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Intercellular transfer of a glycosylphosphatidylinositol (GPI)-linked protein: Release and uptake of CD4-GPI from recombinant adeno-associated virus-transduced HeLa cells

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ABSTRACT **A diverse group of GPI-anchored protein structures are ubiquitously expressed on the external cell membranes of eukaryotes. Whereas the physiological role for these structures is usually defined by their protein component, the precise biological significance of the glycolipid anchors remains vague. In the course of producing a HeLa cell line (JM88) that contained a recombinant adeno-associated virus genome expressing a GPI-anchored CD4-GPI fusion protein on the surface of the cells, we noted the transfer of CD4-GPI to native HeLa cells. Transfer occurred after direct cell contact or exposure to JM88 cell supernatants. The magnitude of contact-mediated CD4-GPI transfer correlated with temperature. Supernatant CD4-GPI also attached to human red blood cells and could be cleaved with phosphatidylinositol-specific phospholipase C. The attached CD4-GPI remained biologically active after transfer and permitted the formation of syncytium when coated HeLa cells were incubated with glycoprotein 160 expressing H9 cells. JM88 cells provide a model for the production, release, and reattachment of CD4-GPI and may furnish insight into a physiologic role of naturally occurring GPI-anchored proteins. This approach may also allow the production of other recombinant GPI-anchored proteins for laboratory and clinical investigation.**

Proteins covalently linked to GPI represent a large group of molecules with ubiquitous expression in eukaryotes (1–4). Their discovery was based on biochemical analyses and demonstrated sensitivity to phosphatidylinositol-specific phospholipase C (PI-PLC) cleavage of the membrane-bound species. Most of these GPI-linked structures are located at the cell surface with their C-terminal amino acid covalently linked to a glycolipid anchor. The protein, carbohydrate, and lipid components of the structures vary widely among species and tissues making generalizations about their biological function difficult. A growing list of biological functions attributed to these molecules include cell adhesion, nutrient uptake, signal transduction, and complement regulation. Decay-accelerating factor (DAF) is a GPI-linked protein present on the surface of peripheral blood cells and numerous epithelial surfaces (5). DAF prevents assembly of C3 convertases to protect the host tissue from complement-mediated lysis (6). In addition, numerous hydrolytic enzymes and proteins with undetermined or pathologic significance like scrapie prion protein have been identified as GPI-linked (7).

Previous studies have shown the usefulness of a fusion protein consisting of the extracellular domain of CD4 linked to the GPI anchor. HIV efficiently infects human cells having endogenously expressed GPI-anchored CD4 receptors (8, 9). Syncytium formation has been demonstrated by cocultivating CD4-GPI expressing HeLa cells with HeLa cells expressing HIV viral glycoproteins (gps; ref. 10). CD4-GPI purified from HeLa cell membranes was able to incorporate into cell membranes and bind gp120, but efficient recombinant HIV-mediated transduction of those cells was not demonstrated (11).

We became interested in the CD4-GPI gene as a marker for recombinant adeno-associated virus (rAAV)-mediated gene transfer. Adeno-associated viruses contain a single-stranded DNA genome and belong to the parvovirus family (12). Their lack of observed pathogenicity, ability to integrate in a site-directed manner, and broad human tissue tropism *in vitro* have lead to an interest in using these virions as vehicles for gene therapy. The availability of reagents, the ease of analyses using flow cytometry, and eventual therapeutic strategies were considered in choosing the CD4-GPI marker gene. rAAV particles containing a CD4-GPI fusion gene efficiently transduced and expressed a CD4-GPI molecule on HeLa cells (13). Those transduction events resulted in both transient and stable expression of the vector genome.

We demonstrate here the isolation of CD4-GPI expressing HeLa cell lines, which stably express the CD4-GPI genome and, surprisingly, release CD4-GPI onto the surfaces of native HeLa cells. Contact-mediated transfer was a rapid and temperature-dependent process. CD4-GPI was also present in culture supernatants. The released CD4-GPI was able to incorporate into other cell membranes and remained biologically active as evidenced by the formation of syncytium with cocultivated gp160 expressing H9 cells.

