Artificially Lipid-anchored Proteins Can Elicit Clustering-induced Intracellular Signaling Events in Jurkat T-Lymphocytes Independent of Lipid Raft Association*

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We have incorporated artificial lipid-anchored streptavidin conjugates with fully saturated or polyunsaturated lipid anchors into the plasma membranes of Jurkat T-lymphocytes to assess previous conclusions that the activation of signaling processes induced in these cells by clustering of endogenous glycosylphosphatidylinositol-anchored proteins or ganglioside GM1 depends specifically on the association of these membrane components with lipid rafts. Lipid-anchored streptavidin conjugates could be incorporated into Jurkat or other mammalian cell surfaces by inserting biotinylated phosphatidylethanolamine-polyethylene-glycols (PE-PEGs) and subsequently binding streptavidin to the cell-incorporated PE-PEGs. Saturated dipalmitoyl-PE-PEG-streptavidin conjugates prepared in this manner partitioned substantially into the detergent-insoluble membrane fraction isolated from Jurkat or fibroblast cells, whereas polyunsaturated dilinoleoyl-PE-PEG-anchored conjugates were wholly excluded from this fraction, consistent with the differences in the affinities of the two types of lipid anchors for liquid-ordered membrane domains. Remarkably, however, antibody-mediated cross-linking of either dipalmitoyl- or dilinoleoyl-PE-PEG-anchored streptavidin conjugates in Jurkat cells induced elevation of cytoplasmic calcium levels and tyrosine phosphorylation of the scaffolding protein linker of T-cell activation in a manner similar to that observed upon cross-linking of endogenous CD59 or ganglioside GM1. The amplitude of the cross-linking-stimulated elevation of cytoplasmic calcium moreover showed an essentially identical dependence on the level of incorporated streptavidin conjugate for either type of lipid anchor. Confocal fluorescence microscopy revealed that PE-PEG-streptavidin conjugates with saturated versus polyunsaturated anchors showed very similar surface distributions vis à vis GM1 or CD59 under conditions where one or both species were cross-linked. These results indicate that cross-linking of diverse proteins anchored only to the outer leaflet of the plasma membrane can induce activation of Jurkat T-cell-signaling responses, but they appear to contradict previous suggestions that this phenomenon rests specifically on the association of such species with lipid rafts.

Although evidence for the existence of specialized membrane domains known as “lipid rafts” was first reported over a decade ago (1–7), many questions remain concerning the organization, properties, and functional importance of rafts in diverse biological contexts. Rafts have long been suggested to play important roles in membrane signaling in hemopoietic cells (see for example Refs. 8–13), yet evidence both for and against such proposals continues to be reported (14–16). Difficulties in assessing the role of rafts in such processes arise in part from the fact that available approaches to study and to modulate the behavior of rafts in biological membranes remain limited.

Antibody-mediated cross-linking of raft-associated molecules such as glycosylphosphatidylinositol (GPI)-anchored proteins and ganglioside GM1 in T-lymphocyte cell lines induces intracellular signaling events (e.g. elevation of cytoplasmic calcium and phosphorylation of specific proteins) that are similar to those observed in the early stages of T-cell activation mediated by the T-cell receptor (8, 9, 17–19). Such findings have been interpreted to suggest that aggregation and/or growth of rafts within the plasma membrane surface may play a key facilitating or triggering role in T-cell receptor-mediated T-cell signaling. It has been suggested that lateral aggregation/ expansion of rafts in the T-cell membrane may locally concentrate key signaling molecules within such domains and favor exclusion of species that normally antagonize the activation process (for review, see Ref. 12). In this picture, the ability of GM1 and GPI-anchored proteins to induce intracellular signaling processes upon antibody-mediated cross-linking rests on their association with lipid rafts and on their consequent ability to induce raft redistribution and/or growth when cross-linked.

Cross-linking or otherwise manipulating endogenous raft-associated molecules to perturb raft organization entails the inherent potential complication that the behavior of these molecules may be influenced by specific interactions with other membrane components as much as by their physical association with rafts per se. In this study we have used a novel approach to introduce into mammalian cells artificial lipid-anchored streptavidin conjugates whose protein component is foreign to the recipient cells and whose lipid anchor can be constructed to carry acyl chains that confer either an affinity for ordered lipid domains or a strong tendency to avoid such domains. We have used lipid-anchored streptavidin conjugates with different lipid anchors to assess previous proposals that the ability of GPI-anchored proteins and ganglioside GM1, 1 The abbreviations used are: GPI, glycosylphosphatidylinositol; CTB, cholera toxin B subunit; di16:0-, di18:2-PE, dipalmitoyl, dilinoleoylphosphatidylinositolamine; FITC, fluorescein isothiocyanate; GM1, ganglioside GM1; Indo-1, acetoxymethyl ester, 4-(6-carboxy-2-indolyl)-4′-methyl-2,2′-ethylenedioxydianiline-N,N,N′,N′-tetraacetic acid (tetraalkylacetoxymethyl) ester; LAT, linker of activation of T-cells; PE, phosphatidylethanolamine; PEG, polyethylene glycol.

