cells transfected with pAFP-PDGFR (Fig. 4B), pAFP-B7 (Fig. 4C), pAFP-DAF (Fig. 4D), and pASGPR(Y)-AFP (Fig. 4E) expressed AFP on the plasma membrane. Surface fluorescence was not observed in untransfected BHK cells treated in an identical fashion, showing that immunostaining for AFP was specific (Fig. 4A). BHK cells transfected with pAFP-B7 also did not display fluorescence after staining with a control polyclonal antibody (Fig. 4F), further confirming the specificity of AFP immunostaining.

To verify that the C-terminal domain of human decay accelerating factor was correctly processed such that a GPI-

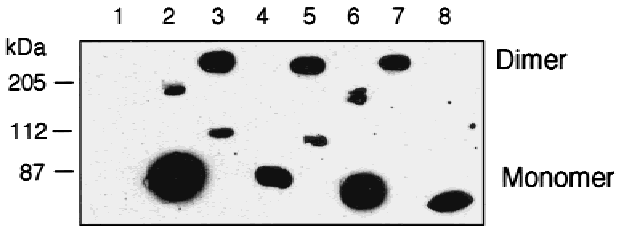

Figure 3. Dimerization of chimeric proteins. Cell lysates were separated on a 5% nonreducing SDS–PAGE and detected by immunoblotting for AFP. Chimeric protein monomers and dimers are indicated. Lane 1, untransfected BHK cells; lane 2, AFP-PDGFR; lane 3, AFP-γ1-PDGFR; lane 4, AFP-B7; lane 5, AFP-γ1-B7; lane 6, AFP-DAF; lane 7, AFP-γ1-DAF; lane 8, purified AFP from HepG2 cells.

anchor was attached to AFP, transfected BHK cells were incubated with PI-PLC to cleave cell surface GPI-linked proteins. The surface fluorescence of BHK cells transfected with the type-I TM constructs (AFP-PDGFR and AFP-B7) was unaffected by treatment with PI-PLC (Figs. 5A,B). In contrast, PI-PLC treatment of BHK cells transfected with pAFP-DAF decreased the surface immunofluorescence of the cells (Fig. 5C), demonstrating that AFP-DAF was anchored to the cell surface by a GPI-anchor.

The surface expression of AFP-TM chimeric proteins was quantified by flow cytometer analysis of transfected BHK cells. Figure 6 shows that all of the chimeric proteins were expressed on the surface of BHK cells. The B7 transmembrane domain (pAFP-B7) directed the most AFP to the cell surface, whereas the ASGPR TM was the least effective. Table II compares the fluorescence intensities of the different AFP-TM chimeric proteins on the cell surface relative to the expression of AFP-PDGFR (100%). The surface expression of AFP-B7 and AFP-DAF were 3-fold and 2.4-fold higher than AFP-PDGFR, respectively. The type-II TM chimeras (ASGPR(Y)-AFP and ASGPR(A)-AFP) were poorly expressed on the surface. Dimerization of the proteins did not significantly change the surface expression of AFP-γ1-PDGFR and AFP-γ1-DAF relative to the AFP-PDGFR and AFP-DAF monomers. The surface expression of AFP-γ1-B7, in contrast, was significantly ($p \leq 0.01$) lower than AFP-B7. Differences in surface expression were not due to differences in transfection efficiency because similar amounts of the internal control protein (βG) were detected in the transfectants (±25%, results not shown).