METHODS

Cell Lines and Recombinant Virus and Cell Supernatants. HeLa and H9 cell lines were obtained from American Type Culture Collection. The following reagent was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Disease, National Institutes of Health: vPE16, gift of Patricia Earl and Bernard Moss (10, 14, 15). All cells were maintained in Richter's medium (Biofluids, Rockville, MD) supplemented with 10% fetal calf serum. Red blood cells (RBCs) were obtained from a volunteer with normal blood indices.

Stocks of the recombinant plasmid and adeno-associated viral vectors, denoted pJM48 and vJM48, were produced and stored as described (13, 16). The pJM48 plasmid was subcloned

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Abbreviations: GPI, glycosylphosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; DAF, decay-accelerating factor; gp, glycoprotein; rAAV, recombinant adeno-associated virus; RBC, red blood cell.

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by ligations of (*i*) the *Xho*I–*Sal*I insert of pMC1neo poly(A) (Stratagene); (*ii*) the *Xho*I–*Bam*HI insert from CMV-CT4D encoding the extracellular domain of CD4 and a DAF-derived signal for the posttranslation attachment of a GPI anchor (17); (*iii*) a *Bam*HI–*Sal*I fragment containing a modified globin poly(A) tail; and (*iv*) the *Xho*I–*Sal*I vector fragment of pJM24 containing the rAAV-inverted terminal repeats (18). The JM88 cell line was obtained by flow cytometry sorting of vJM48-transduced HeLa cell populations for high level CD4-GPI expression followed by clonal selection in 0.5 mg of active geneticin per ml (GIBCO), which is lethal to cells not expressing the neomycin phosphotransferase gene. The JM263 control cell line was isolated after calcium phosphate transfection of pJM48 into HeLa cells. JM88 cell supernatants were collected from cells grown to confluence and centrifuged at 1500 × *g* for 30 min. The resulting supernatants were used immediately or stored at 4°C.

Cell Mixing Conditions, CD4 Staining, and Flow Cytometry. All CD4-GPI expressing cells were grown to confluence, washed with PBS, and collected after a brief exposure to trypsin·EDTA. Serum-containing medium was used to resuspend the cells. Mixing with native HeLa cells was carried out in 1.4-ml Eppendorf tubes by gentle rocking for 15 min. The mixed cells were then centrifuged at 1000 × *g* for 10 min and washed in PBS before analysis.

Immunoenzymatic staining of cells was performed using an alkaline phosphatase anti-alkaline phosphatase staining procedure (Dako). Cells were stained for flow cytometry in serum-free PBS containing phycoerythrin-labeled anti-CD4 antibody or an IgG isotype control (Immunotech, Westbrook, ME) for 30 min at 4°C, washed, and suspended in 4% paraformaldehyde before analysis. Cells mixed for the purpose of temperature comparisons were fixed in 4% paraformaldehyde before CD4 staining. Flow cytometry was performed using an Epics Elite instrument (Coulter) and 10,000 events were collected for analysis. Quantitation of CD4-based cell fluorescence was measured by mean cell fluorescence in populations having a normal fluorescent distribution or by measuring the percentage of cells with CD4 fluorescence at least two standard deviations above the control level in populations staining with an irregular distribution. Calibration of fluorescence relative to microbeads was performed according to the manufacturer's protocol (Flow Cytometry Standards, San Juan, Puerto Rico).

PI-PLC Enzymatic Digestion. Cells (1×10^6) were washed and suspended in 0.1 ml of PBS and 0.005 ml of PI-PLC from *Bacillus cereus* (Boehringer Mannheim) and incubated for 2 hr at 37°C before flow cytometry analysis.