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when cross-linked by antibodies, to activate intracellular signaling events similar to those during T-cell receptor-mediated cell activation rests specifically on their association with lipid rafts. Comparison of the behavior in cell membranes of exog- enously incorporated lipid-anchored species that differ minimally in their overall structure but greatly in their raft-assoc- iating tendencies offers an attractive new strategy to investigate the role of raft association in various aspects of the behavior of endogenous membrane components such as GPI-anchored proteins and glycosphingolipids.

Experimental Procedures

Materials—Unlabeled and Texas Red-labeled streptavidin were obtained from Jackson Immunoresearch Laboratories, and unlabeled and FITC-labeled cholera toxin B subunit (CTB) was from Sigma. Mouse anti-human CD3 monoclonal antibody OKT3 was the generous gift of Dr. Luchino Cohen (Laboratory of Immunology, Université de Montréal). Mouse monoclonal anti-human CD59 antibody was purchased from Serotec, mouse monoclonal anti-human transferrin receptor antibody was from BD Pharmingen, rabbit anti-streptavidin and anti-chol- era toxin antisera was from Sigma, unlabeled goat anti-mouse and anti-rabbit antibodies were from BioCan, and 1:100 and 1:4 goat anti-mouse IgGs were from ImmunoResearch Laboratories. Anti- phosphotyrosine and mouse anti-human LAT antibodies were obtained from Upstate Cell Signaling Solutions. Indo-1 acetoxymethyl ester was purchased from Sigma.

Cell Culture—E6–1 Jurkat T-lymphocytes (American Type Culture Collection) were cultured in RPMI 1640 media with 2 mM l-glutamine (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 10 mM HEPES, 1 mM sodium pyruvate, 100 units/ml penicillin, and 10 µg/ml streptomycin. 3T3-L1 preadipocyte and COS-7 fibroblast cells were cultured in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% serum, 2 mM l-glutamine, and 50 µg/ml gentami- cin (Invitrogen).

Synthesis and Cellular Incorporation of PE-PEG-Biotin—o-Dia- minodiphenyl sulfone (154 g) was reacted in 99.1% methylene chloride/diisopropy- lethylene with 1 mol eq of biotin succinimidyl ester under argon for 2 h at 25 °C, then purified by ion-exchange chromatography on SP-Sephadex C50 (Amersham Biosciences), eluting the desired mono- derivatized product with 10 mM NaCl. The lyophilized product was at 25 °C in 10:1 methylene chloride/dimethylformamide, and the PE-PEG-biotin product was purified by preparative TLC in 70:20:1.5:1.5 methylene chloride/methanol/water/ammonium hydroxide. PE-PEG-biotin stock solutions in methylene chloride/methanol were standardized by phosphorus determination as described previously (21).

For incorporation of biotinylated PE-PEGs into cells, aliquots of PE-PEG-biotin were dried under nitrogen and then under high vacuum, redissolved in a small volume (<1% of final aqueous volume) of di- methylformamide, mixed with 290 µm sucrose, 1 mM HEPES, pH 7.2, and bath-sonicated for 1 min at room temperature. Jurkat cells (7.5 × 10^7 cells/ml) were incubated with PE-PEG-biotin at the indicated final concentrations in sucrose/HEPES for 1 min at 37 °C, then centrifuged, twice resuspended, and centrifuged in serum-free medium and incu- bated for 1 h at 37 °C. The cells were then incubated with 30 µg/ml unlabeled streptavidin or (for microscopy) 15 µg/ml Texas Red-labeled streptavidin and again twice centrifuged from serum-free medium to remove unbound streptavidin. PE-PEG-anchored streptavidin was in- corporated into COS-7 cells (grown to 70–80% conflueney on coverslips or in culture dishes) in a similar manner using serum-free Dulbecco’s modified Eagle’s medium in place of RPMI and carrying out all incubations and washes on adherent cells.

Low Temperature Detergent Fractionation—All reagents and solutions were chilled to 0 °C, and all procedures were conducted on ice. Pelleted cells (1 × 10^7 cells) were lysed in 2 ml of 150 mM NaCl, 25 mM Tris, 5 mM EDTA, pH 7.5, containing 1% Triton X-100, 1 mM phenyl- methanesulfonyl fluoride, and 10 µg/ml each of aprotinin, pepstatin, and leupeptin. After incubation for 10 min the lysate was transferred to a SW41 ultracentrifuge tube, mixed with 2 ml of 80% sucrose in the buffer, and overlaid with 5 ml of 35% and 2.5 ml of 5% sucrose in the same buffer. The samples were centrifuged at 120,000 × g for 16–20 h at 4 °C, and fractions were collected from the gradients and analyzed by SDS-PAGE and immunoblotting.