To investigate whether heterologous proteins could be stably expressed on cells, BHK cells were transfected with pAFP-B7 and selected for resistance to G418. Figure 6

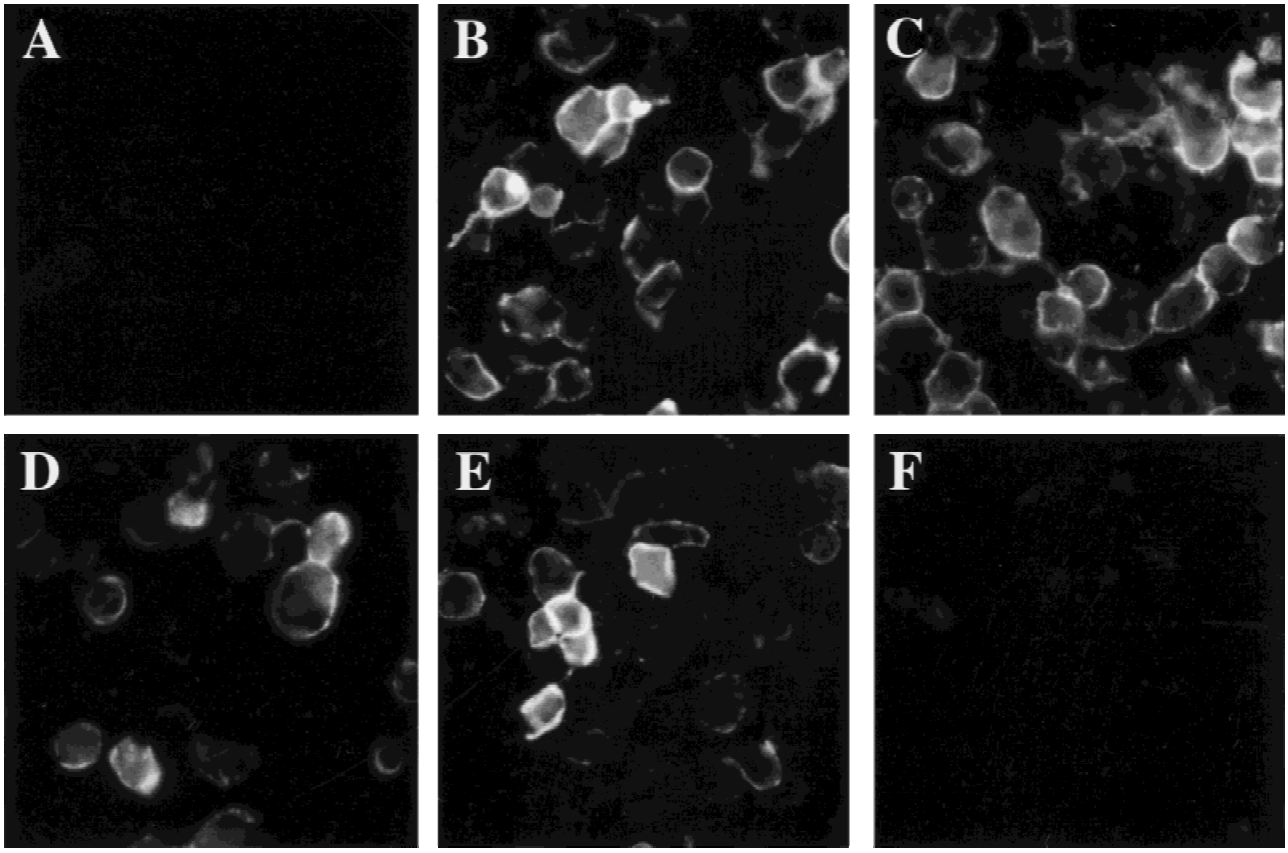

Figure 4. Surface immunofluorescence of AFP-TM chimeric proteins. 48 h after transfection, cells were fixed and incubated with AFP-specific antibody or control antibody (anti-βG) followed by FITC-conjugated second antibody. Cells viewed under fluorescence illumination were photographed. (A) Untransfected BHK cells incubated with AFP-specific antibody. (B—E), pAFP-PDGFR, pAFP-B7, pAFP-DAF, and pASGPR(Y)-AFP transfected cells incubated with AFP-specific antibody. (F) pAFP-B7 transfected cells incubated with control antibody.

