Journal of Cellular Biochemistry 82:234–245 (2001)

Properties of Exogenously Added GPI-Anchored Proteins Following Their Incorporation Into Cells

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Abstract Isolated glycosylphosphatidylinositol (GPI)-anchored proteins, when added to cells in vitro, incorporate into their surface membranes and, once incorporated, exert their native functions. Virtually any protein of interest, if expressed as a GPI-reanchored derivative, can be modified to acquire this capacity. Such transfer of proteins directly to cells, termed ''protein engineering'' or ''painting'' constitutes an alternative to conventional gene transfer for manipulating cell surface composition that has many potential applications. Previous studies with incorporated GPI-anchored proteins have focused almost entirely on their extracellular functions. In this study, biotinylated human erythrocyte (E^{hu}) decay accelerating factor, E^{hu} acetylcholinesterase, and GPI-reanchored murine B7-1 and B7-2 were used as GPI-anchored reporters to characterize their plasma membrane organization and cell signalling properties following addition to Hela or Chinese hamster ovary cells. For each reporter, three types of cell-association were documented; (1) nonphysiological attachment and/or incomplete insertion, (2) uncomplexed membrane integration, and (3) organization into TX-100-resistant microdomains. Transit from the first two compartments into the third, i.e., microdomains, progressed slowly, continuing even after 24 to 36 h and was associated with the acquisition of cell signalling capacity. All four reporters, incorporated in two different detergents, behaved similarly. When organized in microdomains, caveolin and other GPI proteins co-isolated with the incorporated reporter. These results have implications for protein engineering of cells in general, and in particular, for cells such as modified tumor cell immunogens administered to patients for therapeutic purposes. J. Cell. Biochem. 82: 234–245, 2001.

Key words: GPI-anchored protein; incorporation; microdomain

Glycosylphosphatidylinositol (GPI) anchors are posttranslationally-added structures that attach a subset of cell surface proteins to the plasma membrane (reviewed in Englund, 1993; McConville and Ferguson, 1993). These anchoring units are built upon core structures composed of ethanolamine phosphate (EthN-P), three mannose (Man) residues and glusosamine (GlcN) linked glycosidically to phosphatidylinositol (PI) [Ferguson et al., 1988; Homans et al., 1988]. They are preassembled in the ER and are transferred to N-terminally processed nascent polypeptides that contain C-terminal extension sequences with appropriate GPI anchor replacement signals [Medof and Tykocinski, 1990; Moran et al., 1991; Gerber et al., 1992]. According to data available to date, these anchoring moieties are utilized by all eukaryotes. They tether a wide range of functionally diverse proteins to cells and operate ubiquitously irrespective of the extracellular structural properties of the attached surface protein.

A unique feature of GPI-anchored proteins is that following their extraction from cells, they are able to reintegrate into plasma membranes

The first two authors contributed equally to this work.

Grant sponsor: NIH; Grant numbers: DK38181, DK55002, CA81550.

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Received 24 July 2000; Accepted 31 January 2001

when added to other cells. Initial studies [Medof et al., 1984] with sheep erythrocyte (E^{sh}) complement intermediates and the decay accelerating factor (DAF), a GPI-anchored cell surface regulator, showed that once incorporated, the exogenously added protein functioned with efficiency comparable to that of endogenous DAF protein. Subsequent studies with other GPI-anchored proteins including parasite surface coat proteins [Bulow et al., 1988], enzymes [Horta et al., 1991], receptors [Brodsky et al., 1994; Nagarajan et al., 1995], histocompatibility antigens [Huang et al., 1994], adhesion molecules [Selvaraj et al., 1987], as well as other immunologically relevant proteins [Zhang et al., 1992] have shown that, in all cases, the incorporated molecules exhibit their full native extracellular molecular interactive capabilities, establishing that the incorporation process is a general process attributable to the GPI moiety. Studies in which proteins with modified GPI anchor structures have been added to cells [Walter et al., 1992] have shown that stable integration and function in the surface bilayer depends upon the presence of both the sn-1 and sn-2 lipids in the glycerol backbone of the attached PI.

Since the signals that direct GPI anchoring are contained at the C-termini of nascent polypeptides [Medof and Tykocinski, 1990; Moran et al., 1991; Gerber et al., 1992], any protein, in principle, can be converted to a GPI-anchored derivative by preparing a chimeric cDNA in which its endogenous 3′ end-sequence is replaced by that of a native GPI-anchored protein such as DAF. When expressed and purified, the ability of the reanchored protein then can be exploited to attach the reanchored protein to any cell. This technology, termed "protein engineering" or "painting" provides an alternative to conventional gene transfer for modifying cell surface composition which has important practical advantages in a variety of situations (reviewed in Medof et al., 1996). Increasingly, interest has focused on it for clinical applications including the development of cellular tumor vaccines and other immunotherapies (reviewed in Tykocinski et al., 1996). In this regard, studies with GPI-reanchored cytokines [Weber et al., 1994], major histocompatibility complex (MHC) class I proteins [Huang et al., 1994], and co-stimulatory proteins [Brunschwig et al., 1995, 1997; McHugh et al., 1995] such as B7-1 and B7-2 have been undertaken. The production and use of a presumably unlimited range of additional molecules is possible in this and other settings.

In recent years, evidence has been obtained that GPI-anchored proteins are not diffusely expressed on cell surface membranes and that their activities are not entirely limited to the cell exterior as initially hypothesized. Substantial data [Brown and Rose, 1992; Arreaze et al., 1994; Lisanti et al., 1994] indicate that, in many cell types, GPI-anchored proteins localize in sphingomyelin-rich structures termed "rafts" or "TIMs" (Triton-insoluble membranes) which contain caveolin, cytoplasmically oriented kinases, as well as other proteins, and that, under certain conditions, they are able to participate in intracellular signalling. Such signalling has been documented in lymphocyte activation [Shenoy-Scaria et al., 1992] and possibly in cell differentiation [Dunn et al., 1996].

Previous studies concerning the properties of exogenously incorporated GPI-anchored proteins have been concerned, with one exception [van den Berg et al., 1995], exclusively with their extracellular functions. The present study was undertaken to further investigate whether exogenously incorporated GPI-anchored proteins are equivalent to their endogenous counterparts with respect to their cell surface organization and interaction with cellular kinases.