Cytosolic Protein Measurements—For fluorescence monitoring of cytosolic calcium levels, Jurkat cells were loaded with 2 µM Indo-1 acetoxymethyl ester (Molecular Probes/Invitrogen) in serum-free me- dium for 30 min at 37 °C, then washed and incubated further for 30 min. To appropriately pretreated cell samples (6 × 10^5 cells/ml), the following antibodies were added at 37 °C to induce cross-linking of cell surface components while monitoring Indo-1 fluorescence: anti-CD3 antibody OKT3, anti-CD59 antibody (1:250) to cells preincubated with varying amounts of CTB for 10 min at 37 °C, mouse anti-human CD59 at varying concentrations to cells preincubated with mouse anti-human CD59 (10 µg/ml) for 10 min at 37 °C, or anti-streptavidin antiserum (1:250) to cells preincubated with varying concentrations of PE-PEG-biotin followed by streptavidin as described above. Fluorescence measurements were carried out in a PerkinElmer Life Sciences LS50B luminescence spectrometer with a stirred and thermostatted sample holder, continuously recording the ratio of fluorescence emission inten- sities at 480 versus 405 nm with excitation set at 355 nm.

Tyrosine Phosphorylation Analyses—For analysis of tyrosine phos- phorylation stimulated by CD3 cross-linking in Jurkat cells, cell sus- pensions (1.5 × 10^6 cells/ml) in serum-free medium were preincubated on ice with 1 µM mouse anti-human CD3 OKT3 antibody (60 min) and then the addition of anti-mouse IgG. For CD3 cross-linked anti-mouse IgG was from ImmunoResearch Laboratories. Anti- phosphotyrosine and mouse anti-human LAT antibodies were obtained from Upstate Cell Signaling Solutions. Indo-1 acetoxymethyl ester was purchased from Sigma.

RESULTS

We and others have previously shown that long chain diacyl-PE-PEGs, when initially dispersed in pure form in aqueous media, can rapidly integrate into lipid bilayers, with which they remain tightly associated thereafter (22–24). These find- ings led us to explore the strategy illustrated in Fig. 1 to incorporate artificial lipid-anchored streptavidin conjugates, whose lipid anchor structures and properties can be systemat- ically varied, into the surface membranes of living mammalian cells. In this approach, cells are first incubated for short times with a dispersion of a PE-PEG-biotin in an iso-osmotic but low ionic strength medium (which promotes formation of optically clear, presumably micellar (25) suspensions of PE-PEG-biotin species with a variety of acyl chains). After washing and a 1-h post-incubation in serum-free medium, the cells are incubated in the cold with streptavidin, then washed to remove unbound protein. As shown in Fig. 2A, under these conditions immuno- blotting detects streptavidin bound to Jurkat cells only when the cells have been pretreated with PE-PEG-biotin. The amount of bound streptavidin increases steadily with the
amount of PE-PEG-biotin added to the cells. PE-PEG-streptavidin conjugates with a variety of acyl chains can be incorporated into cell surfaces in this manner, although the level of conjugate incorporated (for cells treated with a fixed concentration of different PE-PEG-biotin species) varies to some extent with the acyl chain composition of the biotinylated PE-PEG (Fig. 2B).

Quantitation of the amount of streptavidin bound in cells treated as just described, carried out by SDS-PAGE and immunoblotting of solubilized cell samples alongside known amounts of streptavidin, indicated that cells treated with 0.5–8 μM di16:0PE-PEG-biotin or 0.25–4 μM di18:2PE-PEG-biotin followed by streptavidin (30 μg/ml) incorporated from 5 × 10^5 to 6 × 10^6 molecules of streptavidin/cell depending on the concentration of PE-PEG-biotin used. In analogous experiments we determined that cells treated with CTB under the conditions described under “Experimental Procedures.”

In agreement with the immunoblotting results just described, cells pretreated with PE-PEG-biotin show readily observable surface fluorescence by confocal microscopy after incubation with Texas Red-labeled streptavidin and subsequent washing (Fig. 2C), whereas cells incubated with labeled streptavidin without prior treatment with PE-PEG-biotin showed no detectable fluorescence (not shown). The lipid-anchored streptavidin shows a uniform distribution on the cell surface, both for conjugates with disaturated lipid anchors (as shown in Fig. 2C) and for conjugates with unsaturated anchors (not shown). Similar results were obtained with 3T3-L1 and COS-7 cells in monolayer culture (not shown).