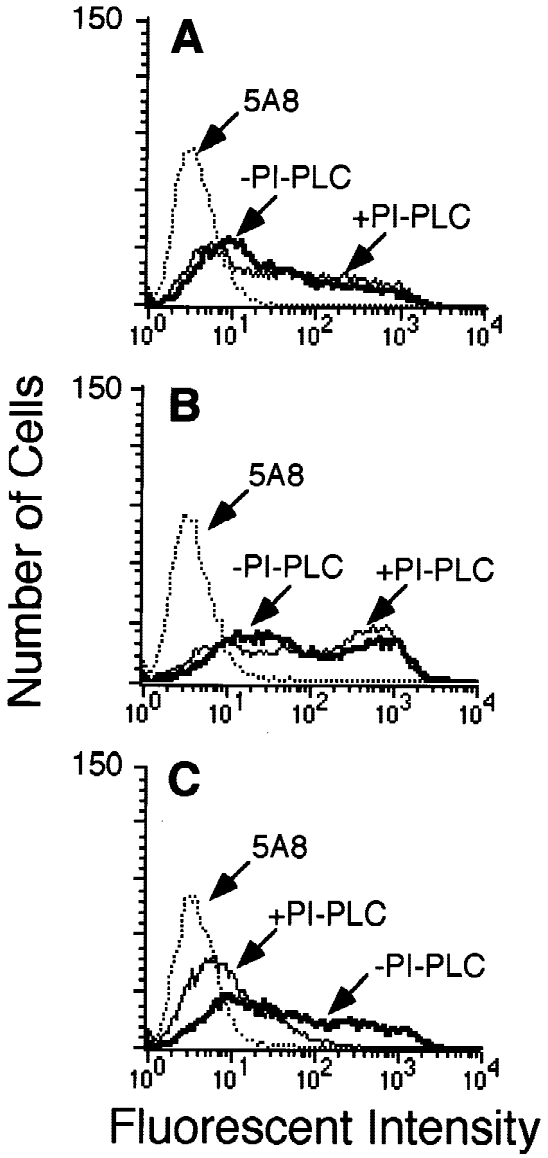

Figure 5. Surface immunofluorescence of cells after PI-PLC treatment. Cells transfected with pAFP-PDGFR (A), pAFP-B7 (B), or pAFP-DAF (C) were incubated with or without PI-PLC at 37°C for 30 min. Surface expression of AFP-TM chimeric proteins was quantified on a flow cytometer after staining with mAb 36.2 or mAb 5A8. (Thick solid lines, mAb 36.2 without PI-PLC treatment; thin solid lines, mAb 36.2 with PI-PLC treatment; dotted lines, control mAb 5A8 without PI-PLC treatment).