Supernatant CD4-GPI Analysis. Affinity chromatography was used to isolate CD4-GPI from the JM88 cell supernatant. Supernatants were adjusted to 1 M NaCl concentration and PEG (M_r 7000; final concentration, 8%) was added before overnight precipitation. PEG precipitates were washed in PBS and resuspended in buffer A [20 mM Tris·HCl (pH 7.2), 1.0 mM EDTA, 0.15 M NaCl, 0.1% Nonidet P-40, 0.01 mg of aprotinin per ml, and 0.01 mg of phenylmethylsulfonyl fluoride per ml] before application to CD4 tresyl-activated agarose beads, prepared according to the manufacturer's protocol (Sigma). After extensive washing with buffer A, the CD4-GPI was eluted with buffer B [20 mM Tris·HCl, 1 mM EDTA, 50 mM glycine, 0.01 mg of aprotinin per ml, and 0.01 mg of phenylmethylsulfonyl fluoride per ml (pH 2.8)] and immediately adjusted to a neutral pH.

Syncytium Formation. The assay for syncytium formation using gp160-expressing H9 cells is described elsewhere (19, 20). VPE16-transduced H9 cells and HeLa, JM88, or CD4-GPI-coated HeLa cells (1×10^5 cells) were cocultivated for 4–16 hr and observed for evidence of cell fusion and syncytium formation.

RESULTS

Transduction of HeLa cells with the vJM48 rAAV resulted in expression of CD4-GPI on their external cell membranes. The CD4-GPI expression was normally distributed and peaked 44 hr after initial viral exposure. The JM88 cell line shown in Fig. 1 was obtained by sorting the 10% of cells with maximum CD4 fluorescence, followed by clonal selection in G418. The resulting clonal population (JM88) expressed the CD4-GPI receptor at uniformly high levels compared to native HeLa cells (Fig. 1 *A* and *B*). In contrast to occasional control cells having nonspecific anti-CD4 staining, the entire JM88 population revealed CD4-specific staining in enzyme-based and flow cytometry analyses. A flow cytometry analysis of peripheral blood lymphocytes is shown in Fig. 1*C* for comparison. Quantitation of the peripheral blood CD4+ lymphocytes fluorescence using calibrated beads revealed 120,000 molecules of equivalent soluble fluorochrome. By comparison, the JM88 cells fluoresced with an extrapolated value of ≈630,000 molecules of equivalent soluble fluorochrome.

When HeLa cells were mixed with CD4-GPI-expressing cells before CD4 staining, a shift in the mean HeLa cell fluorescence was noted. Although the JM88 (transduced) and JM263 (transfected) cells had equivalent fluorescence, mixing JM88 and HeLa populations (Fig. 2*b*) resulted in a larger shift in the HeLa cell fluorescence (average, 5.5 versus 2.5 fluorescent units in duplicate experiments). Mixing cells in medium versus serum-free PBS resulted in an equivalent shift in HeLa cell fluorescence (data not shown). Cellular fixation in 4% paraformaldehyde before mixing prevented the transfer from both cell lines (Fig. 2 *A* and *C*).

The influence of temperature on the transfer of CD4-GPI between cells is shown in Fig. 3. The cell mixtures were maintained at the indicated temperatures for a total of 25 min before fixation in 4% paraformaldehyde. The background fluorescence of native HeLa cells was stable at the temperatures tested. In duplicate mixtures, cells mixed at 4°C doubled their mean fluorescence. Increasing the temperature of the cells to 37°C resulted in a substantial increase in CD4-GPI transfer as measured by a 16-fold increase in HeLa cell fluorescence above background.

