Endocytosis of GPI-anchored Proteins in Human Lymphocytes: Role of Glycolipid-based Domains, Actin Cytoskeleton, and Protein Kinases

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Abstract. GPI-anchored surface proteins mediate many important functions, including transport, signal transduction, adhesion, and protection against complement. They cluster into glycolipid-based membrane domains and caveolae, plasmalemmal vesicles involved in the transcytosis and endocytosis of these surface proteins. However, in lymphocytes, neither the characteristic flask-shaped caveolae nor caveolin, a transmembrane protein typical of caveolae, have been observed. Here, we show that the GPI-anchored CD59 molecule on Jurkat T cells is internalized after cross-linking, a process inhibited by nystatin, a sterol chelating agent. Clustered CD59 molecules mostly accumulate in non-coated invaginations of the lymphocyte membrane before endocytosis, in marked contrast with the pattern of CD3-TCR internalization. Cytochalasin H blocked CD59 internalization in lymphocytes, but neither CD3 internalization nor transferrin uptake. Confocal microscopy analysis of F-actin distribution within lymphocytes showed that CD59 clusters were associated with patches of polymerized actin. Also, we found that internalization of CD59 was prevented by the protein kinase C inhibitor staurosporine and by the protein kinase A activator forskolin. Thus, in lymphocytes, as in other cell types, glycolipid-based domains provide sites of integration of signaling pathways involved in GPI-anchored protein endocytosis. This process, which is regulated by both protein kinase C and A activity, is tightly controlled by the dynamic organization of actin cytoskeleton, and may be critical for polarized contacts of circulating cells.

A set of cell surface proteins is linked to the exoplasmic leaflet of the plasma membrane by a glycosphosphatidylinositol (GPI) motif. These GPI-anchored proteins fulfill numerous functions critical for cell growth and cell-cell communication (Low, 1989; McConville and Ferguson, 1993). Indeed, GPI-anchored proteins initiate signal transduction although the mechanism remains unclear (for reviews see Brown, 1993; Lisanti et al., 1994a). Cross-linking of GPI-anchored molecules is required for the delivery of certain activation signals (Robinson, 1991) and has been shown to trigger their sequestration into specialized glycolipid-based domains (Mayor et al., 1994) on several different cell types, including on epithelial cells, endothelial cells (Chang et al., 1994; Lisanti et al., 1994b; Sargiacomo et al., 1993), and on some circulating cells (Bohuslav et al., 1995; Cinek and Horejsi, 1992).

In epithelial and endothelial cells, these glycolipid-based domains include plasmalemmal vesicles with a characteristic flask-shaped morphology, also termed caveolae (Anderson, 1993a). They are composed of large detergent-insoluble membrane complexes, and are rich in glycosphingolipids such as G_{M1} gangliosides, cholesterol, and GPI-anchored proteins (Parton, 1994a; Ying et al., 1992). Cholesterol plays a critical role in glycolipid-based domain organization since its removing leads to unclustering of GPI-anchored proteins (Rothenberg et al., 1990a). Several molecules have been identified in these detergent-insoluble complexes, including Ca^{2+} pump and channel, actin and actin-binding proteins, signaling molecules such as Src-related tyrosine kinases, protein kinase C (PKC), heterotrimeric and small G-proteins, and the characteristic transmembrane protein caveolin/VIP21 (Fujimoto, 1993; Anderson, 1993b; Chang et al., 1994; Lisanti et al., 1994b; Rothenberg et al., 1992; Sargiacomo et al., 1993). Caveolin was originally identified as an Src tyrosine kinase substrate in transformed fibroblasts (Glenney, 1989; Glenney and Soppet, 1992), and it was suggested that caveolin may play a role as an adaptor for signaling mediated by GPI-anchored proteins (Lisanti et al., 1994a). Caveolae are involved in transcytosis across endothelial cells (Milici et al., 1987), in potocytosis (Ander-
son et al., 1992; Ying et al., 1992), and in endocytosis. They are also seen as organelles able to compartmentalize signal transduction, providing spatial and temporal information as the cell responds to environmental stimuli (Anderson, 1993a).