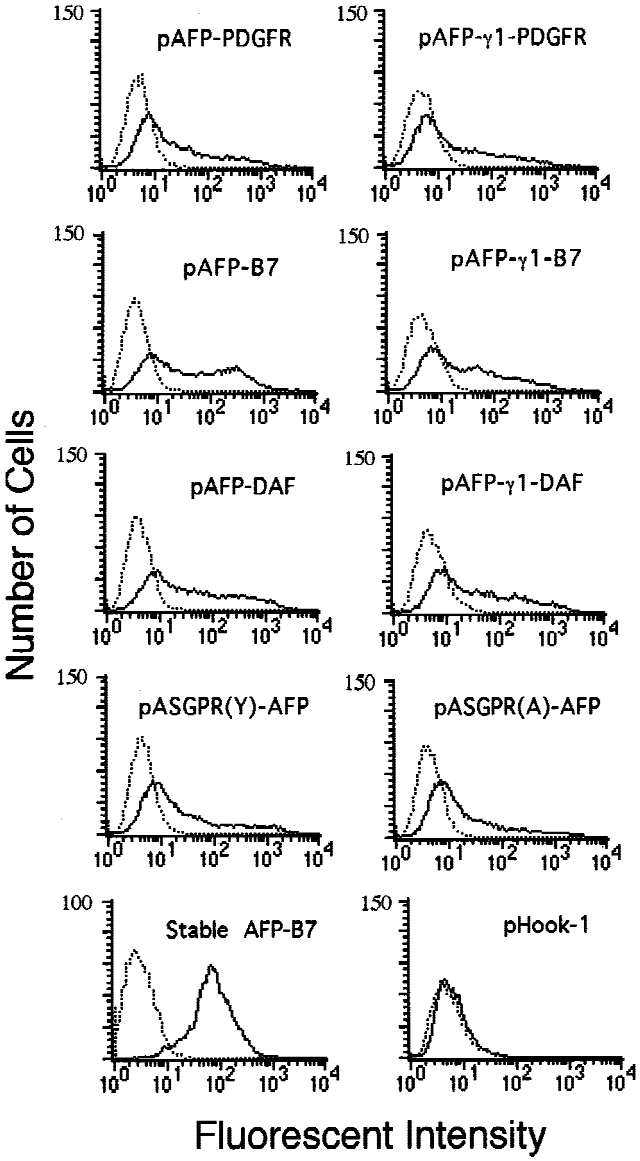

Figure 6. AFP-TM chimeric protein surface expression. Transfected cells were incubated with AFP-specific mAb 36.2 (solid lines) or control mAb (dotted lines) followed by FITC-conjugated second antibody. Stable pAFP-B7 cells were selected in G418 and cloned before analysis whereas all other cells were transient transfectants. The immunofluorescence of 10^4 live cells was determined by flow cytometry.

shows that a single cell clone isolated from the G418 resistant population expressed AFP on the surface of the plasma membrane, demonstrating that long-term expression of heterologous proteins on the cell surface can be achieved. Stable cell transfectants expressing AFP-DAF, AFP-PDGFR, ASGPR(Y)-AFP, and ASGPR(A)-AFP were also successfully established (results not shown).

DISCUSSION

The major goals of this study were to analyze the ability of different transmembrane domains to target proteins to the plasma membrane of cells and determine whether disulfide-linked dimers could be expressed on the cell surface. Human AFP, a 590 amino acid secretory protein with a molecular weight of 69,000 (Morinaga et al., 1983), was employed as a reporter protein in our study because (1) it is secreted as a monomer which should facilitate interpretation of expression studies and prevent complications associated with multisubunit proteins, (2) AFP contains a single glycosylation site that may be employed as a marker of protein maturation (Morinaga et al., 1983), (3) AFP appears to be non-toxic to cells (Chen et al., 1997) and is relatively inert compare to most growth factor, and (4) we have developed immunological reagents that can be utilized for the detec-

Table II. Mean surface fluorescence of chimeric proteins.

Chimeric protein	Mean relative fluorescence (% AFP-PDGFR)[a]
AFP-PDGFR	100
AFP-γ1-PDGFR	110 ± 15
AFP-B7	300 ± 33**
AFP-γ1-B7	135 ± 7.1*
AFP-DAF	240 ± 40*
AFP-γ1-DAF	200 ± 24*
ASGPR(Y)-AFP	70 ± 9.6*
ASGPR(A)-AFP	70 ± 13

[a]The geometric mean fluorescence of cells transfected with AFP-TM transgenes, normalized to cells transfected with AFP-PDGFR. Results represent mean values from 4 independent experiments. Significant differences in the surface fluorescence compared to AFP-PDGFR are indicated (*, $p \leq 0.05$; **, $p \leq 0.005$).

tion of AFP. To control for transfection efficiency and allow valid comparison of the different transgenes, cells were co-transfected with a reporter plasmid to express βG in the cytosol of cells; transgene expression levels were then normalized to βG expression. We found that the efficiency of cell surface expression depended on the choice of TM. In addition, introduction of the γ1 domain allowed efficient dimer formation at the expense of reduced surface expression in some cases.