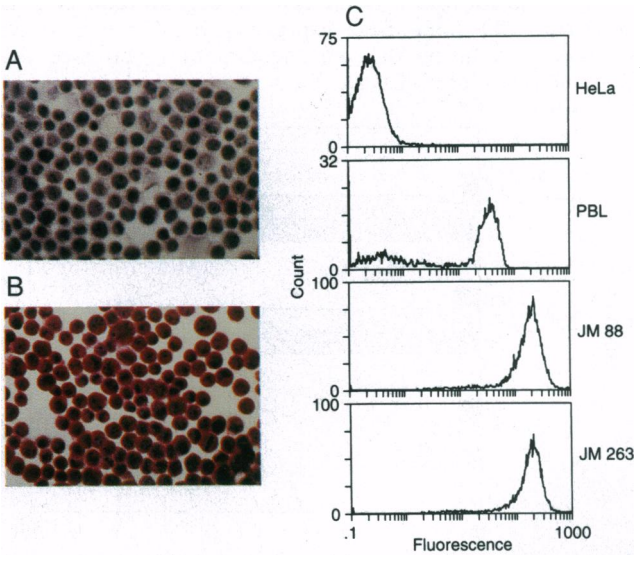

FIG. 1. Immunoenzymatic staining of (*A*) HeLa and (*B*) JM88 cell lines with alkaline phosphatase anti-alkaline phosphatase. Cells binding the anti-CD4 antibody stained red. Cells were removed from their tissue culture plates using trypsin·EDTA cyto-spun slides were fixed with citrate-buffered acetone. (*C*) Flow cytometry analyses of HeLa, peripheral blood lymphocytes, JM88, and JM263 cells stained with phycoerythrin-labeled anti-CD4 antibody.

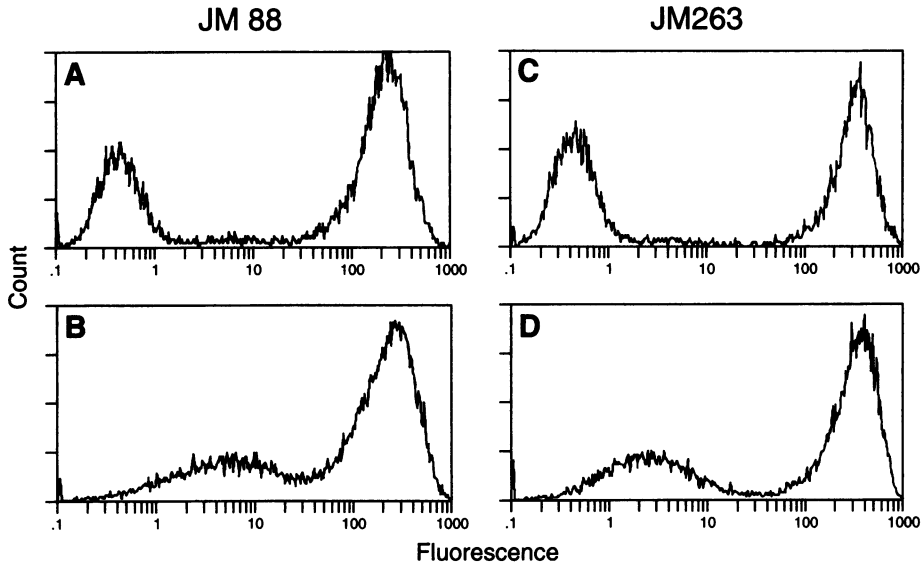

FIG. 2. Flow cytometry analysis of (*A* and *C*) ≈5 × 10^4 HeLa cells and 10^5 JM88 cells versus JM263 cells fixed in 4% paraformaldehyde before mixing and CD4 staining, and (*B* and *D*) cells mixed for 30 min at room temperature before fixation with 4% paraformaldehyde and CD4 staining.

To determine whether direct cellular contact was required for CD4-GPI transfer, we analyzed cell-free supernatants for the presence of CD4-GPI able to incorporate into HeLa cell and RBC membranes. Fig. 4 demonstrates incorporation of the CD4-GPI from JM88 supernatant into RBC membranes. The similarity of fluorescence in cells maintained in JM88 supernatants for 2 hr versus 24 hr suggests stable membrane incorporation of the CD4-GPI receptor in RBCs over this period. Twenty-two percent of the cells fluoresced at levels greater than two standard deviations above the isotypic control at both time points. Most of the CD4-GPI was cleaved with PI-PLC indicating specific GPI linkage of the incorporated CD4-GPI molecules. Supernatant-based transfer was also noted from transfected cell lines expressing CD4-GPI at comparable levels to JM88 cells (data not shown). Supernatant CD4-GPI isolated by affinity chromatography also produced an increase in the incorporated CD4-GPI mean fluorescence (Fig. 5).

