Design of Transgenes for Efficient Expression of Active Chimeric Proteins on Mammalian Cells

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Abstract: Heterologous proteins expressed on the surface of cells may be useful for eliciting therapeutic responses and engineering new extracellular properties. We examined factors that control the membrane targeting of α-fetoprotein (AFP) and a single-chain antibody (scFv). Chimeric proteins were targeted to the plasma membrane by employing the transmembrane domain (TM) and cytosolic tail of murine CD8O (B7-1), the TM of the human platelet-derived growth factor receptor (PDGFR), the glycosylphosphatidylinositol anchor encoded by the C-terminal extension of decay-accelerating factor (DAF), and the TM of the H1 subunit of the human asialoglycoprotein receptor (ASGPR). AFP chimeric proteins containing the B7, DAF, ASGPR, or PDGFR targeting domains displayed half-lives of 12.2, 3.8, 2.4, and 1.6 h, respectively. The newly synthesized B7 chimera was rapidly transported and remained on the cell surface. Glycosylphosphatidylinositol-anchored chimeras reached the surface more slowly and significant amounts were released into the culture medium. PDGFR TM chimeras were rapidly degraded, whereas ASGPR chimeras were retained in the endoplasmic reticulum (ER). The surface expression of both AFP and scFv chimeric proteins followed the order (highest to lowest) of B7 > DAF >> PDGFR. Introduction of a dimerization domain (hinge—CH_2—CH_3 region of human IgG1) between scFv and TM dramatically reduced cleavage of the chimeric protein, increased surface expression, and produced biologically active scFv. Our results indicate that transgenes designed for the expression of active scFv on cells should incorporate a TM that does not undergo endocytosis, include an intact cytoplasmic domain, and possess a spacer to reduce cleavage and retain biological activity. © 2001 John Wiley & Sons, Inc. *Biotechnol Bioeng* **73:** 313–323, 2001.

Keywords: transmembrane domain; spacer; surface expression; chimeric protein; single-chain antibody

INTRODUCTION

Expression of heterologous proteins on the surface of specific target cells may be useful for the therapy of diseases. Surface expression of proteins can decrease systemic side effects and efficiently elicit the biological activities of enzymes, single-chain antibodies (scFv), and cytokines. Several applications employing the surface expression of proteins have recently been developed. We have recently shown that anti-CD3 scFv expressed on the surface of tumor cells can activate naive T cells to kill the genetically modified tumor cells (Liao et al., 2000). In addidon, membrane-bound anti-CD28 scFv molecules with (de Ines et al., 1999) or without (Hayden et al., 1996; Winberg et al., 1996) membrane-bound anti-CD3 scFv generated signals to activate T cells. A surface scFv against CTLA-4 has recently been shown to attenuate the response of activated T cells (Griffin et al., 2000).

Enzymes have been expressed on the surface of mammalian cells for various applications. Carboxypeptidase G2 was recently fused to the TM of c-*erb* B2 for expression on the surface of tumor cells (Marais et al., 1997). Breast carcinoma cells expressing cytosolic carboxypeptidase G2 were only slightly sensitive to a prodrug that can be converted to an antineoplastic agent by carboxypeptidase G2 because the prodrug did not readily enter the cells. In contrast, cells that expressed surface-tethered carboxypeptidase G2 were sensitive to prodrug (Niculescu Duvaz et al., 1998, 1999). Surface expression of enzymes, in contrast to cytosolic expression, should provide bystander killing of non-transfected tumor cells, similar to that found in antibody-directed enzyme prodrug therapy (Cheng et al., 1999). Membrane-anchored β-lactamase (Moore et al., 1997) and neomycin phosphotransferase (Mohler and Blau, 1994) have been engineered and shown to retain enzymatic activity. A novel membrane-bound form of GM-CSF in which this cytokine was fused to the TM of human platelet-derived growth factor receptor (PDGFR) retained biological activity, and elicited protective antitumor immunity (Soo Hoo et al., 1999). A membrane-bound M-CSF precursor produced superior antitumor activity compared with secreted M-CSF (Graf et al., 1999). The N-terminus of the CH_2 and CH_3 region of the human IgG1 region has also been fused to the type II TM of the human transferrin receptor. Membrane-bound Fc was shown to activate superoxide produc-

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tion by macrophages (Stabila et al., 1998). Expression of heterologous or engineered proteins on the surface of cells may therefore represent a rich source for the development of novel biotechnology and therapeutic applications.

A major hurdle to the expression of heterologous proteins on cells is the difficulty of achieving high expression levels. Surface expression of chimeric proteins has ranged from almost undetectable levels (Chesnut et al., 1996; de Ines et al., 1999; Moritz and Groner, 1995; Rode et al., 1996) to high levels (Brocker et al., 1993; Hayden et al.. 1996; Liao et al., 2000; Roberts et al., 1994), but the factors resulting in efficient surface expression have not been characterized. We have generated a panel of chimeric proteins composed of α-fetoprotein (AFP; Chou et al., 1999) or scFv (Liao et al., 2000) fused to different TM. In this study we investigate the mechanisms responsible for the differential expression of these chimeric proteins on mammalian cells. The results of this study may aid in the rational design of chimeric proteins that are efficiently expressed on cells in a biologically active form.

EXPERIMENTAL PROCEDURES

Reagents

Monoclonal antibody (MAb) S5A8 against 38C13 lymphoma cells was kindly provided by Dr. Mi-Hua Tao (Academia Sinica, Taipei, Taiwan). Anti-HA MAb (12CA5, against an epitope of the hemagglutinin protein [HA] of human influenza virus) and anti-c-*myc* MAb (9E10, against the EQKLISEEDL epitope derived from the human c-*myc* protein) were purchased from Boehringer Mannheim (Mannheim, Germany). Polyclonal rabbit serum against AFP and anti-AFP MAb 36.2 were produced as previously described (Chou et al., 1999). Anti-COP (Sigma, St. Louis, MO) and anti-BiP (Affinity Bioreagents, Golden, CO) antibodies were used for intracellular fluorescence immunostaining. FITC-conjugated goat anti-mouse IgG $(Fab')_2$ and FITC-conjugated goat anti-rabbit IgG, IgA, and IgM were from Organon Teknika (Turnhout, Belgium).

Cell Lines and Tissue Culture

BHK-21 (baby hamster kidney) cells were obtained from Veteran's General Hospital (Taipei, Taiwan). BALB/3T3 cells were obtained from the National Health Research Institutes Cell Bank (Hsin Chu, Taiwan). Cells were cultured in Dulbecco's minimal essential medium (Sigma) supplemented with 10% bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in an atmosphere of 5% CO_2.