However, in lymphoid cells, characteristic caveolae have not been observed and caveolin was not detected even at the mRNA level (Fra et al., 1994). Yet lymphocytes display detergent-insoluble membrane domains of ~100 nM in diameter which are enriched in GSL. GPI-anchored proteins, including CD59 (Protectin), CD55 (DAF), and CD48, and two Src-related protein tyrosine kinases, p56

sin H and B, nystatin, forskolin, FITC-conjugated phalloidin, PMSF, leupeptin, aprotinin, and NP-40 were obtained from Sigma Chem. Co.; staurosporine was obtained from Calbiochem (La Jolla, CA). Chemicals for PAGE were obtained from BioRad Labs (Richmond, CA).

**Internalization Assays Using 125I-labeled Antibodies**

Pured mAb were iodinated using the chloramine T (Sigma Chem. Co.) labeling procedure. Briefly, 50-200 μg of antibodies in 0.3 M H2PO4, pH 7.5, were incubated with 1 mCi 125I (Amersham, UK) for 45 at 22°C in the presence of chloramine T (1 mg/ml). The reaction was stopped with sodium metabisulfite and NaI, and labeled antibodies were then filtered through a PD-10 column (Pharmacia). Specific activities of labeled mAb were routinely about 1-10 cpm/μg. For internalization assays, cells were incubated with 1-10 nM of 125I-labeled antibodies in binding medium (RPMI 1640, 5% SVF; 50 mM Hepes, pH 7.5) for 30 min on ice. After washing, cells were incubated with RAM (2 μg/ml) for 30 min on ice, and then washed for 60 min at 37°C. Cells were then chilled on ice and washed two times with cold-binding medium. To distinguish between surface-bound and intracellular radioactivity, cells were treated twice with elution medium (100 mM citrate, 140 mM NaCl, pH 2.0) for 5 min at 23°C. This treatment was shown to remove 95-98% of the surface-bound radioactivity. Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled antibody. The fraction of internalized radioactivity was expressed as (acid-resistant fraction of radiolabel divided by total cell-associated radiolabel) × 100%.

**Electron Microscopy**

Cells were incubated with the indicated mAb (10-20 μg/ml) followed by 10-μm gold-conjugated anti-mouse IgG for 60 min on ice. The cells were then washed for various time intervals at 37°C, and fixed in 2.5% glutaraldehyde in PBS for 1 h at 23°C, and then postfixed in 1% osmium tetroxide. After dehydration in a series of graded ethanol baths (30, 50, 70, 95, and 100%) and propylene oxide, the cells were embedded in Epon. Cell sections (80-200 nm) were obtained using a Reichert Ultracut E microtome and stained with uranyl acetate. The grids were examined using a Philips CM12 electron microscope.

**Conventional and Confocal Immunofluorescence Microscopy**

Cells grown on coverslips were labeled with mAb (10-20 μg/ml) for different time intervals at 4°C, washed, and treated with RAM (2-3 μg/ml) for 30 min on ice. The cells were then warmed for the indicated times at 37°C. After washing in PBS buffer, the cells were fixed and examined under fluorescence microscopy as described above. They were then incubated with FITC-conjugated anti-rabbit IgG for 30 min at 23°C. After three washes, the cells were mounted in Citifluor as an anti-bleaching agent. Immunofluorescence analysis was performed using a Leitz microscope equipped for epifluorescence. Circulating cells were allowed to settle on poly-L-lysine-coated slides (Sigma) for 15 min in the dark before analysis on a Zeiss microscope equipped with a Sony video recorder. Confocal analysis was used for analysis performed on lymphoid cells. Cell suspensions treated as described above, were finally centrifuged and the cell pellets were resuspended in Citifluor as an anti-bleaching agent. Cells were then allowed to settle on poly-L-lysine-coated slides (Sigma) for 15 min in the dark. Fluorescence analyses were performed on a Leitz scanning confocal microscope.