MATERIALS AND METHODS

Proteins and Reagents

Human erythrocyte (E^{hu}) DAF [Medof et al., 1986] and acetylcholinesterase (AChE) [Roberts and Rosenberry, 1986] were purified as described. GPI-reanchored murine B7-1 and B7-2 were prepared in Chinese hamster ovary (CHO) cell transfectants using nucleotides 1020–1124 of DAF's 3′ mRNA end-sequence [Brunschwig et al., 1995]. Anti-DAF monoclonal antibodies (mAb) IA10 and IIH6 were prepared as described [Kinoshita et al., 1985]. Anti-B7-1 and B7-2 mAbs were obtained from N. Nabavi, Hofman La Roche, Nutley, NJ and the ATCC (GL1), respectively. Anti-Caveolin1 mAb was from BD Transduction Lab., Lexington, KY. Anti-mouse DAF mAb (RIKO3) [Ohta et al., 1999] was supplied by Dr. Okada, Nagoya City University, Nagoya, Japan. Streptavidin (SA)-agarose and SA-horseradish peroxidase were purchased from Gibco. Nonidet P-40 (NP-40),

Triton X-100 (TX-100) and 3-[(3-cholamidopropyl) dimethylammonio] 1-propanesulfonate (CHAPS) were bought from Sigma (St. Louis, MO) and NHS-Biotin and Iodogen reagents were purchased from Pierce (Rockford, IL). Na ^{125}I was obtained from Amersham (Arlington Hts., IL) and γ[^{32}P]ATP from New England Nuclear DuPont (Boston, MA). Phosphate buffered saline (PBS) consisted of 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8.1 mM KH_2PO_4, pH 7.4.

Cell Cultures

Hela-S3 cells were grown in a 5% CO_2, humidified, 37°C incubator in Dulbecco's minimum essential medium (DMEM) supplemented with 10% (heat-inactivated) newborn calf serum (NCS) and 2 mM L-glutamine. CHO-K1 cells were grown in DMEM containing 10% fetal bovine serum (FBS). Mouse DAF (GPI-anchored form) transfected CHO cells [Ohta et al., 1999] were grown in DMEM containing 10% FBS and 0.3 mg/ml G418 (Life Technologies, Gaithersburg, MD).

Isolation of Membrane Microdomains

TX-100 insoluble complexes were prepared as described by Brown and Rose [1992]. Briefly, cell monolayers (5×10^7 cells) grown to ~90% confluence in 100 mm plates were harvested using Versene. Pelleted cells were extracted on ice with 1 ml of TNE buffer [25 mM Tris-Cl, pH 7.5, 150 mM NaCl, 5 mM EDTA containing 1.0% TX-100, and a protease inhibitor cocktail (Boehringer Mannheim)]. Following clarification, the extract was homogenized with 20 strokes in a Dounce homogenizer, mixed with an equal volume of 80% sucrose, and placed at the bottom of a 14×89 mm polyallomer tube (Beckman, CA). This mixture was overlayered first with 6.0 ml of 38% sucrose in TNE buffer and then with 3.0 ml of 5% sucrose in TNE buffer. The resulting (three step) discontinuous sucrose gradient was centrifuged at 39K rpm for 19 h at 4°C in a Beckman SW41 rotor. One milliliter fractions were collected from the top using a pipetman.

Radiolabeling and Biotinylation

Fifty microliter of protein (2–5 mg/ml) was radiolabeled using Iodogen (Pierce, Rockford, IL). After addition of the protein to the coated tube, 1.0 mCi of ^{125}I was introduced, the mixture incubated on ice for 5 min, and the reaction stopped by adding 50 µl of saturated KCl, 50 µl of saturated L-tyrosine, and 140 µl of PBS. Following elution from a Sephadex G-25 column with 500 µl of PBS containing 1% bovine serum albumin (BSA), the labeled protein was dialyzed against PBS at 4°C.

For biotinylation, proteins (30–89 µg/ml) in 50 µl of PBS containing either 0.1% NP-40, 0.1% TX-100, or dialysis-depleted CHAPS were mixed with 10 µl of 0.3 M NaH_2CO_3, pH 8.6 and 5 µl of 3 µg/ml NHS-Biotin in DMSO. The mixture was agitated for 2 h at 25°C and dialyzed overnight at 4°C against PBS.

GPI-Anchored Protein Incorporation

Biotinylated GPI-anchored proteins (1–3 µg/ml) were added to 5×10^7 washed cells in 500 µl of 0.1% gelatin-DMEM and the mixtures incubated at 37°C. After 2 h, cells were spun down, washed twice, plated on 100 mm dishes and further incubated for the indicated time intervals. Cells then were cooled to 4°C, extracted with cold TNE buffer containing 1% TX-100 and protease inhibitor cocktail, then the extracts fractionated on the above-described sucrose gradient.

Quantitation of Biotinylated Protein Bound to SA-Agarose

Extracts (500 µl of 5% sucrose, 1% TX-100, 25 mM Hepes, pH 7.4) containing biotinylated proteins were rotated overnight at 4°C in 1.5 ml microfuge tubes with 15 µl of SA-agarose beads. Beads were washed three times in PBS, centrifuged at 10K rpm for 30 sec in a Beckman microfuge, and resuspended in 1.0 ml of PBS containing 1% BSA. [^{125}I]SA or [^{125}I]-labeled specific mAb (1×10^5 cpm/ml) was added, the mixture rotated at 20°C for 2 h, and after centrifugation, the beads were washed three times in PBS and radioactivity counted.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

Proteins were separated on non-reducing 7.5 or 10% SDS-PAGE gels with prestained size markers. For Western blots, proteins were transferred at 40 V/m^2 for 2 h at 20°C to Imobilon membranes (Millipore, Bedford, MA) using a Bio-Rad transblot apparatus. After transfer, the membrane was blocked overnight at 4°C in 5% BSA, 0.1% Tween 20, 20 mM Tris-buffered saline, pH 7.6 (TBST). Biotinylated

proteins were revealed by incubation for 90 min at 20°C with a 1:3000 dilution of SA-horseradish peroxidase conjugate, 3-fold washing for 5 min in TBST, and development using ECL reagent (Amersham) and Kodak XAR film. Mouse DAF and caveolin were stained by hamster anti-mouse DAF (RIKO3) and mouse anti-caveolin mAb, respectively. After incubation for 1 h at room temperature, the membrane was washed with TBST and further incubated with a 1:1000 dilution of HRP-conjugated goat anti-hamster IgG (Jackson ImmunoResearch Lab, West Grove, PA) and HRP-conjugated goat anti-mouse IgG (Sigma), respectively, for 1 h at room temperature. Following washing, the membrane was developed using ECL.