Plasmid Construction

The construction of p2C11-PDGFR, p2C11-γ1-PDGFR, p2C11-B7, p2C11-γ1-B7, and pAFP-TM transgenes has been described previously (Chou et al., 1999; Liao et al., 2000). To create pAFP-PDGFR-AA, the pAFP-PDGFR construct was mutated using the Quick-Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with primer P1 (ATG CTT TGG CAG <u>GCG GCG</u> CCA CGT TAG GCG GCC) and primer P2 (GGC CGC CTA ACG TGG <u>CGC CGC</u> CTG CCA AAG CAT) to change KK (lysine^{558} and lysine^{559}) to AA (underlined) in the truncated cytosolic tail of the PDGFR TM (AVGQDTQEVIVVPH-SLPFK <u>VVVISAILALVVLTIISLIILIMLWQ</u>**KK**PR, TM is underlined). The 2C11scFv cDNA fragment in p2C11-PDGFR was excised with *Sfi*I and *Sal*I restriction enzymes and subcloned into pAFP-DAF in place of the AFP cDNA to obtain p2C11-DAF. A *Sal*I fragment containing the hinge, CH_2, and CH_3 region (γl) of the human IgG1 heavy chain from pAFP-γ1-B7 was inserted into the unique *Sal*I site in p2C11-DAF to produce p2C11-γ1-DAF. All transgenes were fully sequenced.

Transfection of Transgenes

A total of 2.5×10^5 BHK or BALB/3T3 cells were cultured overnight in six-well plates before transfection with 3 μg plasmid and 10 μL lipofectamine according to the manufacturer's instructions (Gibco Laboratories, Grand Island, NY). BALB/3T3 cells transfected with pcDNA3, p2C11-B7, or p2C11-γ1-B7 and BHK cells transfected with AFP-TM transgenes were also selected in 0.5 mg/mL G418 (Calbiochem, San Diego, CA) for 2 weeks to produce stable transfectants.

Flow Cytometer Analysis

A total of 5×10^5 transfected cells were washed and suspended in DMEM containing 0.5% bovine serum and first antibody for 60 min at 4°C. Cells then were washed and incubated with FITC-conjugated second antibody for 60 min, and subsequently washed and suspended in PBS containing 5 μg/mL propidium iodide before the surface immunofluorescence of 10^4 viable cells was measured with a FACScaliber flow cytometer (Becton Dickinson, Mountain View, CA). Dead cells, identified by red propidium iodide fluorescence, were gated out. Fluorescence intensities were analyzed with CELLQUEST software (Becton Dickinson).

Internalization of AFP-TM Chimeric Proteins from the Plasma Membrane

Stable transfectants were incubated with MAb AFP 36.2 (5 μg/mL) or control antibody MAb 5A8 (5 μg/mL) on ice for 60 min. The cells were then split into three fractions. The first fraction was incubated at 4°C, whereas the other two were incubated at 37°C for 30 min or 60 min, respectively. Cells were then placed on ice for 30 min before FITC-conjugated goat anti-mouse IgG was added for 60 min. The

cells were washed and the surface fluorescence of the cells was measured by flow cytometry.

Fluorescence Immunostaining of Intracellular Chimeric Proteins

One day after 5×10^4 cells were seeded on a glass coverslip, the cells were washed with PBS, fixed with 4% *p*-formaldehyde, and permeabilized with 1% Triton X-l00 in PBS at room temperature for 10 min. Cells were washed with PBS and poly-anti-AFP (1:500), anti-BiP (8 μg/mL), or mouse anti-COP (1:500) antibodies were added for 60 min. Cells were further washed extensively in PBS before FITC-conjugated second antibodies (1:500) were added for 60 min. Finally, cells were washed in PBS, mounted on slides in 50% glycerol/PBS, and viewed under a confocal microscope.

Pulse Chase and Immunoprecipitation

A total of 5×10^6 stable transfectants were labeled with 100 μCi/mL Pro-mix L-[^{35}S] in vitro cell labeling mix (Amersham Pharmacia Biotech, Piscataway, NJ) in cysteine- and methionine-free DMEM (Sigma) for 3 h at 37°C. Cells were washed with PBS and harvested by treatment with vercene (0.6 m*M* ethylene-diamine tetraacetic acid [EDTA], 140 m*M* NaCl, and 5.4 m*M* KCl [pH 7.4]) or incubated in DMEM supplemented with 100 m*M* methionine and 10% bovine serum for 2 to 8 h. Cells were washed three times in 1 mL ice-cold buffer (10 m*M* Tris-HCl [pH 7.4] and 0.125 *M* NaCl) and suspended for 2 h in 300 μL of ice-cold lysis buffer (AFP-B7: 20 m*M* sodium phosphate [pH 7.4] 0.68 *M* sucrose, 0.15 *M* NaCl, 5 m*M* EDTA, 5 m*M* sodium pyrophosphate, 1 mg/mL BSA, 1% Triton X-100, and protease inhibitor cocktail [Boehringer Mannheim]; AFP-PDGFR and ASGPR[Y]-AFP: 0.5 *M* NaCl, 0.02 *M* Tris-HCl [7.4], 0.5% Triton X-100, protease inhibitor cocktail, and 1 m*M* DTT; AFP-DAF: 150 m*M* NaCl, 10 m*M* Tris-HCl [pH 7.4], 2% Triton X-114, and protease inhibitor cocktail). Insoluble proteins were removed by centrifugation at 100,000 *g* for 45 min. Under these conditions, >90% of chimeric protein was solubilized as determined by immunoblotting. Samples were precleared twice with 2 μL normal rabbit serum before 30 μL of Protein A Sepharose CL-4B gel (Pharmacia, Uppsala, Sweden) was added for 1 h to reduce nonspecific binding. Five micrograms of rabbit anti-AFP Ab (IgG fraction) was added for 1 h before 50 μL of Protein A Sepharose CL-4B Gel was added overnight. The Protein A Gel was washed once with 1 mL of wash buffer (100 m*M* Tris-HCl [pH 8.0], 1% Triton X-100, 0.2% sodium deoxycholate, 10 m*M* EDTA, 1 mg/mL BSA, 0.5 *M* NaCl, and protease inhibitor cocktail) and five times with 1 mL of wash buffer containing 0.125 *M* NaCl and 0.05% sodium dodecylsulfate (SDS). The Protein A Gel was boiled in 20 μL of reducing SDS-polyacrylamide gel electrophoresis (PAGE) buffer before electrophoresis on a 10% SDS-PAGE. The polyacrylamide gel was treated with En^{3}hance (NEN, Life Science, Boston, MA) following the manufacturer's instructions before drying. Gels were exposed to Kodak X-ray film for 1 to 3 days. Band intensities were measured by scanning the X-ray films with a computing densitometer (Molecular Dynamics, Mountain View, CA).