**Internalization Assays of FITC-conjugated Ligands**

The uptake of FITC-conjugated transferrin (FITC-Tf) was performed as follows: cells were washed twice with HBS 1% BSA, and incubated for 45 min at 37°C in the same medium in order to deprive cells of free and bound transferrin. Cells, adjusted to 106 per ml in the above medium, were incubated with 1 μM of Cyto H, and then labeled with 2 μg/ml of FITC-Tf for 60 min on ice. Cells were then washed for 30 min at 37°C, allowing transferrin to internalize, or left at 4°C. After three washes, the cells were fixed and examined under fluorescence microscopy as described above.

**Measurement of F-Actin**

Activated Jurkat cells were washed in PBS buffer, and then fixed for 10...
min at 23°C in PBS, 3.7% paraformaldehyde. After two washes in PBSB, fixed cells were permeabilized for 30 min at 23°C in PBSB, 0.05% saponin. The cells were then stained with FITC-phalloidin (1 μg/ml) in PBSB, 0.05% saponin for 30 min at 23°C in the dark. After three extensive washes in PBSB, F-actin fluorescence was determined using a FACScan flow cytometer. The data are reported as the ratio of the mean of fluorescence intensity of stimulated cell to control cells, or the relative fluorescence index (RFI). In some experiments, F-actin fluorescence was examined using confocal microscopy as described above.

Results

Internalization of CD59 GPI-anchored Protein Is Induced by Cross-linking and Blocked by Nystatin

We have investigated internalization of the CD59 GPI-anchored protein. Jurkat cells were labeled with either [125I] CD59 or CD3 mAbs at 4°C, washed, incubated again at the same temperature with anti-mouse Ig, and then warmed to 37°C for various periods of time. CD59 internalization occurred only upon cross-linking with a second antibody, was detectable as soon as 30 min after the beginning of the cross-linking, and reached a maximum at 2 h. By contrast, CD3 internalization reached its maximum at 30 min (Fig. 1 A).

To determine whether CD59 internalization is dependent on cholesterol-rich membrane domains, we used the cholesterol binding drug, nystatin. Nystatin has been shown to selectively disrupt caveolae, but to have no effect on clathrin-coated pits, actin cables, or other submembraneous structures (Rothberg et al., 1992). For example, nystatin reduced the rate of plasmin formation by the complexes of urokinase plasminogen activator with their receptors, which localized in caveolae (Stahl and Mueller, 1995). In our preliminary experiments, nystatin proved to have minimal toxic effects on lymphocytes: 25 mg/ml of nystatin in serum-free culture medium for up to 24 h had no effect on cell morphology or viability, nor did it modify the binding of [125I] CD59 mAb "H19" to those cells (results not shown). Jurkat cells were preincubated for 30 min with or without nystatin, and then labeled with either [125I] CD59 or CD3 mAbs before cross-linking. Under these conditions, CD59 internalization was decreased up to 30% in the presence of nystatin (Fig. 1 B). In contrast, T cell antigen receptor (TCR) downmodulation was unaffected by nystatin (Fig. 1 B). These data suggest that in lymphoid cells, GPI-anchored proteins are clustered upon cross-linking in sterol-enriched domains of the plasma membrane, and these domains are required for internalization of the receptor.

Internalization of CD59 GPI-anchored Protein Occurs Preferentially in Uncoated Vesicles

The fate of CD59 following antibody-induced cross-linking on the lymphocytic T cell line Jurkat was next examined by electron microscopy. Cells were labeled with CD59 mAb followed by incubation with colloidal gold-labeled anti-mouse IgG at 4°C or for various times at 37°C. Cells incubated at 4°C showed uniform distribution of CD59 on the plasma membrane, and only 10% of the total particles were clustered at the cell surface (Fig. 2 and Table I). Upon warming at 37°C, gold particles associated with CD59 were clustered (up to 45% of the particles observed on the plasma membrane). After 15 min at 37°C, they were detectable within the cells (Table I), and in large endocytic vesicles (1–10 particles per vesicle) associated with internal material (Fig. 2). Quantitation indicated that after 60 min of cross-linking at 37°C, 33% of the gold particles were internalized (Table I), and could be seen associated with large endocytic vesicles and multivesicular bodies (Fig. 2). Notably, gold particles associated with CD59 were seen most often in noncoated invaginations of the plasma membrane. After 15 min of cross-linking at 37°C, quantitative determination showed that up to 6% of particles were found in coated vesicles, whereas 30% were found in noncoated invaginations (Fig. 2 and Table I). Thus, these results show that upon clustering, CD59 preferentially accumulates in noncoated structures.