The commercial vector, pHook-1, was employed as the backbone to construct all of the AFP-TM transgenes examined in our study. The human PDGFR TM present in pHook-1 allowed expression of high amounts of chimeric protein in BHK cells but only intermediate levels of reporter protein on the cell surface. The amino acid sequence of the PDGFR TM in pHook-1 contains a di-lysine ER retention signal (Nilsson et al., 1989) at its C-teminus (VVVISILALVVLTIISLIILIMLWQKKPR, the TM is underlined). At least 18 amino acids, however, are required between the TM and the di-lysine signal for efficient ER retention (Vincent et al., 1998). The low ratio of AFP-PDGFR surface expression to total chimeric protein production suggests that the short distance between the KKXX retention signal and the PDGFR TM may still allow partial binding of the signal to β-COP in the ER, resulting in retarded transport of the chimeric protein to the cell surface.

A chimeric protein composed of the type-II TM of the human ASGPR H1 subunit fused to the N-terminal of AFP resulted in high protein expression but poor surface accumulation. The ASGPR is composed of a complex of H1 and H2 subunits in liver cells (Bischoff et al., 1988) and transfected fibroblasts (Fuhrer et al., 1991). The human ASGPR H1 subunit has been shown to form homodimers and trimers in HepG2 cells (Lodish et al., 1992). The N-terminal 60 amino acids of the chicken ASGPR, which encompassed the cytoplasmic, TM and a small portion of the exoplasmic domain, was sufficient for receptor oligomerization (Loeb and Drickamer, 1987). These results suggest that the N-terminal cytoplasmic tail and TM of the human ASGPR H1 subunit is sufficient for receptor homo-oligomerization. The large size of AFP (69 kDa) may interfere with oligomerization of the ASGPR TM, resulting in improper protein folding, processing, or sorting, leading to reduced surface expression. Tyr5 in the cytosolic tail of the ASGPR has been implicated in receptor endocytosis upon binding to asialyglycoproteins (Fuhrer et al., 1991). The similar surface expression of ASGPR(Y)-AFP and ASGPR(A)-AFP, in which Tyr5 was mutated to alanine, however, argues against endocytosis of the chimeric protein as a major mechanism of low surface expression.

The murine B7-1 TM targeted the most monomeric AFP to the plasma membrane of cells even though the expression of total AFP-B7 protein was significantly lower than AFP-PDGFR. The efficient routing of AFP-B7 to the cell surface may reflect the absence of ER and golgi retention signals (Rothman and Wieland, 1996) in the cytosolic tail of B7-1. The lack of B7-1 internalization (June et al., 1994) may also contribute to the high surface expression of AFP-B7. The low levels of total cellular AFP-B7 protein, however, indicates that some chimeric protein was degraded, possibly due to TM-mediated conformational restraints in the folding of the normally soluble AFP protein. The significantly lower surface expression of dimerized AFP-γ1-B7 compared to AFP-B7 monomer is also likely due to conformational restraints on protein folding or transport. Further increases in the expression and surface targeting of B7-1 TM chimeric proteins may be possible by introducing a flexible linker between the extracellular and TM domains to diminish restrictions on protein folding. The expression of AFP-B7 on the surface of cells selected for stable expression of the pAFP-B7 plasmid shows that chimeric protein expression is not toxic to cells and indicates that long-term expression of heterologous proteins on the cell membrane can be achieved.