Protein Sorting Rate

Next, 10^6 stable baby hamster kidney (BHK) transfectants were labeled with 150 μCi of Pro-mix L-[^{35}S] for 30 min before the cells were chased for 0 to 6 h. The cells were then washed three times with physiological buffer (120 m*M* NaCl, 4.4 m*M* KH_2PO_4, and 20.6 m*M* Na_2HPO_4) and surface proteins were biotinylated with 1 mg sulfo-NHS-biotin (Pierce, Rockford, IL) at 4°C for 30 min. The cells were washed with physiological buffer three times before they were harvested by vercence treatment and lysed in lysis buffer. After immunoprecipitation with poly-anti-AFP Ab, Protein A Gel-bound AFP-TM chimeric proteins were eluted by boiling in 50 μL of reducing SDS-PAGE buffer. The supernatant was incubated with 50 μL streptavidin-agarose beads (Pierce) overnight. Supernatant (unbound AFP-TM chimeric protein) was collected and streptavidin-agarose beads were washed in 1 mL of wash buffer and 1 mL wash buffer containing 0.125 *M* NaCl and 0.05% SDS. Bound biotinylated AFP-TM chimeric proteins were eluted by boiling in reducing SDS-PAGE buffer. Total proteins (streptavidin-agarose bound and unbound fractions) were separated on 10% SDS-PAGE.

Immunoprecipitation of AFP-DAF from Culture Medium

A total of 7×10^5 cells were labeled with 300 μCi of Pro-mix L [^{35}S] for 10 h and then chased for 15 h. Culture medium was collected and the cells were lysed with lysis buffer. The cell lysate and culture medium were centrifuged at 100,000 *g* for 45 min before the supernatant was immunoprecipitated with anti-AFP antibody.

Immunoblotting of Chimeric Proteins

BHK cells were harvested 48 h after transfection with 2C11scFv-TM transgenes. The cells were washed in PBS and 5×10^4 cells were boiled in reducing SDS-PAGE buffer. Proteins were electrophoresed on 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, and 0.5 m*M* 2-mercaptoethanol) by capillary diffusion. The blots were blocked with 5% skim milk and probed with 5 μg/mL anti-HA or anti-*myc* antibodies. The blots were washed extensively, incubated with HRP-conjugated goat anti-mouse IgG $(Fab')_2$ (l:2000), and washed and visualized by ECL detection according to the manufacturer's instructions (Pierce).

Splenocyte Proliferation

BALB/3T3 cells were transfected with pcDNA3, p2C11-B7, or p2C11-γ1-B7 48 h before they were lethally irradiated and serially diluted into round-bottom 96-well plates. Splenocytes from BALB/c mice (5×10^5 cells/well in RPMI-1640 supplemented with 10% FBS and 10 m*M* HEPES) were added to wells for 2 days. One microcurie of [^{3}H]-thymidine was added to wells for 16 h, cells were harvested, and the radioactivity was measured on a Top-Count Microplate Scintillation Counter (Packard, Meriden, CT).

Statistical Analysis

Statistical significance of differences between mean values was estimated with the shareware program, SCHOOLSTAT (White Ant Occasional Publishing, West Melbourne, Australia), using the independent *t*-test for unequal variances.

RESULTS

Surface Display of Chimeric AFP-TM Proteins

AFP was employed as a reporter protein to investigate the ability of different TM to direct chimeric proteins to the surface of mammalian cells. The TM and cytosolic tail of murine B7-1 and the GPI-anchor signal sequence from decay-accelerating factor (DAF) allowed high surface expression, whereas the TM of the human platelet-derived growth factor receptor (PDGFR) or the human asialoglycoprotein receptor (ASGPR) H1 subunit produced low surface expression of AFP (Fig. 1). Mutation of a tyrosine residue (Tyr5) in the cytoplasmic tail of the ASGPR H1 subunit to alanine (ASGPR[A]-AFP) did not increase the surface expression of chimeric protein (Fig. 1). Accumulation of chimeric proteins on the cell surface did not correlate with the level of protein expression (Chou et al., 1999). These results indicate that the TM can dramatically alter surface expression of chimeric proteins.

Internalization of ASGPR(Y)-AFP and ASGPR(A)-AFP from the Cell Surface

Because the ASGPR can undergo endocytosis, the internalization of ASGPR(Y)-AFP and ASGPR(A)-AFP from the plasma membrane was examined. AFP-B7 surface expression was relatively stable with 76% of the chimeric protein remaining on the surface after 1 h at 37°C (Table I). In contrast, only 29% of ASGPR(Y)-AFP was retained on the plasma membrane after 1 h. ASGPR(A)-AFP was internalized at a slower rate, with 42% of the chimeric protein remaining on the cell surface after 1 h. Comparison of the fluorescent intensities of antibody-coated cells after 1 h at 37°C showed that the surface expression of AFP-B7 was greatest, followed by ASGPR(A)-AFP and ASGPR(Y)-AFP (Table I).

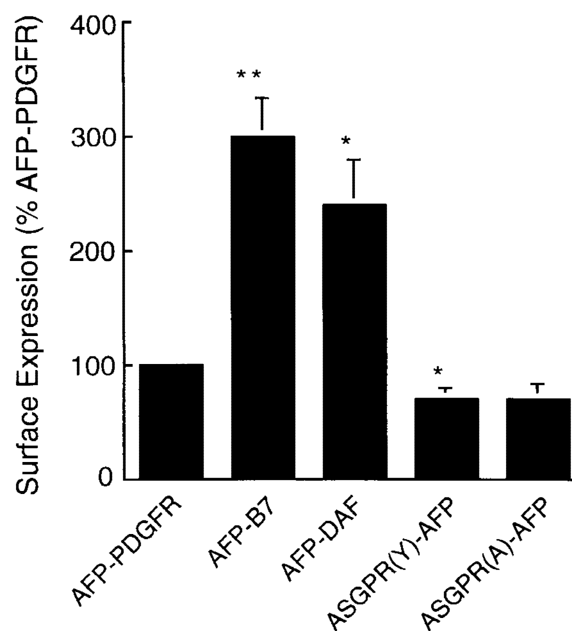

Figure 1. Mean surface fluorescence of chimeric proteins. BHK cells were transfected with AFP-TM transgenes and 48 h later the surface immunofluorescence of 10,000 cells was determined. Results show mean fluorescent intensities of three independent experiments relative to AFP-PDGFR (100%). Significant differences between surface expression compared with AFP-PDGFR are indicated: $*P \leq 0.05$; $**P \leq 0.005$. Bars indicate SE.

KKXX Signal at C-Terminal of PDGFR TM Is Not Involved in ER Retention of AFP-PDGFR

To determine whether the low surface expression of AFP-PDGFR was related to ER retention by a KKXX motif located at the C-terminus of the truncated cytosolic tail of the PDGFR TM employed in this study, KK was mutated to AA. Flow-cytometric analysis of BHK cells transfected with these constructs, however, did not reveal a statistically significant difference between their surface expression (Fig. 2).