To investigate whether disaturated long chain PE-PEG-streptavidin conjugates, once integrated into cellular membranes, could also incorporate into “raft” domains, we examined the distributions of cell-incorporated (saturated) di16:0PE- or (tetrainsaturated) di18:2PE-streptavidin conjugates on sucrose density gradients after low temperature cell solubilization with Triton X-100. Replicate aliquots of Jurkat cells were treated with either di16:0PE-PEG-biotin or di18:2PE-PEG-biotin followed by streptavidin as described above. Two additional aliquots of cells were labeled with CTB, a GM1-binding protein that is widely used as a raft marker in these cells (8, 10, 26–28). Samples of cells labeled with either lipid-PEG-streptavidin conjugate were combined with CTB-treated cells and incubated at 0 °C with Triton X-100 (1%), then loaded under sucrose gradients and centrifuged at 4 °C to separate a low density raft fraction from the bulk of cellular protein, which remains near the bottom of the gradient (26, 29). Fractions from the gradient were analyzed by Western blotting for streptavidin or CTB or for transferrin receptor as a non-raft marker (30–32). Results from a representative experiment are shown in Fig. 3A. A substantial proportion of the di16:0PE-PEG-streptavidin conjugate localizes to low density fractions, as does the CTB-GM1 complex. By contrast, the di18:2PE-streptavidin conjugate is entirely found in higher density fractions enriched in the transferrin receptor. The fractionation behavior of the di18:2-streptavidin conjugate was not altered when the cell-incorporated conjugates were cross-linked with anti-streptavidin before detergent fractionation (not shown). A marked difference in the distributions of the di16:0PE- and di18:2PE-PEG-streptavidin conjugates was also observed when 3T3-L1 cells (Fig. 3B) or COS-7 cells (not shown) incorporating these markers were fractionated by cold Triton treatment and gradient centrifugation. The divergent fractionation behavior observed for the di16:0PE- and di18:2PE-PEG-streptavidin...
complexes in the above experiments is in accord with the differing physical properties of their lipid anchors and indicates that remodeling of the lipid moieties must be minimal over the time scale of the experiments described in the remainder of the “Results” section.

When PE-PEG-streptavidin conjugates incorporated into sonicated liquid-disordered 1-palmitoyl-2-oleoyl-phosphatidylcholine vesicles were added to Jurkat cells (under which condition incorporation of the conjugates into cellular membranes is expected to be negligible (22)), no di16:0-PE-PEG-streptavidin conjugate was detected in low density fractions upon subsequent cold Triton X-100 treatment and gradient centrifugation as described above (Fig. 3A, bottom blot). This result indicates that the behavior of the di16:0 PE conjugate in the above fractionation experiments genuinely reflects the distribution of this species in cellular membranes before Triton addition rather than a redistribution occurring after the addition of detergent. This conclusion agrees with those reported previously (33) for saturated lipids in model membrane systems containing coexisting liquid-ordered and liquid-disordered domains.

Cytoplasmic Calcium Measurements—Antibody-mediated cross-linking of either the T-cell receptor complex (using anti-CD3 antibodies) or GM1-bound CTB in Jurkat cells has been reported to trigger intracellular signaling processes mimicking early events in T-cell activation induced by MHCII-mediated antigen presentation (8, 34), including elevation of cytoplasmic calcium levels and enhanced tyrosine phosphorylation of specific cellular signaling proteins. On the basis of detergent fractionation and fluorescence-microscopic experiments which suggest that both the T-cell receptor and CTB-bound GM1 are associated with lipid rafts under these conditions (8, 30, 31), it has been suggested that cross-linking of these membrane components leads to lateral clustering or growth of rafts on the T-cell surface, which in turn stimulates intracellular signaling events such as those just noted. We therefore examined whether anti(streptavidin)-mediated cross-linking of the cell membrane-inserted PE-PEG-streptavidin conjugates could stimulate these same signaling events, and if so, whether the efficiency of such stimulation was affected by the acyl chain composition of the inserted conjugates, as expected if such stimulation reflects enhanced clustering of lipid rafts induced by antibody-mediated cross-linking of raft-associated conjugate molecules.

As reported previously (8, 34) and as illustrated in Fig. 4A, cross-linking of the T-cell receptor using the anti-CD3 antibody OKT3 induces a rapid initial elevation of cytoplasmic calcium, as monitored by fluorescence measurements on Jurkat cells loaded with the calcium binding dye Indo-1 acetoxyethyl ester. After peaking within 1–2 min after the addition of anti-CD3, cytoplasmic calcium levels subsequently declined markedly, although they remained above prestimulation levels for at least 30 min after antibody addition. The time courses of elevation of $[Ca^{2+}]_{cyto}$ upon the addition of anti-CD3 were essentially identical in untreated Jurkat cells or in cells pretreated with di16:0- or di18:2-PE-PEG-biotin followed by streptavidin (not shown). The addition of anti-CTB antiserum to Jurkat cells pretreated with CTB also leads to a rapid elevation of cytoplasmic calcium, as illustrated in Fig. 4C and also as reported previously (8). Cross-linking of the endogenous GPI-anchored protein CD59 likewise stimulates an elevation of cytoplasmic calcium, albeit of somewhat lower magnitude even at high doses of antibody (Fig. 4D).