Two-Site Immunoradiometric Assay (IRMA)

Concentrations of native or biotinylated proteins were quantified by two-site IRMA using mAbs specific to the protein, e.g., in the case of DAF, IA10 as capturing reagent and [^{125}I]labeled IIH6 (against a different epitope) or [^{125}I]SA as detecting reagent. Purified native or biotinylated proteins of known concentrations were used as standards.

AChE Enzymatic Assay

Aliquots (500 μl) of sucrose gradient fractions were added to 2.5 ml of a buffer prepared by mixing 100 ml of 75 mM acetylthiocholine iodide (AcTCh), 1.0 ml of 0.2 M sodium phosphate, pH 7.4, 10 mM 5,5′, dithio-bis(2-nitrobenzoic acid) (DTNB) in 0.1 M sodium phosphate, and 28.8 ml of 0.02 M sodium phosphate containing 0.1% TX-100. AChE activity was measured by the change of absorbance over 1 min at 412 nM.

Detection of G_{M1} in the Sucrose Gradient Fractions

For analyses of G_{M1}, CHO cells (2×10^7) were incubated with 5 μg/ml of HRP-conjugated cholera toxin β subunit (List Biological Lab, Campbell, CA) for 30 min in DMEM, 10 mM Hepes and 0.2% BSA. The treated cells then were extracted with 1% Triton X-100 in TNE buffer with a protease inhibitor cocktail (Boehringer Mannheim) and the extract subjected to sucrose gradient fractionation as described above. The separated fractions were assayed for peroxidase activity using a commercial ImmunoPure® TMB Substrate Kit (Pierce, Rockford, IL).

Distribution of [^{3}H]Choline-Labeled Sphingomyelin (SM) and Phosphatidylcholine (PC) in TIMs

Analysis of the distribution of SM and PC was done essentially as reported elsewhere [Sevlever et al., 1999]. Briefly, with ~80% confluent CHO cells in logarithmic growth in five 150 mm culture plates then were labeled for 48 h with 10 μCi of [^{3}H]choline (ARC). The radiolabeled cells and unlabeled cells from four additional 150 mm plates were harvested, combined, and extracted with 1% Triton X-100. After centrifugation, the twelve sucrose gradient fractions were dialyzed, dried, and the residues extracted with 600 μl of water, 720 μl of methanol containing 2% acetic acid, and 720 μl of chloroform. The organic phases were separated, dried, redissolved in methanol, and analyzed on TLC. Lipids were resolved using chloroform:acetone:methanol:acetic acid:water (60:24:18:12:6, by volume) as the developing solvent. The positions of [^{3}H]-labeled SM and [^{3}H]-labeled PC were determined by comigration with commercial standards.

In Vitro Kinase Assays

Prior to extraction, incorporated proteins were crosslinked with anti-B7 or anti-DAF mAB. Fractions (1.0 ml), harvested from sucrose gradients and added to 1 ml of 1% Triton X-100 in TNE buffer were incubated at 4°C for 8 h with anti-DAF mAB-IA10/protein A-Agarose or 15 μl of SA-agarose beads. Beads then were pelleted, washed twice with 25 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM sodium orthovanadate, 1% Triton X-100, and resuspended in 20 μl of kinase reaction buffer (25 mM Hepes, pH 7.4, 3 mM $MgCl_2$, 5 mM $MnCl_2$, 0.1 mM sodium orthovanadate). Reactions were initiated by the addition of 5 μCi γ[^{32}P]ATP and, after incubation for 15 min at 25°C with intermittent mixing, were stopped by addition of 20 μl of 2X Laemmli buffer and boiling for 3 min. [^{32}P]-labeled products were resolved on 7.5% SDS-PAGE gels and visualized by autoradiography.

RESULTS

Properties of Endogenous Surface DAF Protein in Hela Cells

To obtain baseline data allowing comparative analyses of the properties of incorporated and