Localization of AFP-TM Chimeric Proteins in Transfected Cells

The cellular location of AFP-TM chimeric proteins was examined in fixed and permeabilized transfected BHK cells by immunofluorescence detection under a confocal micro-

Table I. Internalization of AFP-TM chimeric proteins.

Cell line	Surface expression[a] (% initial)		Mean fluorescence intensity[b] at 60 min
	30 min	60 min	
AFP-B7	84	76	65
ASGPR(Y)-AFP	45	29	2.0
ASGPR(A)-AFP	54	42	9.8

[a]Surface fluorescence after 30 or 60 min at 37°C compared with surface fluorescence of cells maintained at 4°C.

[b]Mean fluorescent intensity of cells after 60 min at 37°C.

scope. Figure 3 shows that AFP-PDGFR (A), AFP-B7 (B), and AFP-DAF (C) were present in the Golgi apparatus as shown by the similarity of their staining pattern to β-COP (F), a resident Golgi protein. AFP-B7 (B) and AFP-DAF (C) were also evident on the plasma membrane. In contrast, ASGPR (Y)-AFP (D) was primarily present in the endoplasmic reticulum as shown by the similar localization of BiP (E), a resident ER protein.

AFP-TM Chimeric Protein Half-Lives

To determine whether the different TM influenced the half-lives of the AFP-TM chimeric proteins, stable BHK transfectants were pulsed with ^{35}S-[Met + Cys] and chased for 0 to 8 h. AFP-TM chimeric proteins were solubilized and precipitated by anti-AFP polyclonal antibodies. Specific bands on X-ray film (Fig. 4A) were scanned on a densitometer and the intensities were plotted versus time. No significant band was present in lane 6 in which untransfected BHK cells were immunoprecipitated with anti-AFP antibodies, showing that imrnunoprecipitation was specific. All chimeric proteins exhibited apparent first-order half-lives (Fig. 4B). The half-lives of AFP-PDGFR, AFP-B7, AFP-DAF, and ASGPR(Y)-AFP were 1.6, 12.2, 3.8, and 2.4 h, respectively.

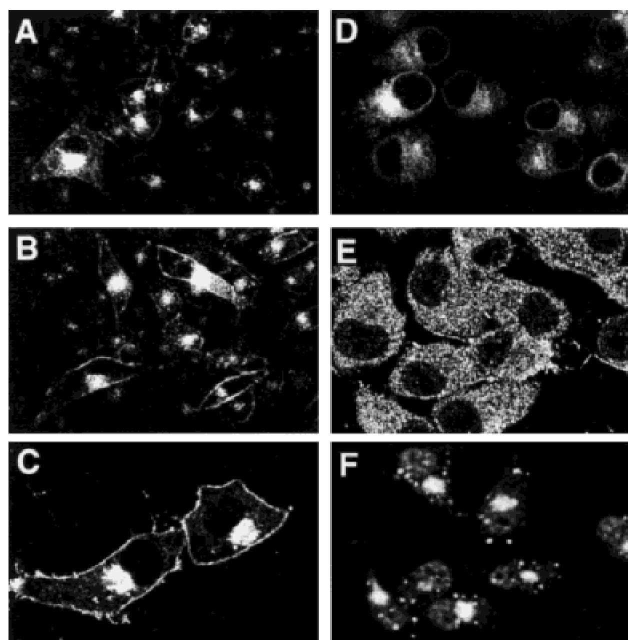

Figure 3. Intracellular localization of AFP-TM chimeric proteins. Stable AFP-PDGFR (A), AFP-B7 (B), AFP-DAF (C), and ASGPR(Y)-AFP (D) transfectants were fixed, permeabilized, and stained with anti-AFP antibody and FITC-conjugated second antibody. The stable ASGPR(Y)-AFP transfectant was also stained with anti-BiP (E) or anti-COP (F) antibody. The cells were viewed under a confocal microscope.

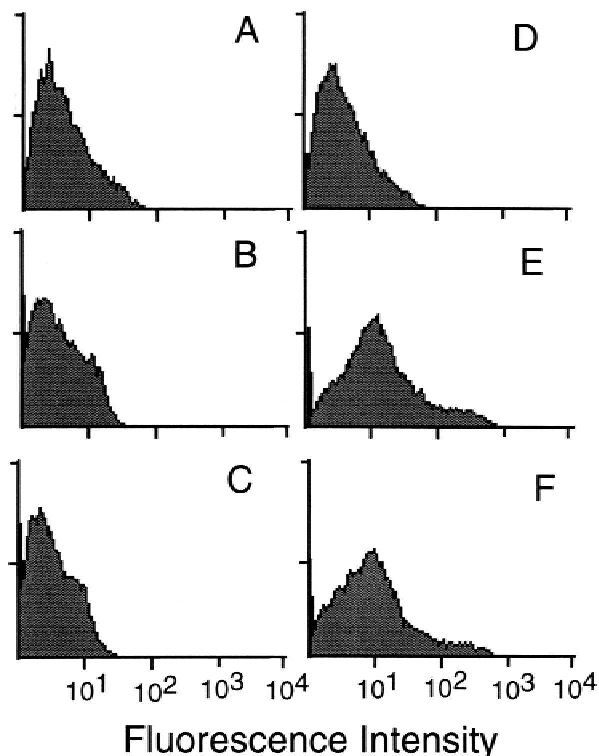

Figure 2. Surface expression of AFP-PDGFR-AA. Untransfected BHK cells (A, D) or BHK cells that were transfected with pAFP-PDGFR (B, E) or pAFP-PDGFR-AA (C, F) were stained with control MAb S5A8 (A–C) or MAb 36.2 (D–F), followed by FITC-conjugated second antibody. Cell surface expression of 10,000 cells was determined on a flow cytometer.

Sorting Rate of Chimeric Proteins to Plasma Membrane

To investigate the rate that chimeric proteins were sorted to the plasma membrane, AFP-TM transfectants were labeled with ^{35}S-[Met + Cys] for 30 min and cell surface proteins were then biotinylated after different chase periods. AFP-TM chimeric proteins on the cell surface were separated from the intracellular chimeric proteins by binding to streptavidin-agarose beads. The upper part of each panel in Figure 5 shows surface chimeric proteins, whereas the lower panel shows intracellular AFP-TM proteins. AFP-PDGFR was slowly transported to the cell surface; surface AFP was only detected after a 2-h chase. Although surface expression of AFP-PDGFR was minimal, this chimeric protein did not accumulate intracellularly, suggesting that rapid intercellular degradation of AFP-PDGFR contributed to low surface expression. In contrast, the majority of newly translated AFP-B7 was transported to the cell surface within 1 h. In addition, substantial amounts of AFP-B7 remained on the plasma membrane for at least 6 h. AFP-DAF was transported to the cell surface more slowly than AFP-B7, but the majority of newly synthesized chimeric protein reached the surface within 2 h. Surface expression of AFP-DAF, however, decayed rapidly, with only marginal AFP-DAF remaining on the plasma membrane 6 h after translation. A minor fraction of ASGPR(Y)-AFP was transported rapidly to the cell surface within 1 h of translation, but the majority of this chimeric protein was retained and degraded intracellularly. No band was apparent when untransfected BHK