Having confirmed through the above experiments the previous findings that cross-linking of either CD3- or GM1-bound CTB can stimulate elevation of $[Ca^{2+}]_{cyto}$ in Jurkat cells, we next examined the response of cytoplasmic calcium levels upon the addition of anti-streptavidin antiserum to Jurkat cells incorporating PE-PEG-streptavidin conjugates. As shown in Fig. 4E, anti-streptavidin addition to cells incorporating di16:0-PE-PEG-streptavidin conjugates leads to a rapid elevation in cytoplasmic calcium levels, which is comparable in magnitude to that induced by anti-CD3. Interestingly, however, the addition of anti-streptavidin to cells incorporating di18:2-PE-PEG-streptavidin conjugates induces an elevation of $[Ca^{2+}]_{cyto}$ very similar to that observed for cells incorporating the analogous di16:0-conjugate (Fig. 4F). Similar results were also obtained using cells incorporating a diunsaturated dipalmityloyleoyl-(di-cis-9-hexadecenoyl) -PE-PEG-streptavidin conjugate (not shown). The addition of anti-streptavidin to cells not pretreated with PE-PEG-biotin and streptavidin did not cause any change in cytoplasmic calcium levels (Fig. 4E, bottom trace).

The results just described suggested that the ability of anti-streptavidin antibody to induce rapid elevation of $[Ca^{2+}]_{cyto}$ in Jurkat cells incorporating PE-PEG-streptavidin conjugates is not correlated with the acyl chain structure (long chain saturated versus polyunsaturated) of the phospholipid anchor. To examine this question more quantitatively, replicate aliquots of cells were incubated first with varying concentrations of either di16:0- or di18:2-PE-PEG-biotin, then with streptavidin, and the amplitude of the elevation of $[Ca^{2+}]_{cyto}$ upon subsequent
addition of anti-streptavidin antiserum was determined. The amount of cell-bound streptavidin was also determined for each sample by immunoblotting, and the relationship between the level of bound streptavidin and the peak amplitude of the anti-streptavidin-induced calcium response was determined as a function of the amount of cell-incorporated PE-PEG-streptavidin conjugate. As shown in Fig. 5, the maximum amplitude of the streptavidin-induced calcium response increased with the level of incorporated PE-PEG-streptavidin conjugate in a manner that is indistinguishable for cells incorporating either the di16:0- or the di18:2-anchored conjugate.

Tyrosine Phosphorylation Induced by Conjugate Cross-linking—Antibody-mediated cross-linking of either CD3- or GM1-bound cholera toxin in Jurkat cells has been reported to induce a rapid enhancement of tyrosine phosphorylation of specific signaling proteins, including notably the scaffolding protein linker of activation of T-cells (LAT (8, 35)). We therefore examined whether cross-linking of cell surface-incorporated lipid-PEG-streptavidin conjugates could similarly stimulate tyrosine phosphorylation of LAT, and if so, whether this ability was dependent on the saturated versus unsaturated nature of the conjugate lipid “anchors.” As shown in Fig. 6 and in agreement with previous reports (8, 35), treatment of cells with anti-CD3 and secondary antibody leads to a rapid but transient elevation of the level of LAT tyrosine phosphorylation detected by antiphosphotyrosine immunoblotting. The addition of anti-(cholera toxin) antiserum to CTB-pretreated cells likewise stimulates tyrosine phosphorylation of LAT, again in accord with previous findings (8), although the time course is somewhat slower and more sustained than is the response to CD3 cross-linking. Antibody-induced cross-linking of GPI-anchored CD59 induces a similarly prolonged elevation of LAT tyrosine phosphorylation. The addition of anti-streptavidin antiserum to Jurkat cells incorporating either di16:0- or di18:2PE-PEG-streptavidin conjugates at similar levels (determined by anti-streptavidin immunoblotting) also stimulated LAT phosphorylation, again with more sustained time courses that were observed after CD3 cross-linking.

Cell Surface Distribution of Diacyl PE-PEG-Streptavidin Conjugates—Antibody-mediated cross-linking of certain raft-associating membrane components has been reported to induce at least a partial colocalization with other raft markers as observed by confocal microscopy in Jurkat cells (8, 11, 36). We accordingly used confocal imaging to compare the cell surface distributions of lipid-PEG-streptavidin conjugates with those of GM1-bound CTB and endogenous CD59 in live cells when
angles

concentrations of either PE-PEG-biotin species followed by streptavidin (open triangles). Replicate aliquots of cells were preincubated with varying concentrations of either PE-PEG-biotin species followed by streptavidin (30 μg/ml). The peak Indo-1 fluorescence measured upon subsequent addition of anti-streptavidin to an aliquot of each treated cell sample was then determined from time courses like those shown in Fig. 4, and the relative level of cell-bound streptavidin (normalized to sample protein content) was measured for a replicate aliquot by anti-streptavidin immunoblotting and densitometry.