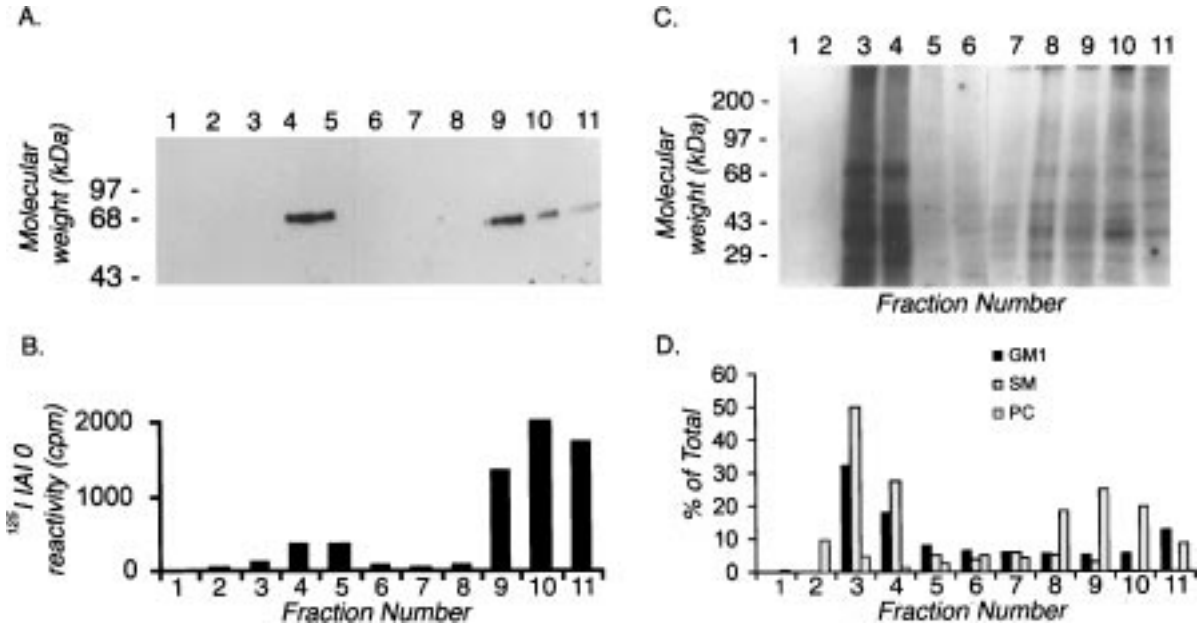

Fig. 1. Surface distribution and activation properties of endogenous DAF protein in Hela cells. Hela cells were extracted at 4°C with 1% TX-100 detergent and subjected to discontinuous sucrose gradient fractionation as described in the Methods. **A:** Western blot analysis of 15 μl of each gradient fraction revealed with anti-DAF mAb, IA10. **B:** Apparent DAF concentrations in each gradient fraction as measured by two-site DAF IRMA. **C:** Autoradiographs of [^{32}P]-labeled bands generated in a separate experiment on washed anti-DAF mAb/protein A-agarose beads in the presence of surface DAF crosslinking prior to extraction. **D:** Distribution of microdomain components in the sucrose fractions. Cells were preincubated with the HRP-labeled β subunit of cholera toxin which specifically binds to G_{M1} and following gradient separation the fractions containing G_{M1} were quantitated as in the Methods. Alternated cells were labeled with [^{3}H]choline which specifically labels SM and PC. Following gradient separation the fractions containing each component were determined by TLC as described in the Methods.

native GPI-anchored proteins in cells, the plasma membrane characteristics of endogenous DAF protein in Hela-S3 cells first were evaluated. Figure 1 shows a discontinuous sucrose gradient fractionation profile of the protein following extraction at 4°C in 1% TX-100 detergent (see Materials and Methods). As seen, ~80% of the native molecule localized in fractions 3, 4, and 5 as described for TX-100 detergent-resistant membrane microdomains [Brown and Rose, 1992; Lisanti et al., 1994]. The remaining 20% localized in fractions 9–11 appropriate for uncomplexed protein. When the concentration of DAF protein in each fraction was quantitated by two-site IRMA, the quantities in fractions 4 and 5 were underestimated (panel B). This presumably reflected inaccessibility of the detecting antibody to the captured DAF antigen due to its association with other microdomain components [Shenoy-Scaria et al., 1992; Arreaze et al., 1994; Lisanti et al., 1994]. Antibody crosslinking of the surface protein prior to extraction, subsequent immunoprecipitation of DAF in each fraction with anti-DAF mAb IA10/protein A-agarose, and addition of γ[^{32}P]ATP to immunoprecipitates showed that the crosslinked (panel C) but not uncrosslinked (not shown) DAF protein complexes in fractions 3 and 4 contained kinase activity which gave rise to phosphorylated bands migrating at ~75, ~55, and most prominently 40–43 kDa. The dependence of phosphorylation on crosslinking is in accordance with previously reported findings [Shenoy-Scaria et al., 1992; Arreaze et al., 1994].

To confirm the distribution of microdomains in the sucrose gradient fractions, two types of experiments were done. In one, the position of the ganglioside G_{M1} which has been shown to be a characteristic marker of "rafts" [Streuli et al., 1981] was determined. For this purpose, the HRP-labeled β subunit cholera toxin which specifically binds to G_{M1} was incubated with cells. After detergent extraction and sucrose gradient separation, HRP activity in each gradient fraction was measured. As shown in Figure 1D, essentially all of G_{M1} was detected in fractions 3–5. In the second experiment,

cells were labeled with ^{3}H-choline which specifically labels SM and PC, the former of which is concentrated in the "rafts" and the latter of which is concentrated in non-"raft" plasma membrane. Following TX-100 extraction of cells, sucrose gradient centrifugation, and separation of fractions organic extracts of the fractions were analyzed on TLC. As shown in Figure 1D, the majority of SM colocalized with G_{M1} fractions 3–5. On the other hand, PC which does not associate with microdomains was detected in fractions 8–10. The results taken together verified that GPI proteins in fractions 3–5 are colocalized with microdomains.

Methodology for Analysis of Incorporated GPI-Anchored Proteins

To permit accurate and sensitive assessment of the properties of exogenously added GPI-anchored proteins, biotinylated GPI-anchored reporters and SA-based detection were utilized. Four different biotinylated reporters, E^{hu} DAF, E^{hu} AChE, and GPI-reanchored murine B7-1 and B7-2 (Fig. 2), were prepared and methods for recovery of these incorporated reporters were systematically evaluated. For developing these methods, biotinylated E^{hu} DAF was employed as the test reporter, SA-agarose used as a capturing vehicle, and ^{125}I[SA] employed as the detecting agent. Utilizing 50 ng of biotinylated DAF (the theoretically calculated maximal amount of incorporated protein assuming 5% incorporation [Walter et al., 1992] that would be present in any given gradient fraction), (1) the amount of SA-agarose required for quantitative recovery of this added DAF reporter, (2) the kinetics of its uptake onto SA-agarose beads, and (3) the proportionality of binding of [^{125}I]SA to the agarose-captured DAF protein were measured. As seen in Figure 3, 15 μl of SA-Agarose provided $>$2-fold excess capacity for uptake of the DAF reporter (panel A), 8 h of incubation allowed for maximal binding (panel B), and following adsorption to the beads, the uptake of [^{125}I]SA increased nearly linearly with the amount of biotinylated DAF reporter offered. As little as 0.4 ng of biotinylated DAF was reproducibly detectable. Parallel results were obtained if [^{125}I]-labeled anti-DAF mAb IA10 or IIH6 was used in place of [^{125}I]SA as detecting reagent but the minimum detectable level was ~10-fold higher (not shown).