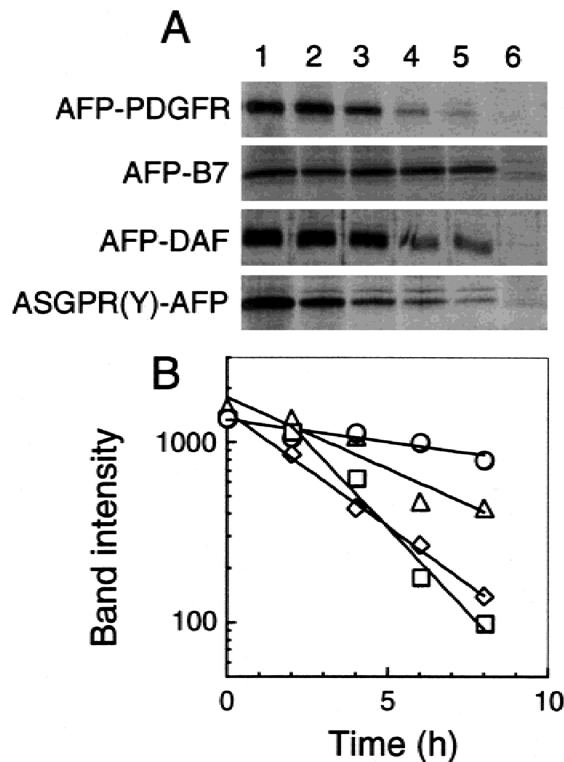

Figure 4. Half-lives of AFP-TM chimeric proteins. (A) Stable BHK transfectants were pulsed with ^{35}S-[Met + Cys] for 3 h and chased for 0 h (lane 1), 1 h (lane 2), 2 h (lane 3), 4 h (lane 4), or 6 h (lane 5) before solubilized proteins were immunoprecipitated with AFP-specific antibody. Untransfected BHK cells were also labeled, chased for 4 h, and immunoprecipitated (lane 6). (B) Specific bands on X-ray film were scanned on a densitometer and intensities were quantified by ImageQuant software. Results show the band intensities of AFP-PDGFR (□), AFP-B7 (○), AFP-DAF (△), and ASGPR(y)-AFP (◇) versus time.

cells were processed in an identical fashion (lane 1), showing that immunoprecipitation reactions were specific.

AFP-DAF Is Released into the Culture Medium

The stability of AFP-DAF and AFP-B7 on the cell surface was examined by labeling stable AFP-B7 and AFP-DAF transfectants and untransfected BHK cells with ^{35}S-[Met + Cys] for 10 h and then chasing overnight in methionine-containing medium. Immunoprecipitation of the culture medium with anti-AFP antibody revealed specific bands for AFP-B7 and AFP-DAF (Fig. 6, lanes 2 and 3). No band was visible in lane 1 in which the culture medium of untransfected BHK cells was immunoprecipitated. AFP-B7 and AFP-DAF could also be immunoprecipitated from cell lysates (Fig. 6, lanes 5 and 6). The molecular weights of AFP-B7 and AFP-DAF in the culture medium (lanes 2 and 3) were smaller than the same proteins immunoprecipitated from total cell lysates (lanes 5 and 6), indicating that the release of these chimeric proteins into the culture medium was due to cleavage at a site between AFP and the B7 TM GPI anchors. The amount of AFP-DAF in the culture medium was about 2.8-fold greater than the amount of AFP-B7 released into the medium.

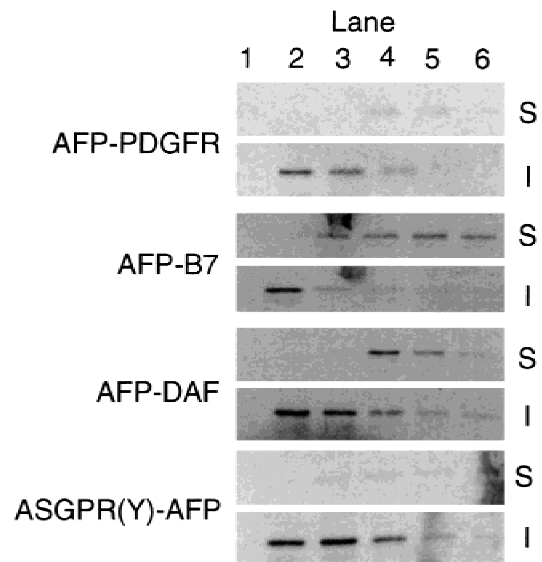

Figure 5. AFP-TM chimeric protein sorting rate is TM-dependent. BHK cells (lane 1) or stable BHK transfectants (lanes 2–6) were pulsed with ^{35}S-[Met + Cys] for 30 min and surface proteins were biotinylated after 4 h (lane 1), 0 h (lane 2), 1 h (lane 3), 2 h (lane 4), 4 h (lane 5), or 6 h (lane 6). Solubilized AFP-TM proteins were immunoprecipitated with anti-AFP antibody and surface AFP chimeric protein was separated by binding to streptavidin agarose beads. AFP-TM chimeric proteins were electrophoresed on a 10% reducing SDS-PAGE and exposed on X-ray film. The upper part of each panel shows the biotinylated surface chimeric protein (S), whereas the lower part of each panel shows intracellular chimeric protein (I).

Surface Display of Chimeric 2C11-TM Proteins

To examine the generality of the differential surface expression produced by different TM, the cDNA of a single-chain antibody (2C11 scFv) was fused to the PDGFR TM, the GPI signal sequence of DAF, and the TM and cytosolic tail of murine B7-1. In addition, the hinge—CH_2—CH_3 region (γl) of human IgG1 was inserted between the scFv and the TM to allow expression of disulfide-linked dimers on the plasma membrane. These transgenes were transfected into

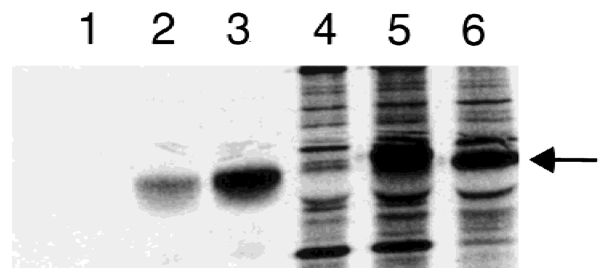

Figure 6. AFP-DAF chimeric protein is released into the culture medium. Stable transfected BHK cells were pulsed with ^{35}S-[Met + Cys] and chased overnight. Cell lysates and culture medium were immunoprecipitated with poly-anti-AFP antibody. Lane 1, untransfected BHK cell culture medium; lane 2, AFP-B7 transfectant culture medium; lane 3, AFP-DAF transfectant culture medium; lane 4, untransfected BHK cell lysate; lane 5, AFP-B7 transfectant cell lysate; lane 6, AFP-DAF transfectant cell lysate. The position of the intact AFP-DAF protein is indicated by an arrow.