Syncytium formation resulting from cocultivation of cells expressing CD4 and those expressing gp160 requires cell adhesion and fusion of their membranes to produce the multinucleated protoplasmic mass. We used the vaccinia-transduced H9 syncytium model to test the biological activity of the incorporated CD4-GPI. HeLa and JM88 cells were used as controls for the formation of syncytium with gp160-expressing H9 cells (Fig. 6 *A* and *B*). The incorporation of JM88-released CD4-GPI into native HeLa cell membranes permitted the formation of syncytium in those cells (Fig. 6*C*). The process was specific for the presence of CD4-GPI and was blocked by the addition of CD4 antibodies (Fig. 6*D*). These results, together with the sensitivity of incorporated CD4-GPI to PI-PLC cleavage (Fig. 3), provide strong evidence for the ability of CD4-GPI to become an integral, biologically active component of the recipient cell's membrane.

DISCUSSION

We have described a cell line able to release CD4-GPI molecules, which incorporate into recipient cell membranes and retain their biological activity. The cells were originally cloned to study rAAV biology in the context of surface receptor expression (unpublished data). They demonstrate the capability of rAAV to stably introduce and express proteins

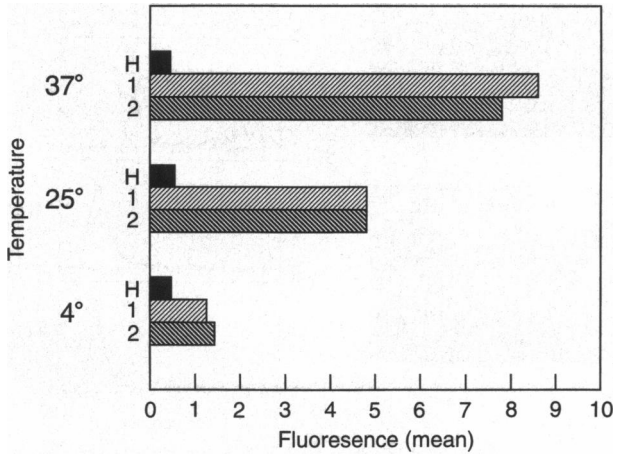

FIG. 3. Temperature dependence of CD4-GPI transfer between cells. HeLa cells (5 × 10^4) and 10^5 JM88 cells were mixed at the temperatures shown for 30 min before fixation, staining, and analysis. Horizontal bars represent mean fluorescence of native HeLa cells (bars labeled H) and JM88-exposed HeLa cells in duplicate experiments (bars labeled 1 and 2).

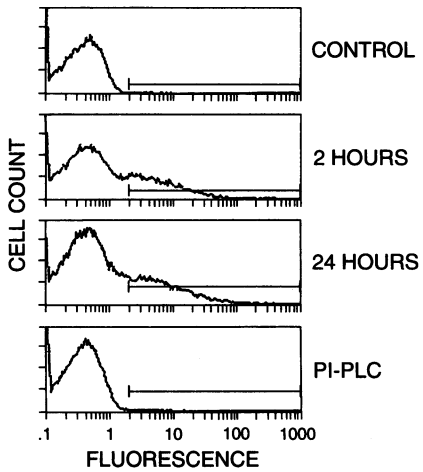

FIG. 4. RBCs incubated with JM88 supernatants. RBCs (1 × 10^6) incubated for 2 hr and 24 hr at 37°C with supernatant CD4-GPI and PI-PLC before CD4 staining and flow cytometry analysis. The bars indicate relative fluorescence greater than two standard deviations above the isotypic control.