Appendage of the C-terminal extension of DAF to AFP resulted in the attachment of a GPI anchor to AFP. AFP-DAF was highly expressed on the cell surface although at slightly lower levels than AFP-B7. It is possible that the overexpression of AFP-DAF saturated cellular machinery required for cleavage of the C-terminal extension or attachment of the GPI-anchor. GPI-anchored proteins possess potential advantages and disadvantages compared to chimeric proteins containing integral membrane TM. The soluble C-terminal extension may place fewer constraints on the folding of chimeric proteins compared to proteins fixed to the lumen of the ER by a TM. The high surface expression of AFP-γ1-DAF compared to AFP-γ1-B7 may reflect the more physiological folding of soluble AFP-γ1-DAF dimers in the ER. GPI-anchored proteins can spontaneously release from the membrane of cells and reinsert in neighboring cells (Low, 1987). This could be useful in applications where a "bystander" effect is advantageous, for example with in vivo transduced cells. However, GPI-anchored proteins can also be cleaved and released from the cell surface by cell-associated GPI-specific phospholipase D (Metz et al., 1994). Immunoprecipitation of ^{35}S-methionine-labeled cells transfected with pAFP-DAF detected AFP in the culture

supernatant (unpublished results), indicating that AFP-DAF is released from the cell surface. GPI-anchored chimeric proteins may therefor be less desirable for applications requiring stable surface attachment such as the expression of prodrug-activating enzymes on the cell surface (Marais et al., 1997) or for tissue engineering.

Dimer formation may be useful to increase the avidity of surface-expressed peptides (Longo et al., 1997; Carrithers and Lerner, 1996) or proteins (Pepinsky et al., 1991; Schodin and Kranz, 1993), to maintain the biological activity of natural dimeric proteins (Agou et al., 1996; Zhang and Rollins, 1995; Kolbeck et al, 1994), to increase the stability of proteins (Mainfroid et al., 1996), change the substrate specificity of enzymes (Muraki et al., 1994), and allow increased cross-linking and activation of cell surface receptors (Yee et al., 1994; Ledbetter et al., 1986). Insertion of the γ1 domain between AFP and TM allowed efficient formation of disulfide-linked dimers. The γ1 domain, however, impaired the surface expression of AFP-γ1-B7 compared with the monomeric protein. Our results suggest that the GPI-anchor of DAF is preferred for the surface expression of dimers.

The vectors developed in this study may be useful for several applications. For example, tumor cells transduced with specific enzymes are susceptible to killing by antineoplastic prodrugs (Deonarain et al., 1995). Springer and colleagues recently demonstrated that carboxypeptidase G2 expressed on the surface of tumor cells as a fusion protein with the erbB2 transmembrane domain (TM) efficiently activated CMDA (4-([2-chloroethyl][2-mesyloxyethyl]amino)-benzoyl-L-glutamic acid) prodrugs (Marais et al., 1997). Cell surface expression of enzymes, in contrast to cytosolic expression, may provide potent bystander killing of non-transduced tumor cells, similar to that found in antibody-directed enzyme prodrug therapy (Ejigu Retta et al., 1996; Cheng et al., 1999). Tissue engineering research has primarily focused on the development of protein or polymer scaffolds for attachment and growth of tissues (Niklason and Langer, 1997; Bhatia et al., 1997). Expression of peptides, proteins, or antibodies on the surface of cells to create artificial receptors may allow additional control of homotypic and heterotypic cell interactions. Surface expression of antigens may also allow development of more potent vaccines. For example, the specific antibody titer against *Plasmodium falciparum* was increased by surface expression of antigens (Vijaya et al., 1988; Yang et al., 1997). Surface expression of reporter proteins may also be useful for sorting transfected cells from a population (Chesnut et al., 1996), for screening combinatorial libraries (Georgiou et al., 1997) and for basic genetic research (Moore at al., 1997). In addition, the expression of single-chain antibodies (Winberg et al., 1996) or others immunoregulatory molecules on the surface of cells may be useful for modulating specific or local immune responses.

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NOMENCLATURE

AFP	α feto-protein
ASGPR	asialoglycoprotein receptor
βG	β-glucuronidase derived from *E. coli*
DAF	decay accelerating factor
ELISA	enzyme-linked immunosorbent assay
TM	transmembrane domain
PDGFR	platelet-derived growth factor receptor
scFv	single-chain antibody
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
γ1	hinge-CH2-CH3 domain of human IgG1 heavy chain

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