BHK cells and surface expression of scFv was measured on a flow cytometer. Similar to the results found for AFP, the GPI signal sequence of DAF and the TM of murine B7-1 allowed high surface expression (Table II). Only minor surface expression of 2C11-PDGFR chimeric protein was observed. Introduction of γl between the scFv and TM produced significantly higher surface expression of all chimeric scFv compared with the monomeric proteins.

Stability of 2C11-B7 on Stable Transfectants

2C11-B7 chimeric protein was expressed on the surface of transfected BALB/3T3 cells as determined by flow cytometry 48 h after transfection (Fig. 7A). After selection of stable transfectants in G418 for 2 weeks, however, 2C11-B7 could not be detected on the cells (Fig. 7B). 2C11-γ1-B7 dimers were highly expressed on the surface of BALB/3T3 fibroblasts 48 h after transfection (Fig. 7C). In contrast to 2C11-B7, 2C11-γ1-B7 was also present on the surface of fibroblasts after cells were selected in G418 medium for 2 weeks (Fig. 7D).

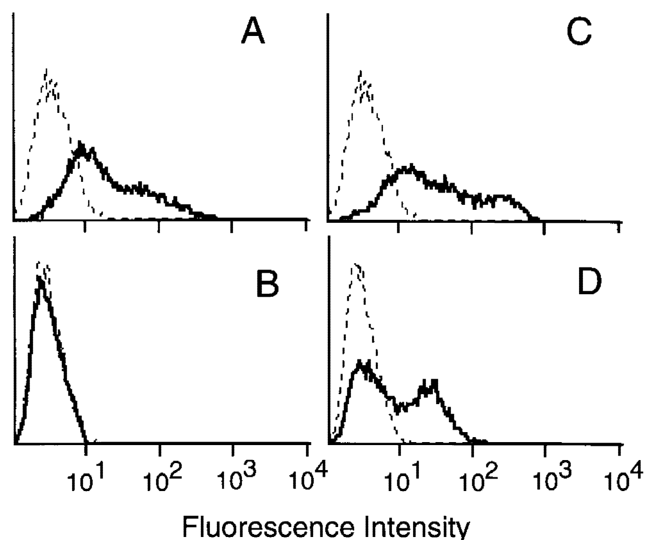

Figure 7. Surface imunofluorescence of transient and stable 2C11-B7 and 2C11-γ1-B7 transfectants. BALB/3T3 cells were transfected with pcDNA3 (dashed line), p2C11-B7 (A and B, solid line), or p2C11-γ1-B7 (C and D, solid line). The transfectants were selected in G418 for 2 weeks (B, D) or were immediately stained (A, C) with MAb 12CA5 against the HA epitope followed by FITC-conjugated goat anti-mouse IgG $(Fab')_2$ before the immunofluorescence of 10^4 viable cells was measured with a FACScaliber flow cytometer.

γ1 Spacer Reduces Cleavage of 2C11 scFv Chimeric Proteins

The cleavage of chimeric scFv proteins was examined by transfecting scFv transgenes into BHK cells and immunoblotting total cell lysates 48 h later. Figure 8A shows that only about 10% of 2C11-B7 (lane 2) possessed the expected molecular size of 34 kDa, whereas 90% was present in a degraded form with a molecular size of 26 kDa. In contrast, about 60% of 2C11-γ1-B7 (lane 1) was present in the intact form (60 kDa) with 40% present as a degradated product (52 kDa). scFv chimeric proteins were cleaved at the C-terminal end because the degradation products retained the HA epitope present at the N-terminal of the chimeric proteins. To further localize the cleavage site, chimeric proteins containing a c-*myc* epitope between the scFv and PDGFR TM were immunoblotted with anti-*myc* antibody. Figure 8B shows that, similar to B7 chimeric proteins, about 90% of 2C11-PDGFR (lane 2) was degraded, whereas 50% of 2C11-γ1-PDGFR (lane 1) was present in the intact form. The degradation products retained the *myc* epitope, indicating that the cleavage site was between the *myc* epitope and TM.

Table II. Surface display of 2C11-TM chimeric proteins.

Transgene	Surface display[a]
2C11-PDGFR	1.7 ± 0.9
2C11-γ1-PDGFR	3.6 ± 0.2[b,c]
2C11-DAF	5.2 ± 0.6[b]
2C11-γ1-DAF	8.3 ± 1.0[b,c]
2C11-B7	8.2 ± 0.34[b]
2C11-γ1-B7	12.2 ± 1.2[b–d]

[a]Mean fluorescence of cells transfected with the indicated transgene divided by the mean fluorescence of cells transfected with pcDNA3.

[b]Significantly higher than 2C11-PDGFR, $P \leq 0.05$.

[c]Significantly higher than the corresponding transgene without a γ1 domain, $P \leq 0.05$.

[d]Significantly higher than 2C11-γ1-DAF.

γ1 Spacer Allows Expression of Active scFv Chimeric Protein

BALB/3T3 cells transiently transfected with p2C11-γ1-B7 induced significant ($P \leq 0.0005$) proliferation of naive sple-

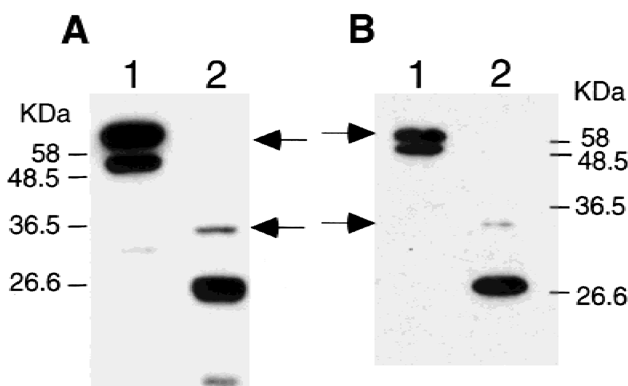

Figure 8. Degradation of membrane-bound scFv. (A) p2C11-γ1-B7 (lane 1) or p2C11-B7 (lane 2) were transfected into BHK cells. Forty-eight hours later, 5×10^4 cells were electrophoresed on an 8% reducing gel, transferred to nitrocellulose paper, and probed with anti-HA antibody. (B) BHK cells were transfected with p2C11-γ1-PDGFR (lane 1) or p2C11-PDGFR (lane 2) and 48 h later total cell lysates were electrophoresed on an 8% gel. A blot was probed with anti-*myc* antibody. The positions of the intact chimeric scFv are indicated by arrows.

nocytes compared with pcDNA3 (Fig. 9). In contrast, 2C11-B7 did not induce significant T-cell proliferation compared with pcDNA3, even though 2C11-B7 fusion protein was expressed on the surface of transfected cells (results not shown). T cells bound to BALB/3T3 cells transfected with p2C11-γ1-B7, but not to cells tansfected with p2C11-B7, indicating that 2C11-B7 was unable to bind CD3 on T cells (results not shown).