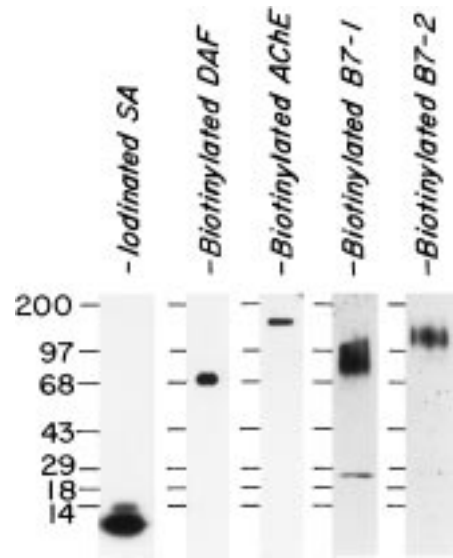

Fig. 2. GPI-anchored reporter proteins used in experimental studies. E^{hu} DAF and AChE (**lanes 2** and **3**) and GPI-reanchored murine B7-1 and B7-2 (**lanes 3** and **4**) biotinylated as described in the Methods were examined on Western blots probed with SA horse radish peroxidase (HRP). Each protein is seen at its characteristic migration position and each preparation is free of biotinylated contaminants. [^{125}I]-labeled SA is shown in **lane 1**.

Properties of Exogenously Added GPI-Anchored Proteins

With the use of the above methodology, investigations into the behavior of the biotinylated reporters following their addition to cells next were initiated. In the first series of studies, biotinylated GPI-anchored B7-1 and nonbiotinylated AChE were used as reporters, and incorporation for 2 h at 37°C into Hela cells was arbitrarily adopted. Following this incorporation period either without further incubation or after an additional 6 h of 37°C chase, the surface distributions of the added biotinylated B7-1 and nonbiotinylated AChE then were analyzed. As seen in Figure 4 panel A for biotinylated B7-1, after incorporation without chase, most of the B7-1 reporter was recovered in fractions 9–10 and in the fraction 12 pellet. Only a small amount (16%) was recovered in fractions 3 and 4. In contrast, with 6 h of chase at 37°C but not 0°C, a much larger proportion of the protein (42%) moved into fractions 3 and 4 (Fig. 4, panel B). Comparable results (21%→53%) were obtained with nonbiotinylated AChE in which the protein was assayed by enzymatic analysis of whole sucrose fractions (Fig. 4C and D). The kinetics of movement of the

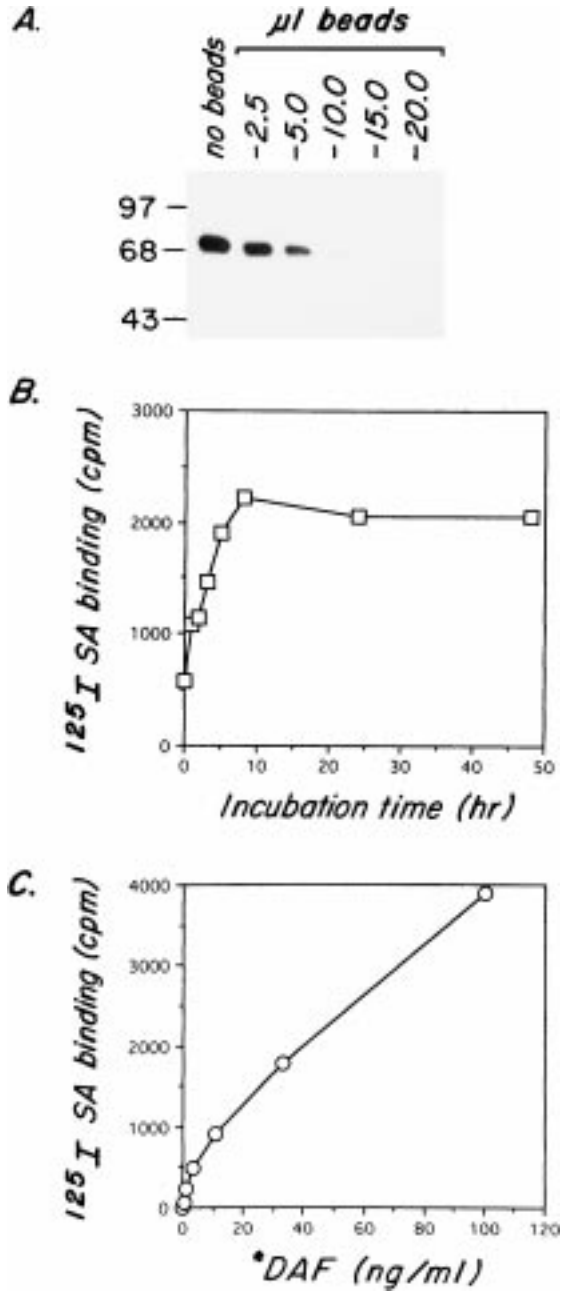

Fig. 3. Conditions for recovery of biotinylated GPI-anchored protein from gradient fractions with SA-agarose beads. **A:** Increasing volumes of SA-agarose beads were incubated overnight at 4°C with 50 ng of biotinylated DAF and following centrifugation, the supernatants were assayed for unbound biotinylated DAF by Western blotting with SA-peroxidase. **B:** 15 µl of SA-agarose beads were incubated at 4°C for increasing times with 50 ng of biotinylated DAF, and after centrifugation, the amount of agarose-bound DAF quantitated by addition of [^{125}I] SA and counting the washed beads. **C:** Increasing concentrations of biotinylated DAF were incubated for 8 h at 4°C with 15 µl of SA-agarose beads and bound DAF protein quantitated as in Panel B.