The indicated one or more of these components were cross-linked with appropriate antibodies. As illustrated in Fig. 7, panels A and B, anti-cholera toxin-induced cross-linking of CTB in cells incorporating di16:0PE-PEG-streptavidin conjugates resulted in a markedly inhomogeneous distribution of CTB around the cell periphery but had little effect on the distribution of CTB (panel C). Simultaneous addition of anti-cholera toxin and anti-streptavidin antisera to the cells led to a marked co-clustering of bound CTB and di16:0PE-PEG-streptavidin conjugates (Fig. 7, panels A–C). Interestingly, although the two proteins redistribute to similar regions of the cell surface, leaving large areas depleted of these species, their distributions within the regions of concentration are not entirely coincident. Similar results are observed in analogous experiments using cells treated with CTB and incorporating di18:2PE-PEG-streptavidin conjugates (Fig. 7, panels D–F). In experiments parallel to those just described we examined the effects of cross-linking the endogenous GPI-anchored protein CD59 on the surface distributions of GM1-bound cholera toxin or lipid-anchored streptavidin conjugates. As shown in Fig. 8A, cross-linking of CD59 alone induces a highly inhomogeneous distribution of the protein around the cell periphery. In cells pretreated with fluorescent CTB, in some regions of the cell periphery where CD59-associated fluorescence is concentrated one can observe a local accumulation of CTB fluorescence as well (Fig. 8, panels B and C). Nonetheless, under these conditions most of the CTB fluorescence remains homogeneously distributed around the cell periphery. Similarly, when CD59 was cross-linked in cells incorporating di16:0PE-PEG-Texas Red-streptavidin conjugates, the anchored streptavidin...
beled secondary antibody. Red-streptavidin were incubated with anti-CD59 and fluorescein-la-
labeled secondary antibody.

A

Red streptavidin (merged image. A–C, panels

distributions of cross-linked CD59 and GM1-bound
CTB or lipid-anchored streptavidin conjugates in Jurkat cells. A–C, cells labeled with CTB-FITC were incubated with anti-CD59 and rhodamine-labeled secondary antibody. A, CD59. B, CTB-FITC. C, merged image. D–F, cells incorporating di16:0PE-PEG-anchored Texas Red streptavidin (SAv) were incubated with anti-CD59 and fluorescein-labeled secondary antibody. D, CD59. E, Texas Red-streptavidin. F, merged image. G–I, cells incorporating di18:2PE-PEG-anchored Texas Red-streptavidin were incubated with anti-CD59 and fluorescein-la-
labeled secondary antibody. G, CD59. H, Texas Red-streptavidin. I, merged image.

showed an apparent enrichment in regions of the cell periphery where CD59 fluorescence was concentrated (Fig. 8, panels D–F). Very similar results were observed when CD59 was cross-linked in cells incorporating di18:2PE-PEG-anchored Texas Red-streptavidin (Fig. 8, panels G–I).

DISCUSSION

The method described here to incorporate exogenous lipid-anchored soluble proteins into cells is similar in some respects to the previously described technique of "cell painting" by which purified exogenous GPI-anchored proteins can be incor-
porated into cell surfaces (37–42). However, for applications like those illustrated here it is advantageous to utilize proteins tethered at the membrane surface to lipid anchors that have simple and unnatural structures and, hence, are less likely to interact in a biospecific manner with other membrane compo-
nents than is for example a GPI-anchor, which is native to all mammalian cells. The artificial lipid anchors used here also allow wide variation in the structures of their hydrocarbon chains, allowing comparison of the behavior of cell-incorpo-
rated "matched sets" of conjugates with similar overall structures but very different physical properties, as exemplified by the dipalmityloyl- and dilineoylphospholipid-PEG-streptavidin conjugates examined in the present study. While this work was in progress a similar approach to anchoring soluble proteins to cell membranes using artificial lipid anchors was reported by Kato et al. (43), analogous to methods previously developed to incorporate lipid-grafted polyethyleneglycol derivatives (and proteins that can subsequently be linked to such derivatives) into liposomes (23). As noted by Kato et al. (43) this approach has considerable potential for cell surface engineering, particular-
ly given that a variety of means can be envisioned as alternatives to the biotin-streptavidin interaction used here to tether proteins to suitably modified lipid-PEG anchors.

As already described, the implication of lipid raft redistribution in T-cell signaling either as an initiator or as a conse-
quence of early steps in T-cell activation remains unclear. Early evidence (summarized in Ref. 12) suggested that reclo-
alization of lipid rafts to the immune synapse might constitute a critical early step in antigen-dependent lymphocyte activation. This conclusion has since been challenged by various findings (14, 16, 44–46), whereas other results suggest that raft reor-
ganization indeed plays a key role in physiological T-cell activation (13, 47, 48). Part of the initial evidence suggesting involvement of rafts in T-cell signaling was provided by observ-
ations that antibody-induced cross-linking of GPI-proteins or GM1-bound CTB can trigger intracellular signaling events re-
sembling some of the early events occurring during physiological T-cell activation (8, 17, 19, 49, 50). It has been suggested that cross-linking of these molecules on the lymphocyte surface triggers intracellular signaling events by causing lateral redis-
tribution and/or growth of rafts within the plasma membrane, favoring the enrichment of proteins promoting cell activation, and the depletion of species that antagonize such activation (e.g. CD45) within regions where rafts become concentrated. In this model the ability of GPI-proteins and glycosphingolipids to trigger cell signaling events upon cross-linking rests directly on their abilities to associate with lipid rafts.