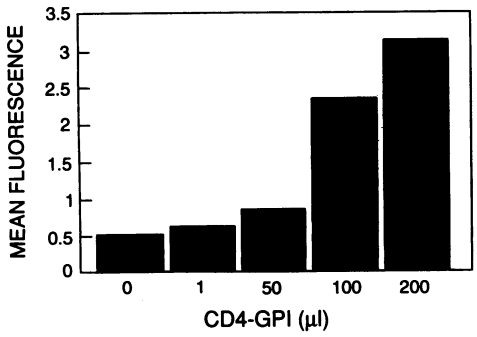

FIG. 5. Mean fluorescence of 1×10^6 HeLa cells with CD4-GPI isolated by affinity chromatography. An equal number of HeLa cells were incubated in serum containing culture medium with increasing volumes of CD4-GPI for 2 hr at 37°C before the addition of anti-CD4 antibody and flow cytometry analysis. Addition of CD4-GPI resulted in a right shift in flow cytometric pattern of cell fluorescence. The pattern of fluorescence remained normally distributed in all samples and represented an semiquantitative increase in surface CD4-GPI per cell.

that require significant posttranslational modifications at very high levels in eukaryotic cells. Our results were not obvious and grew from the unexpected initial observation of a shift in the CD4-stained fluorescence of HeLa cells cocultivated for days with JM88 cells (data not shown). Others have described a soluble form of DAF detected in HeLa cell supernatants and body fluids including urine (21). The secreted DAF did not incorporate into recipient cell membranes and may not be

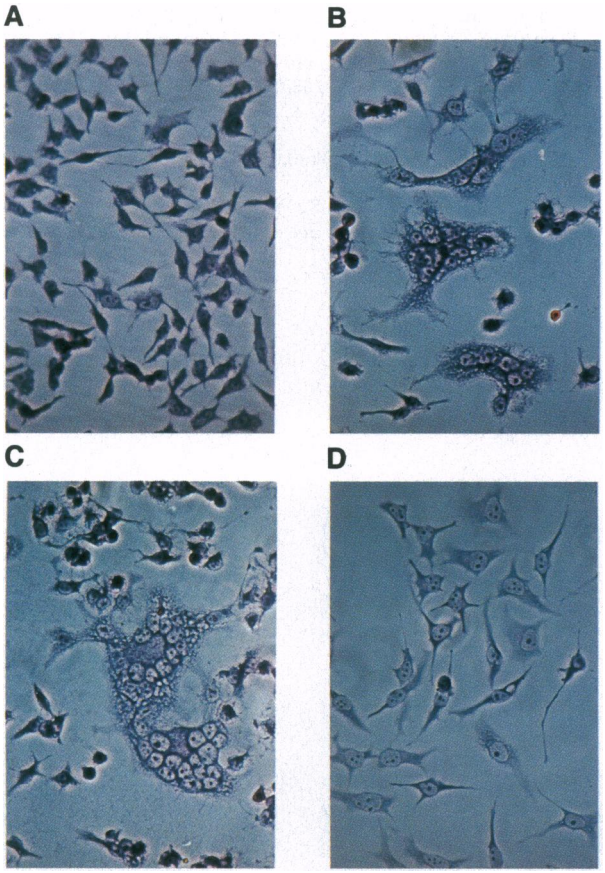

FIG. 6. Syncytium formation between gp160- and CD4-expressing cells. Panels show Wright–Giemsa-stained populations of cells after gp160-expressing H9 cells were incubated with (*A*) HeLa cells, (*B*) JM88 cells, (*C*) HeLa cells coated with CD4-GPI, and (D) CD4-GPI-coated HeLa cells preincubated with anti-CD4 monoclonal antibody.

associated with a GPI anchor due to an alternately spliced form of endogenous DAF mRNA (22). Another cell line expressed a CD4-GPI fusion gene but failed to release detectable CD4-GPI of any form (23). While all the cells express a GPI-anchored CD4 receptor on their plasma membranes, differences in the cell lines include the CD4-GPI-containing genome itself and the absolute level of CD4-GPI expression. These variables may in part explain the novel biological properties of the JM88 cell line.