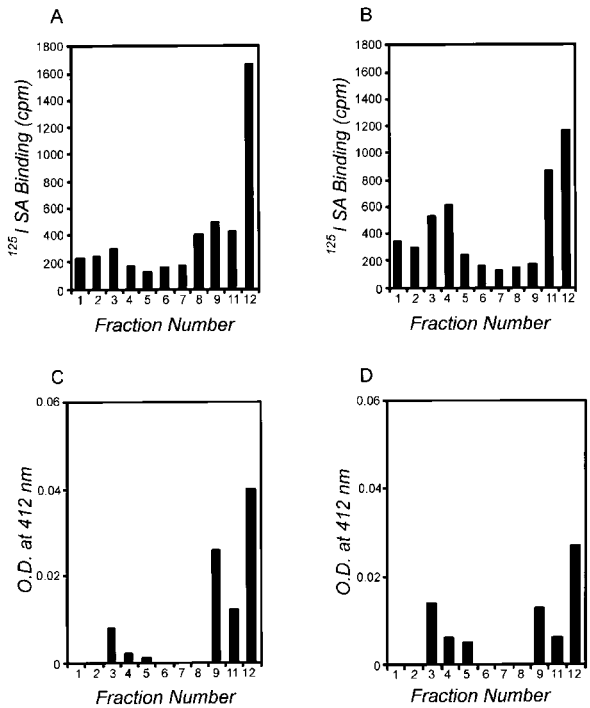

Fig. 4. Surface distribution of exogenous GPI-anchored B7-1 and AChE following incorporation into Hela cells without or with chase. Biotinylated GPI-anchored murine B7 (3 µg/µl in detergent-depleted CHAPS) and AChE (2 µg/µl in 0.005% TX-100) were incubated for 2 h at 37°C with 5×10^7 Hela cells. TX-100 4°C extracts of the washed cell then, either prepared immediately (**A** and **C**) or after 6 h of further 37°C chase in 100 mm plates in DMEM containing NCS (**B** and **D**), were separated on sucrose gradients and the added proteins in each gradient fraction quantitated. B7-1 was quantitated by SA-agarose/^{125}I-SA binding and AChE by enzymatic assay. The total amounts of B7-1 recovered from the gradient were 4,400 CPM and 4,600 CPM for 0 time and 6 h respectively. The total activity recovered was 0.088 and 0.0072 U for 0 time and 6 h, respectively.

biotinylated and nonbiotinylated reporters from the lower to the upper fractions was similar, and parallel results were obtained with the two independent assay systems, thereby (1) confirming the validity of the SA-Sepharose/[^{125}I]SA methodology for recovery/quantification of the GPI-reporter added to cells and (2) establishing that different incorporated GPI-anchored reporters exhibit the same localization behavior. Similar results were obtained with biotinylated DAF and GPI-anchored B7-2 (not shown). These findings of initial, presumably unorganized GPI-anchored protein membrane attachment and subsequent association with microdomains confirm previous findings by van den Berg et al. [1995] with purified CD59

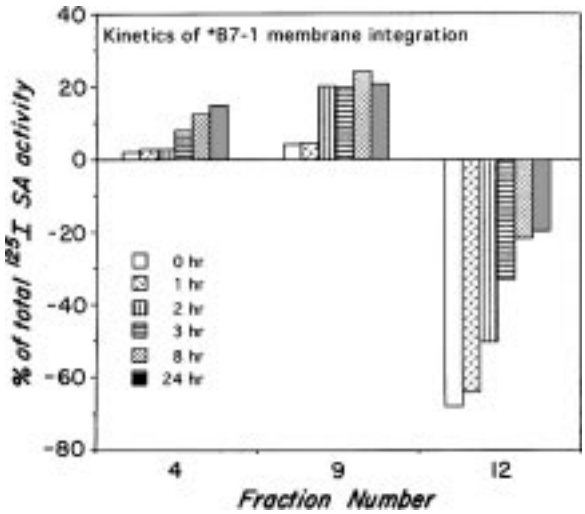

Fig. 5. Kinetics of membrane reorganization of GPI-reanchored B7-1 following association with Hela S3 cells. GPI-reanchored murine B7-1 protein (2 μg/ml) was incubated at 37°C for 2 h with Hela S3, and the cells chased at 37°C for the indicated times. At each time point, 4°C TX-100 extracts of the washed cells were then separated on discontinuous sucrose gradient, and DAF in each fraction quantitated by binding to SA agarose beads and quantitating with [^{125}I] SA.

protein in Brij58 detergent exogenously added to CD59-deficient U937 cells (see Discussion).

Reorganization of Incorporated GPI-Anchored Proteins

Based on the above validation of the biotinylated reporter methodology and on the above initial findings, the effect of chase time on the reorganization of the exogenously added GPI-anchored proteins was further investigated. For these experiments, biotinylated GPI-anchored B7-1 was employed and the kinetics of the change in cell surface organization was analyzed using shorter and longer chase times. As seen in Figure 5, only a small amount (<5%) of added protein was in fraction 4 until 3 h. With increasing time, there was (1) a progressive decrease in fraction 12 (pellet-associated) protein, (2) a plateau in fraction 9 (uncomplexed) protein, and (3) a progressive increase in fraction 4-associated protein with movement from the former compartment to the latter continuing even after 24 h of chase. At this time point (24 h), ~17% of the cell-associated B7-1 was recoverable in fraction 4, 22% in fraction 9, 18% in fraction 12, and ~40% lost or otherwise unaccounted for. In a second series of experiments, chase times were further extended. As seen in Table I, even further movement into fractions 3–4 could be documented at 36 h. In similar experiments utilizing AChE added to CHO-KI cells and enzymatic quantitation of whole gradient fractions, a similar effect of extended chase time was observed (not shown).

TABLE I. Movement of Incorporated GPI-Anchored Reporter Into Microdomains

B7-1/Hela-S3 Fractions	Chase time		
	12 h	24 h	36 h
3–4	313*	439	533
3–4/9–10	0.733	1.02	1.13

*cpm.

In the next series of studies (Fig. 6), the capacity of incorporated GPI-anchored reporters upon crosslinking with antibodies to induce kinase activity similar to that observed for endogenous GPI-anchored proteins (see Fig. 1) was assessed. For these analyses, (1) biotinylated GPI-anchored B7-1 added to Hela cells and (2) biotinylated DAF added to CHO cells were studied. In each case, the requirement for extended chase time (24 h) allowing organization of the reporter into fraction 4 microdomains was investigated. As shown in Figure 6A for GPI-anchored B7-1 added to Hela cells, after 24 h of 37°C chase, specific kinase activity with crosslinked B7-1 was demonstrable in fraction 4, whereas it was weakly detectable with B7-1 in fractions 9, 10, or 11. The kinase activity in fractions 3/4 gave rise to prominent phosphorylated bands of ~75 and 40–43 kDa, most of which corresponded in size to those bands seen for endogenous DAF in Hela cells (see Fig. 1). Only minimal kinase activity in fractions 3/4 was recovered immediately following incorporation at 2 h without extended chase, or in the absence of antibody crosslinking (not shown). Similarly to B7-1 added to Hela cells, for DAF added to CHO cells (Fig. 6B), kinase activity was readily demonstrable in large amounts in fractions 3/4 after crosslinking again only following 24 h of chase. In this latter case with CHO cells, the kinase activity in this fraction gave rise to phosphorylated bands of 36 and 50–55 kDa in fraction 4, products similar in size to those detected previously in analyses of GPI-anchored F3-transfected CHO cells [Cervello et al., 1996]. As has been reported previously [Stefanova et al., 1991], the phosphorylated proteins produced by kinases co-precipitated with GPI-