In this study we have sought to evaluate the hypothesis just noted by examining the surface distributions and signaling potential in Jurkat cells of artificial lipid-anchored protein conjugates whose lipid residues can be chosen to enhance or to minimize their expected abilities to interact with liquid-ordered lipid microdomains. We find that upon cross-linking on the cell surface, PE-PEG-anchored streptavidin conjugates can induce intracellular signaling events (elevation of cytoplasmic calcium levels and enhanced tyrosine phosphorylation of pro-
teins such as LAT) similar to those observed upon cross-linking of endogenous raft constituents such as CD59 or ganglioside GM1. Surprisingly, however, these effects are quantitatively very similar whether PE-PEG-streptavidin conjugates with a saturated dipalmitoyl anchor or a tetraunsaturated dillinoleoyl anchor are incorporated into the cells. Similarly, the surface distributions of streptavidin conjugates with saturated versus polyunsaturated anchors, as observed by confocal microscopy, vary in a very similar manner vis à vis those of GM1 or CD59 under conditions where the anchored streptavidin and/or these endogenous membrane components are cross-linked. Nonetheless, the detergent fractionation behavior of the saturated and disaturated conjugates when incorporated into Jurkat or fibro-
blast cells differs markedly, in the manner expected given the vastly different affinities of their phospholipid anchors for li-
quid-ordered lipid domains as determined by a direct fluores-
cence assay in lipid bilayer systems modeling the plasma mem-
bane outer leaflet (51). This result indicates not only that the lipid anchors of the PE-PEG-streptavidin conjugates exhibit distinct physical properties when incorporated into mamma-
lian cell membranes but also, importantly, that the lipid an-
chors do not undergo extensive remodeling of their fatty acyl groups on the time scale of the experiments described here.

As we observed, the activation of calcium signaling and protein tyrosine phosphorylation induced by cross-linking of cell surface components such as CD59 and GM1-bound CTB in Jurkat cells may reflect a general property of proteins that are anchored only to the outer membrane leaflet rather than a property specific to raft-associating pro-
teins. This finding challenges previous suggestions that lateral clustering of GPI proteins or GM1 triggers intracellular re-
sponses such as cytoplasmic calcium elevation and protein tyrosine phosphorylation by clustering lipid rafts within the lymphocyte cell membrane (8, 18). It could be argued that antibody-mediated cross-linking of streptavidin anchored to the Jurkat cell plasma membrane by unsaturated as well as saturated PE-PEGs could induce lateral redistribution of rafts, in the former case by an indirect mechanism in which redistribution of the unsaturated PE-PEG-streptavidin conjugates causes a reorganization of non-raft regions of the bilayer, which
in turn affects the distribution of raft domains. However, this proposal appears unlikely to explain the finding that at any given level of incorporation into Jurkat cells, PE-PEG-streptavidin conjugates with saturated or polyunsaturated lipid anchors induce very similar elevations of cytoplasmic calcium levels upon cross-linking (Fig. 5). Moreover, if this proposal was correct, the raft association or exclusion of a given membrane component would have no predictive value for understanding its ability to trigger signaling events in lymphocytes upon cross-linking.

Our current results of course do not negate the potential importance of rafts in physiological T-cell signaling. However, they clearly indicate that inducing lateral redistribution of non-raft-associated species such as unsaturated PE-PEG-streptavidin conjugates within the Jurkat cell plasma membrane can induce intracellular signaling events similar to those that are observed when endogenous raft-associating molecules are artificially clustered. It remains to define how lateral clustering of a non-raft-associated species such as the di18:2PEG-streptavidin conjugate examined here can activate signaling events within Jurkat cells. We suggest that phenomena such as steric “cross-talk” between different protein species on the crowded cell surface may offer alternatives to raft aggregation as mechanisms by which lateral redistribution of cell surface proteins could trigger intracellular signaling events.

Our confocal-microscopic observation that streptavidin molecules anchored to the Jurkat cell plasma membrane via a polyunsaturated (and hence raft-avoiding) lipid anchor appear to co-enrich with antibody-clustered CD59 to a similar extent as do analogous saturated lipid-streptavidin conjugates under-reporting as mechanisms by which lateral redistribution of cell surface proteins could trigger intracellular signaling events.

The present results demonstrate the potential utility of artificial lipid-anchored protein conjugates as tools to assess the extent to which raft association per se determines the physical and functional properties of raft-associated molecules such as glycosphingolipids and GPI-anchored proteins. It will be of interest to employ strategies similar to that employed here to investigate such questions in other experimental systems as well.