Contact-mediated transfer of CD4-GPI was demonstrated from high CD4-GPI-expressing clones derived from rAAV-transduced and transfected cell lines (Fig. 2). In both cases, the transfer occurred after a brief exposure (15-min mix followed by a 10-min low-speed centrifugation). The low level of transfer at 4°C suggests an energy-dependent mechanism. Conversely, the higher level of transfer at 37°C suggests that transfer of GPI-anchored surface proteins between living cells is a rapid process that may be initiated simply by cell–cell contact at physiologic temperatures.

The conclusion that CD4-GPI is released and specifically incorporates into recipient membranes is strongly supported by our data. Incorporation of CD4-GPI into RBCs ruled out the possibility that the CD4-GPI was being expressed from contaminating rAAV or other nucleic acids. The sensitivity of the incorporated CD4-GPI molecules to PI-PLC digestion was specific for anchoring by GPI. The syncytium assay demonstrated that the GPI-anchored CD4-GPI detected by flow cytometry represented integrated, biologically active molecules rather than artifactual or nonspecific binding.

Many questions remain, including the ease with which other cell lines that release biologically active, GPI-linked proteins can be created and the physical nature, stability, and metabolism of released and membrane reincorporated CD4-GPI. The CD4-GPI particles in the cell supernatants could represent shed membrane, vesicular, or micelle forms. Alternatively, the supernatant CD4-GPI may have been transported by a component of the bovine serum medium supplement (24). To precisely understand the molecular mechanism of GPI-linked intercellular transfer, cell culture in defined medium preparations, eventual purification, and precise biochemical identification of the supernatant CD4-GPI will be required.

Our *in vitro* model for the release and reincorporation of GPI-linked proteins to lipid bilayers may enhance the interpretation of other reports. These include the finding that schistosomes in contact with plasma evade the alternative complement pathway by incorporating functional host DAF into their membranes (25). GPI anchor-bearing alkaline phosphatase has been detected in human serum (26), and alkaline phosphatase found in calf intestinal lumen hypothetically concentrates the enzyme by GPI incorporation into chyme (27). Scrapie prion protein is thought to originate and replicate from endocytosis and conversion of a GPI-anchored membrane isoform (28). Recently, a transgenic mouse model demonstrated GPI-linked protein transfer between cells by an undefined mechanism (29). Our data support a hypothesis that these *in vivo* observations reflect direct or distant intercellular transfer of GPI-linked proteins. This type of protein transit may be a common biological process in eukaryotes.

Release and reincorporation of CD4-GPI illustrates a novel model of intercellular communication. The model may facilitate additional studies of natural or engineered proteins associated with phosphatidylinositol structures. Obvious parallels linking the supernatant CD4-GPI protein and prions are intriguing. Endogenously produced GPI-linked receptors have many unique biological properties relevant to membrane metabolism and signal transduction, including apical membrane sorting, caveolae clustering, and association with tyrosine kinases (30–34). Experiments should be performed to determine whether these properties persist after exogenous incorporation in the membrane. Since membrane-purified

DAF and CD59 partially correct the defect in RBCs from patients with paroxysmal nocturnal hemoglobinuria (35, 36), GPI-linked receptor transfer from normal to diseased cells should be tested directly by cocultivation experiments. The demonstration of cell adhesion and syncytium formation suggests a role for recombinant GPI-linked proteins in future immunomodulation and antiviral strategies. Notably, strategies directed toward the development of GPI-linked tumor vaccines (37) should consider the possibility of intercellular receptor transfer in their prediction of a desired immune response. Novel strategies aimed at cell-targeted delivery of nucleic acids and other biologically-active substances are also possible. The ability to specifically modify the membrane receptor milieu of living cells has broad implications for the laboratory and clinic.

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