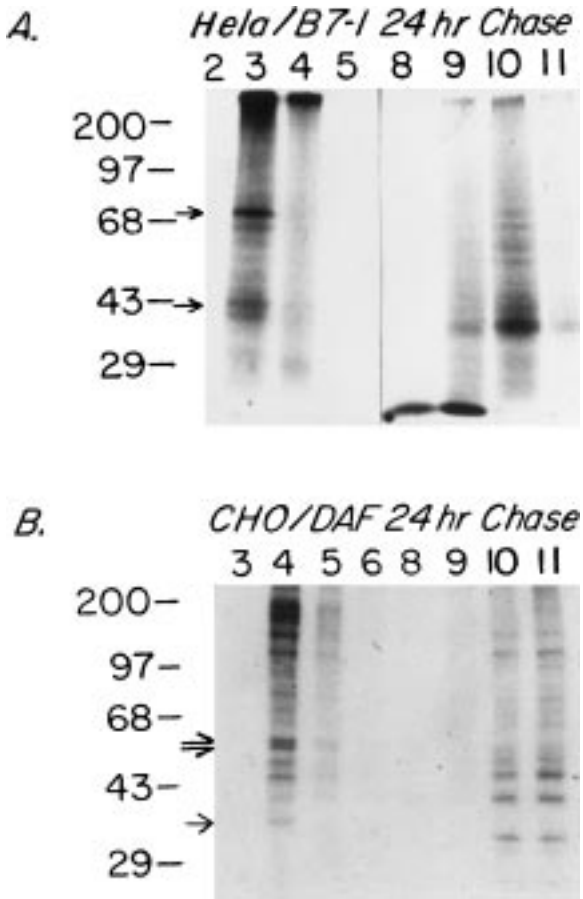

Fig. 6. Kinase activity of proteins associated with incorporated GPI-anchored reporters. **A:** Biotinylated murine B7-1 was incubated with Hela S3 cells as in Figure 5 and 4°C TX-100 extracts of washed cells after 24 h of chase then separated on sucrose gradients, and biotinylated B7-1 protein isolated from each fraction by addition of SA agarose beads. After thorough washing, proteins on the beads were analyzed for kinase activity as described in the Methods. **B:** Biotinylated DAF was incubated with CHO cells and analyses conducted as in Panel A. Note that some bands were seen in fractions 9 and 10 as well as in 3 and 4. However, several new bands were observed in fractions 3 and 4 as indicated by arrows.

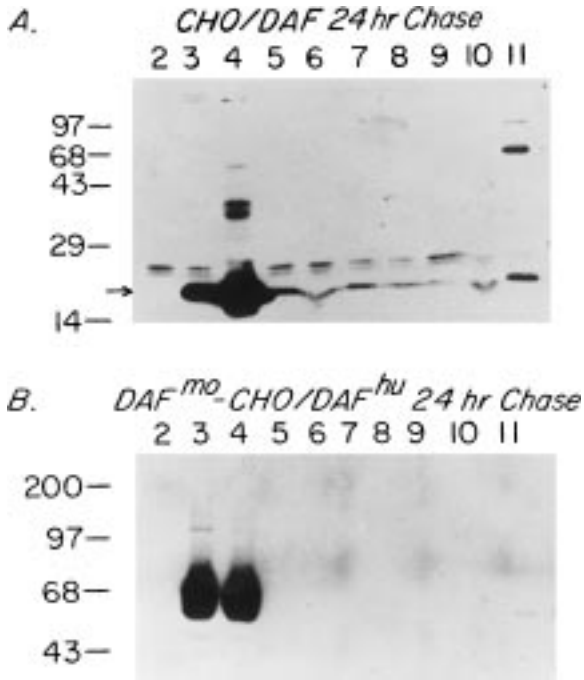

Fig. 7. Association of incorporated DAF with the components of rafts that co-isolate with endogenous GPI-anchored proteins. **A:** Biotinylated DAF was incubated with CHO cells and the cells extracted after 24 h of chase. After separation on sucrose gradients, SA agarose beads were added to each fraction, the bead washed, and the eluted protein analyzed on Western blots stained with anti-caveolin mAb. **B:** Biotinylated DAF was incubated with the CHO cells stably transfected with cDNA encoding GPI-anchored mouse DAF. Cells were treated as is Panel A and membranes were probed with anti-mouse DAF mAb.

anchored protein differ depending on the cell type used.

To examine the association of incorporated GPI-anchored reporters with other components of rafts that have been described in studies of endogenous GPI-anchored proteins [Anderson, 1998], association of incorporated DAF with caveolin and other GPI-anchored proteins was assessed. For this purpose two experiments were done. In the first, biotinylated DAF was incorporated into CHO cells and following incubation of sucrose fractions with SA-Agarose, proteins were eluted from the beads and Western blots examined with anti-caveolin antibody. As shown in Figure 7A, a major band of 20 kDa was detected in fractions 3 and 4, the fractions containing incorporated biotinylated DAF (Fig. 6B). No bands were recovered in the absence of SA-agarose beads. In the second experiment, biotinylated human DAF was incorporated into CHO cells which were stably transfected with cDNA encoding the GPI-anchored form of mouse DAF. Following incubation of sucrose fractions with SA-Agarose, proteins were eluted and Western blots probed with anti-mouse DAF mAb. As shown in Figure 7B, a band of 60 kDa appeared in fractions 3 and 4 indicating that incorporated biotinylated human DAF was associated with endogenous mouse DAF protein in the transfectants.