A Role for Protein Kinases in GPI-anchored Protein Internalization within Lymphoid Cells

Since cytoplasmic kinases have been found to be involved in caveolae internalization (Smart et al., 1995), we examined the effects of pharmaceutical agents on the endocytic pathway of GPI-anchored proteins. Confocal analysis showed that staurosporine, a PKC inhibitor, and forskolin, an activator of protein kinase A (PKA), abrogated the internalization of CD59 (Fig. 3). Of note, no significant alteration of the clustering of CD59 was observed (Fig. 3, C and D), indicating that kinase inhibition acts on the internalization process of GPI-anchored proteins rather than on a prerequisite phase of cross-linking. Other PKC inhibitors, namely calphostine C and sphingosine, also inhibited CD59 internalization at concentrations consistent with PKC inhibition, whereas herbimycin A, a tyrosine kinase inhibitor, did not inhibit CD59 internalization (not shown). By contrast, forskolin almost completely inhibited CD59 but not CD3 downmodulation (Fig. 4). This indicates that cAMP-dependent serine/threonine kinases are involved in regulating internalization of GPI-anchored proteins within lymphoid cells.
Figure 2. Localization of CD59 in Jurkat cells by immunoelectron microscopy. Cells were labeled with CD59 mAb followed by secondary antibody conjugated with 10 nm gold for 60 min at 4°C. The cells were then warmed to 37°C for 0-60 min before glutaraldehyde fixation. A-I show semi-thick Epon sections representative of the distribution of gold particles at 4°C (A), after 15 min at 37°C (B-E), 30 min (F and G), or 60 min (H and I). Gold label has clustered on plasma membrane (B) near (C and D) or in (E) uncoated vesicles. Arrowheads show gold label in uncoated vesicles near the membrane (F and G) and in multivesicular bodies (H and I). The arrow shows an empty coated pit. Bars, 100 nm.

Disruption of Actin Filaments Inhibits Internalization of GPI-anchored Proteins in Lymphocytes

The integrity of the actin cytoskeleton can be required for endocytosis, depending on the cell type, and, most likely, on the mechanism of endocytosis (Anderson, 1991). We therefore examined, within lymphocytes, the influence of the actin cytoskeleton on the endocytosis of GPI-anchored proteins and proteins requiring a clathrin-dependent mechanism of endocytosis.

First, we measured endocytosis with [125I] CD59 mAb. When cells were incubated with Cyto H, the internalization of [125I] CD59 mAb was abrogated, whereas [125I] CD3 mAb was internalized to the same extent as in the controls (Fig. 5). Scanning confocal microscopy performed on saponin-permeabilized cells showed that time-dependent internalization of CD59 was completely abolished by 1 μM of Cyto H. Importantly, it should be noted that Cyto H neither inhibited the initial patching nor the capping of CD59, suggesting that the inhibition of GPI-anchored protein internalization cannot be accounted for by the inhibition of their clustering. By contrast neither CD3/TCR internalization (results not shown) nor Tf-FITC uptake by Jurkat cells (Fig. 6) was affected.

We next examined CD59 internalization by electron microscopy. Upon Cyto H treatment, cells exhibited a patched distribution of gold particles on the plasma membrane (2–10 particles per patch) (Fig. 7). Although the amount of clustered CD59-associated gold particles were identical in untreated or Cyto H-treated cells, only 4% of the particles were found within Cyto H-treated cells, even after 60 min of clustering at 37°C (Table I, Fig. 7, A–D). By contrast, the internalization of gold particles associated with the CD3 complex was not inhibited by Cyto H treatment, and particles (3–12 per vesicle) were still detectable in multivesicular structures (Fig. 7 E). Thus, in lymphocytes, structural integrity of actin filaments is required for the internalization pathway mediated by glycolipid-based domains.