DISCUSSION

In the present study, we found that the murine B7 TM and cytosolic tail tethered the most AFP or scFv protein to the cell surface. The C-terminal extension of DAF also resulted in high expression of AFP and scFv on cells. The type II TM and cytosolic tail of the human ASGPR H1 subunit, as well as the TM and truncated cytosolic tail of PDGFR, directed only low levels of chimeric protein to the cell surface. The various TM affected ER retention, half-life, sorting rate, and degradation of the chimeric proteins. In addition, γ1 reduced cleavage of scFv chimeric proteins and enhanced their surface expression.

Previous studies have shown that mutation of Tyr5 to Ala5 in the N-terminus of the ASGPR H1 subunit can reduce its internalization (Fuhrer et al., 1991). We analyzed the internalization of ASGPR(Y)-AFP and ASGPR(A)-AFP from the plasma membrane and found that mutation of Tyr5 to Ala5 at the N-terminus of the ASGPR H1 subunit decreased the internalization of ASGPR(A)-AFP and allowed higher surface expression of ASGPR(A)-AFP relative to ASGPR(Y)-AFP (Fig. 4). In this experiment, internalization of ASGPR-AFP proteins was likely induced by crosslinking of chimeric protein with anti-AFP antibody. No difference in the expression of ASGPR(Y)-AFP and ASGPR(A)-AFP was found on cells when the proteins were not crosslinked (Fig. 1), suggesting that basal internalization of the ASGPR chimeras is low. Internalization of ASGPR(Y)-AFP, therefore, does not account for the low surface expression of this chimeric protein. Rather, low surface expression is likely due to improper folding and retention of the chimeric protein in the ER (Fig. 3D). This hypothesis is supported by the results of pulse-chase experiments that showed the majority of newly synthesized ASGPR(Y)-AFP was retained within cells (Fig. 5).

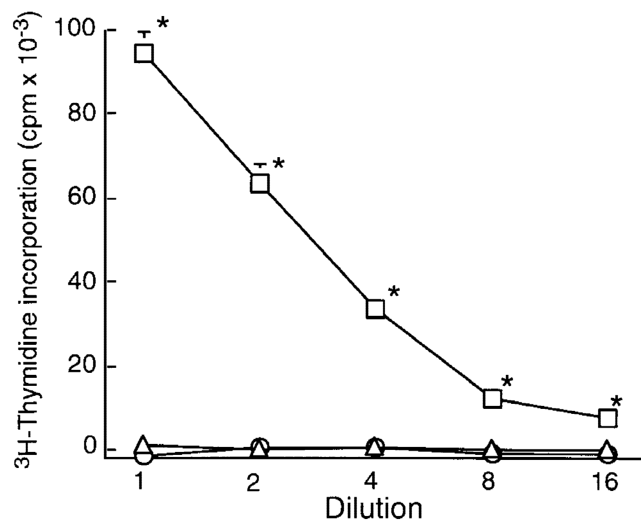

Figure 9. The bioactivity of 2C11-B7 chimeric proteins. Serial dilutions of lethally irradiated BALB/3T3 cells transfected with p2C11-γ1-B7 (□), p2C11-B7 (△), or pcDNA3 (○) were cultured with syngeneic splenocytes for 2 days. Cells were pulsed with 1 μCi of [^{3}H]-thymidine for 16 h before the nonattached cells were harvested and radioactivity was measured. Significant differences between splenocyte proliferation induced by BALB/3T3 cells transfected with p2C11-γ1-B7 or p2C11-B7 are indicated (*$P \leq 0.0005$). Bars indicate SE.

AFP-PDGFR possesses a KKXX signal at the C-terminal end of the truncated cytoplasmic tail of the PDGFR TM. Because membrane proteins with a KKXX signal located at the C-terminus can be retained in the ER (Jackson et al., 1990, 1993), we examined whether this motif caused ER retention of AFP-PDGFR by mutagenesis of KKXX to AAXX. The surface expression of AFP-PDGFR-AA, however, was not greater than that of AFP-PDGFR (Fig. 2), demonstrating that the low surface expression of AFP-PDFGR was not due to ER retention of the chimeric protein by the KKXX signal. Lack of ER retention can be attributed to the proximity of the KKXX motif to the PDGFR TM (Vijaya et al., 1988). Confocal image analysis also confirmed that AFP-PDGFR was not retained in the ER (Fig. 3A). The low surface expression of PDGFR chimeric proteins could be attributed to the short half-life (1.6 h), slow sorting to the surface (Fig. 5), and poor retention of chimeric protein on the plasma membrane (Fig. 5). Several investigators have suggested that sorting elements in the cytoplasmic domain of proteins assist in the translocation of proteins (Bremnes et al., 1994; Hobert and Carlin, 1995, Odorizzi et al., 1994; Potter et al., 1999). Thus, low surface expression of PDGFR TM-chimeric proteins may be due to the truncated cytoplasmic tail of the receptor.

Formation of GPI-anchored chimeric proteins by appendage of the C-terminal sequence of DAF to AFP and scFv allowed relatively high surface expression, although at lower levels than B7 TM chimeras. AFP-DAF required twice as long as other AFP-chimeric proteins to reach the cell surface (Fig. 5). This may reflect the time required for proteolytic processing of the C-terminal tail and synthesis of the GPI anchor. Pulse-chase experiments revealed that AFP-DAF was rapidly modulated from the plasma membrane (Fig. 5). Previous studies found that intact GPI-anchored protein can be transferred to neighboring cells in vitro and in vivo (Anderson et al., 1996; McHugh et al., 1995). The decreased molecular weight of soluble AFP-DAF (Fig. 6, lane 3), however, shows that intact AFP-DAF was not released in detectable quantities. GPI-anchored proteins can be enzymatically cleaved from the cell surface by GPI-specific phospholipase D (Metz et al., 1994). Because GPI-anchored proteins can be easily released from the cell surface, this anchor is not suitable for applications that require stable surface expression. However, GPI-fusion pro-

teins can be purified from transfected cells and subsequently incorporated into the plasma membrane of target cells (Brunschwig et al., 1995; McHugh et al., 1995, 1999). The GPI anchor may therefore be useful for ex vivo incorporation of proteins on cells; for example, to coat primary cells that are difficult to culture and transduce with a transgene.