DISCUSSION

Previous studies have shown that exogenous GPI-anchored proteins, when reincorporated

into cells, are indistinguishable from endogenous GPI-anchored proteins with respect to their extracellular functions [Medof et al., 1984; Selvaraj et al., 1987; Bulow et al., 1988; Horta et al., 1991; Zhang et al., 1992; Brodsky et al., 1994; Huang et al., 1994; Nagarajan et al., 1995], membrane mobilities [Selvaraj et al., 1987], and lack of susceptibility to agents such as liposomes [Walter et al., 1992] or GPI-specific phospholipase D [Walter et al., 1992] which act upon unprotected phospholipids. Based on these findings, incorporated proteins have been presumed to be fully reintegrated into plasma membrane lipid bilayers. Whether they indeed are integrated in their physiological orientations previously has not been rigorously assessed by other criteria. The data presented in this study extend previous work by van den Berg et al. [1995] (see below) by showing that added GPI-anchored proteins in fact can be associated with membranes in three ways: (1) incompletely or otherwise incorrectly inserted, (2) inserted as independent apparently unassociated molecules, or (3) integrated into membrane microdomains, in this latter state, presumably as they predominantly exist in situ in cells. The data further indicate that following uptake by cells, GPI-anchored proteins undergo changes in their state of membrane attachment and that their transition into the microdomain-associated state is a time- and temperature-dependent process.

Our initial characterizations of endogenous DAF in Hela cells showed that, upon extraction with TX-100 detergent at 4°C, the protein is associated with detergent-resistant membrane microdomains that float in sucrose gradients (fractions 3–5). This finding parallels those of Brown and Rose [1992] also in Hela cells and of other investigators with other GPI-anchored proteins in other cell types [Lisanti et al., 1989, 1994; Arreaze et al., 1994; ; Schell et al., 1992; Shenoy-Scaria et al., 1992; Wollner et al., 1992]. In accordance with the findings of these previous investigations, studies with our system (presented elsewhere) have shown that TX-100 extraction at 37°C does not fully preserve these microdomains but liberates DAF as unassociated molecules. Our findings (Fig. 1A and B) that much lower levels of 4°C TX-100 extracted DAF were detectable by IRMA than on Western blots prepared from SDS-treated proteins are in accordance with inaccessibility of all the individual DAF molecules due to their organization with associated microdomain components. As reported in other studies [Shenoy-Scaria et al., 1992, 1993a, 1993b; Lisanti et al., 1994], the presence of kinases among the associated components was documented by protein phosphorylation upon crosslinking with anti-DAF antibody.

The strategy that we have developed to study incorporated proteins offers a number of opportunities for analysis of the process of assembly of incorporated GPI-anchored proteins into microdomains, as well as for analysis of microdomain structure itself. In conjunction with SA-based detection, the use of biotinylated reporters allows monitoring of low levels of the incorporated proteins either by Western blots or IRMA. That biotinylation does not alter the behavior of the incorporated proteins (although free GlcN and EthN amines are present in GPIs) was documented by the identical behavior in all studies of the biotinylated reporters and non-biotinylated AChE which was assayed enzymatically. A further advantage of the biotin labeling method is that through the use of SA-agarose it allows isolation of components associated with the incorporated biotinylated GPI protein. Moreover, in this context, it could permit analysis of the kinetics of association of individual proteins in the course of microdomain formation. In previous work using purified CD59 protein and CD59-deficient U937 cells, van den Berg et al. [1995] showed that 4 h after addition to the cells, the added CD59 protein chased into microdomains containing kinase activity, and that upon crosslinking, the incorporated CD59 was able to induce a Ca^{++} signal. Our results extend these findings in showing that similar reorganization of incorporated proteins is applicable to four other GPI-anchored reporters in two other cell types, that three types of cell association can occur, and that the reorganization process is not complete after 4 h but rather continues for more than 24 to 36 h. Although the precise organization of endogenous GPI-anchored proteins and kinases in natural microdomains in situ is not yet known, our kinase analyses showed that incorporated GPI-anchored proteins gradually migrate to these same microdomains where, after crosslinking by antibodies, their interaction with cellular kinases can be observed. Furthermore, we could show direct evidence that biotinylated exogenous DAF is associated with both endogenous caveolin and endogenous mouse GPI-

anchored DAF in transfectants. Recovery of caveolin and mouse DAF by biotinylated DAF and SA-beads strongly supports the proposition that following adequate time exogenous DAF is integrated into microdomains in the same manner as is endogenous DAF. Of note, in the study of van den Berg et al. [1995], 5–15 μg/ml of CD59 was used for incorporation, and the majority (up to 70%) of the initially-attached protein was rapidly lost from the cells. In our study, 1–3 μg/ml of GPI-anchored reporter in detergent depleted CHAPS or dilute TX-100 was used and only 10% was lost. It is likely that upon GPI-anchored protein addition to cells, the initial state of the cell-associated protein, and the time required for microdomain integration may depend on the conditions used for incorporation, and in particular, the type and concentration of detergent.

The results of our studies are relevant to recently initiated efforts to develop tumor cell vaccines. One mechanism by which tumors evade cellular immune responses is by their failure to express cell surface co-stimulators which normally provide second signals for T cell activation. In support of this, it has been shown in studies with B7 tumor cell transfectants that expression of B7 costimulator molecules in certain tumors restores anti-tumor T cell activation and induces subsequent curative tumor rejection in vivo [Chen et al., 1992, 1994]. In other investigations [Brunschwig et al., 1997], with the use of the alternative technique of GPI protein transfer, the two GPI anchored murine B7 proteins employed in this study have been incorporated into EL-4, SMMUCC-1, BW5147.3, P815, Ag104A, and EMT6 tumor lines and the incorporated proteins have been shown to confer costimulator activity to the tumor lines in in vitro assays. The perfection of GPI protein transfer methods that provide for maximal longevity of the incorporated molecules and for optimal physiologic T cell activation and other components of T cell activation will surely be an important prerequisite for producing optimally engineered tumor cell vaccines for maximizing success with this therapeutic approach.

ACKNOWLEDGMENTS

We thank Dr. H. Okada, Nagoya City University, for kindly providing monoclonal anti-mouse DAF antibody.

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