REFERENCES

1. Cinek, T., and Horejsi V. (1992) J. Immunol. 149, 2262–2270
2. Brown, D. A., and Rose, J. K. (1992) Cell 68, 533–544
3. Simons, K., and Ikonen, E. (1997) Nature 387, 569–572
4. Brown, D. A., and London, E. (1998) Annu. Rev. Cell Dev. Biol. 14, 111–136
5. Brown, D. A., and London, E. (1998) J. Membr. Biol. 164, 103–114
6. Simons, K., and Toomre, D. (2000) Nat. Rev. Mol. Cell Biol. 1, 31–39
7. Edelstein, M. (2000) Annu. Rev. Biophys. Biomol. Struct. 32, 257–283
8. James, P. W., Ley, S. C., and Magee, A. I. (1999) J. Cell Biol. 147, 447–461
9. James, P. W., Ley, S. C., and Magee, A. I. (2000) Semin. Immunol. 12, 23–34
10. Tuosto, L., Parolini, I., Schroder, S., Sargiacomo, M., Lanzavecchia, A., and Viola, A. (2001) Eur. J. Immunol. 31, 345–349
11. Jordan, S., and Rodgers, W. (2003) J. Immunol. 71, 78–87
12. Dykstra, M., Cherukuri, A., Sohn, H. W., Tseung, S. J., and Pierce, S. K. (2003) Annu. Rev. Immunol. 21, 451–487
13. Pizzo, P., and Viola A. (2004) Microbes Infect 6, 686–692
14. Pizzo, P., Giuriato, E., Tassi, M., Benedetti, A., Pozzan, T., and Viola, A. (2002) Eur. J. Immunol. 32, 3082–3090
15. Munro S. (2003) Cell 115, 377–388
16. Glebov, O. O., and Nichio, B. J. (2004) Nat. Cell Biol. 6, 238–243
17. Brown, D. (1993) Curr. Opin. Immunol. 5, 349–354
18. Horejsi, V., Drbal, K., Cebecauer, M., Cerny, J., Brdicka, T., Angelisova, P., and Stockinger, H. (1999) Immunol. Today 20, 356–361
19. Harder, T., and Simons, K. (1999) Eur. J. Immunol. 29, 556–562
20. Shahinian, S., and Silvius, R. J. (1995) Biochemistry 34, 5813–5822
21. Lowry, R. R., and Tinsley, I. J. (1974) Lipids 9, 491–492
22. Silvius, R. J., and Zuckermann, M. J. (1993) Biochemistry 32, 1315–1316
23. Fenske, D. R., Palmer, L. R., Chen, T., Wong, K. F., and Cullis, P. R. (2001) Biochem. Biophys. Acta 1512, 259–272
24. Palmer, L. R., Chen, T., Lam, A. M., Fenske, D. B., Wong, K. F., MacLachlan, I., and Cullis, P. R. (2003) Biochem. Biophys. Acta 1611, 204–216
25. Johnson, M., and Edwards, K. (2003) Biophys. J. 85, 3839–3847
26. Fra, A. M., Williamson, E., Simons, K., and Parton, R. G. (1994) J. Biol. Chem. 269, 30745–30748
27. Tangamanur, S., Brodbeck, U., and Hoeslius, D. C. (1997) Biochem. Biophys. Acta 1328, 227–236
28. Parmar, C., Parley, D., Job, P., and Magee, A. I. (2003) Exp. Cell Res. 285, 27–38
29. Lisanti, M. P., Scherer, P. E., Vlodavsky, J., Zeng, T., Hermansen-Vosatch, A., Chen, T., Weis, W. I., and Sargiacomo, M. (1994) J. Cell Biol. 126, 11–128
30. Montici, C., Langlet, C., and Bertrand, A., 1998 EMBO J. 17, 5334–5348
31. Xavier, R., Brennan, T., Li, Q., McCormack, C., and Seed, B. (1998) Immunity 8, 723–732
32. Giuriato, E., McIntosh, D. P., Tassi, M., Gamberucci, A., and Benedetti, A. (2000) J. Biol. Chem. 275, 111–117
33. Schroeder, R., London, E., and Brown, D (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12130–12134
34. Weiss, A., and Littman, D. R. (1994) Cell 76, 263–274
35. Zhang, W., Sloan-Lancaster, J., and Cullis, P. R. (1994) Science 264, 27–38
36. Rodgers, W., and Zavazavadijn, J. (2001) Exp. Cell Res. 267, 173–188
37. Fenske, D. R., Palmer, L. R., Chen, T., Wong, K. F., and Cullis, P. R. (2003) Annu. Rev. Immunol. 21, 723–732
38. Pizzo, P., Giuriato, E., Tassi, M., Gamberucci, A., and Benedetti, A. (2000) J. Biol. Chem. 275, 111–126
39. Schroeder, R., London, E., and Brown, D (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12130–12134
40. Bronschweig, E. B., Van den Berg, C. W., Cinek, T., Hallett, M. B., Horejsi, V., Morgan, B. P., and Magee, A. I. (2002) J. Immunother. 25, 390–400
41. Premkumar, D. R., Fukuzawa, Y., Selevier, D., Bronschweig, E., Rosenberry, T. L., Tykocinski, M., and Magee, A. I. (2001) J. Cell. Biochem. 82, 234–245
42. Simons, K., and Ikonen, E. (1997) Nature 387, 569–572
43. Edelstein, M. (2000) Annu. Rev. Biophys. Biomol. Struct. 32, 257–283
44. James, P. W., Ley, S. C., and Magee, A. I. (2000) Semin. Immunol. 12, 23–34