The high levels of surface expression achieved with the chimeric proteins that were fused to the murine B7 TM and cytosolic tail can be attributed to several factors. First, the chimeric protein was not retained in the ER/Golgi as shown by the rapid sorting of AFP-B7 to the cell surface (Fig. 5). Examination of the cytoplasmic tail of B7-1 did not reveal ER/Golgi retention signals (Pfeffer and Rothman, 1987; Rothman, 1987). Second, AFP-B7 displayed a long half-life (12.2 h), indicating that the chimeric protein was stable and correctly folded. Although some AFP was released into the medium of cells transfected with pAFP-B7, this chimeric protein was stably expressed on cells for a prolonged period (Fig. 5). The major difference between the PDGFR and B7-1 constructs was the presence of an intact cytoplasmic domain in the B7-1 chimeras. As previously mentioned, elements present in the cytoplasmic domain of TM proteins may be important for efficient translocation of proteins to the cell surface (Bremnes et al., 1994; Hobert and Carlin, 1995; Odorizzi et al., 1994; Potter et al., 1999). The intact cytoplasmic domain of B7-1 may thus be required to allow high expression of heterologous proteins on the plasma membrane. This suggestion is supported by the observation that chimeric proteins that exhibit high surface expression usually contain an intact cytoplasmic tail (Brocker et al., 1993; Hayden et al., 1996; Liao et al., 2000; Roberts et al., 1994). In contrast, truncation of the cytoplasmic domain often results in low surface expression (Alvarez Vallina and Hawkins, 1996; Chesnut et al., 1996; de Ines et al., 1999; Liao et al., 2000; Rode et al., 1996). Definitive confirmation of the role of an intact cytoplasmic tail on the efficient surface expression of B7 chimeric proteins will require swapping the B7 and PDGFR cytosolic tails.

We previously found that AFP-TM chimeric proteins were susceptible to proteolytic cleavage (Chou et al., 1999). In the present study, we found that scFv–TM proteins were also cleaved at a site between the scFv and TM based on immunoblotting analysis of 2C11-PDGFR (Fig. 8B). de Ines and colleagues also found that chimeric proteins composed of an scFv and the interleukin-6 (IL-6) TM were susceptible to degradation at a site near the IL-6 TM, resulting in undetectable surface expression of chimeric protein (de Ines et al., 1999). We found that introduction of γ1 between the scFv and TM dramatically reduced cleavage (Fig. 8), allowing increased expression of chimeric protein on the cell surface. This was especially evident after transfected cells were drug-selected for stable expression; 2C11-γ1-B7 was expressed on stable transfectants, whereas 2C11-B7 could not be detected on cells (Fig. 7). There are several possible mechanisms that may explain how the γ1 reduces degradation of the chimeric scFv. First, carbohydrate chains present in the CH_2 domain of γ1 may sterically hinder protease binding to the chimeric protein. Removal of carbohydrate chains often increases the degradation of glycoproteins (de Virgilio et al., 1999; McCracken and Brodsky, 1996; Toki et al., 1997; Wicker Planquart et al., 1999). Second, dimerization of chimeric proteins by γ1 (Chou et al., 1999) may help conceal susceptible cleavage sites from proteases. Finally, the flexibility of γ1 may enhance correct folding of scFv, reducing degradation in the ER. These possibilities are currently under investigation. Reduction of proteolytic cleavage is important because functional scFv released from cells could interfere with the activity of surface scFv. For example, soluble anti-CD3 $(Fab')_2$ blocks T-cell activation, whereas immobilized (surface-bound) anti-CD3 antibodies activate T cells (Geppert and Lipsky, 1987; Woodle et al., 1991).

The γ1 spacer was also required for surface scFv activity; 2C11-γ1-B7 induced T-cell proliferation, whereas 2C11-B7 was inactive (Fig. 9). Addition of a 42-amino-acid spacer was found to increase the binding of an anti-phOx scFv to phOx-coated magnetic beads (Rode et al., 1999). Moritz and colleagues also demonstrated that spacers (derived from CD4 and CD8 molecules) were required to increase the distance of an anti-ErbB2 scFv from the plasma membrane to maintain scFv binding activity (Moritz and Groner, 1995). The requirement for a spacer to allow expression of active scFv on cells may reflect the need to extend the scFv from the cell membrane to allow unrestricted access with its cellular target. This hypothesis is supported by the finding that the activity of chimeric scFv is cell-dependent (Moritz and Groner, 1995). Alternatively, a flexible spacer may help retain proper folding and activity of surface scFv.

In summary, the choice of TM affects the half-life, sorting rate, and surface retention of chimeric proteins resulting in significant differences in surface expression. Introduction of γ1 between scFv and TM dramatically reduced cleavage of chimeric protein and resulted in increased surface expression of scFv. The γ1 domain was also required for retention of scFv activity. Our results indicate that transgenes designed for high surface expression of active chimeric scFv should incorporate a TM that does not undergo endocytosis, include an intact cytoplasmic domain, and contain a spacer between the scFv and TM to reduce cleavage and retain biological activity.

NOMENCLATURE

2C11	scFv constructed from the 145.2C11 antibody
2C11-B7	fusion protein between 2C11 scFv with B7 TM and cytosolic tail
2C11-γ1-B7	fusion protein between 2C11 scFv, γ1, and B7 TM and cytosolic tail
2C11-DAF	fusion protein between 2C11 scFv and DAF C-terminal extension
2C11-γ1-DAF	fusion protein between 2C11 scFv, γ1, and DAF C-terminal extension
2C11-PDGFR	fusion protein between 2C11 scFv and PDGFR TM and truncated cytosolic tail
2C11-γ1-PDGFR	fusion protein between 2C11 scFv, γ1, and PDGFR

	TM and truncated cytosolic tail
AFP	α-fetoprotein
AFP-B7	fusion protein between AFP and B7 TM and cytosolic tail
AFP-DAF	fusion protein between AFP and DAF C-terminal extension
AFP-PDGFR	fusion protein between AFP and PDGFR TM and truncated cytosolic tail
AFP-PDGFR-AA	fusion protein between AFP and PDGFR TM and mutant ($K^{558}K^{559}$ to $A^{558}A^{559}$) truncated cytosolic tail
ASGPR	asialoglycoprotein receptor
ASGPR(A)-AFP	fusion protein of ASGPR TM with mutant (Y^5 to A^5) cytosolic tail and AFP
ASGPR(Y)-AFP	fusion protein of ASGPR TM with wild-type cytosolic tail and AFP
B7	CD80
DAF	decay-accelerating factor
GPI	glycosylphosphatidylinositol
HA	hemagglutinin
PBS	phosphate-buffered saline
PDGFR	platelet-derived growth factor receptor
TM	transmembrane domain
scFv	single-chain antibody
γ_1	H—CH_2—CH_3 region of human IgG1

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