Reorganization of the Actin Cytoskeleton and CD59 Clustering

Given the effects of Cyto H on CD59 internalization, we examined the organization of the actin cytoskeleton following CD59 cross-linking. CD59 was cross-linked for different times at 37°C, cells were fixed, and then permeabilized with saponin. Incubation with FITC-conjugated phalloidin allowed detection of the cellular content of F-actin; quantification was performed by FACS analysis. CD59 cross-linking induced a transient augmentation of F-actin (1.4-fold augmentation of RFI at 10 min), peaking at 15 min, and decreasing after 30 min (Fig. 8 A). This process was abolished by incubating the cells with 1 μM Cyto H (Fig. 8 A). Scanning confocal microscopy showed that CD59 cross-linking increased the level of F-actin, but also induced the formation of actin patches (Fig. 8 B, panel b). Cross-linking of CD59 mAb with a TRITC-labeled GAM IgG for 10 min at 37°C, followed by FITC-conjugated phalloidin and confocal analysis, showed that F-actin closely localized with the CD59 clusters at the cell surface (Fig. 9).

Discussion

In this report, we have investigated the endocytosis of CD59, a typical GPI-anchored protein from human lymphocytes (Deckert et al., 1992, 1995). We show that it first requires clustering within glycolipid-based membrane domains in a sterol-dependent manner. Next, a dynamic reorganization of the actin cytoskeleton is required. In addition, we show that the signaling pathway leading to endocytosis is controlled both by PKC and PKA activity. It appears quite distinct from the signaling pathway initiated through CD59 cross-linking on lymphocytes, which requires protein tyrosine kinase activities.
### Table 1. Quantitative Analysis of Gold-labeled Protecin Distribution after Adding a Second Ab; Influence of Cytochalazine H

| Conditions                | Gold on surface | Gold internalized | Percent of gold particles in invaginations |
|---------------------------|-----------------|-------------------|-------------------------------------------|
|                           | %               | %                 | Coated | Noncoated |
| 0°C                       | 100 (10)*       | 0                 | 0      | 14        |
| 37°C 15 min               | 90 (45)         | 10                | 0      | 6         |
| 37°C 60 min               | 67 (44)         | 33                | 9      | 27        |
| 37°C 60 min + Cyto H      | 96 (41)         | 4                 | 8      | 40        |

*Numbers in parrentheses show the percentage of clustered gold particles at the cell surface. A cluster of gold particles was defined as three or more associated gold particles. 

**250–300 individual gold particles were counted for each condition, representing 20–40 cells sections (representative of two different experiments).**

In lymphoid cells, caveolae and caveolin are not detectable (Fra et al., 1994; Gorodinsky et al., 1995). Instead, lymphocytes display large, glycolipid-based domains where GPI-anchored proteins are found (Stefanova et al., 1991; Cinek and Horejsi, 1992). Recently, Parton and Simons proposed the term of detergent-insoluble glycosphingolipid-enriched complexes to distinguish such regions from classical morphological entity termed caveolae (Parton and Simons, 1995). However, in the absence of caveolae in lymphocytes, the endocytic process of GPI-anchored proteins remains unclear. We observed under electron microscopy analysis that the internalization of CD59 on lymphocytes involves an initial step of accumulation in uncoated membrane invaginations. After clustering, CD59 proteins undergo internalization via uncoated pits and vesicles of ~60–100 nm in diameter. Most of the CD59-labeled material was found within such uncoated vesicles near the membrane, although a small proportion was also seen in coated pits. Our observations are consistent with the biochemical studies from Horejsi and colleagues about the dimension of the detergent-insoluble GPI-domains in immune cells, including lymphocytes (Cinek and Horejsi, 1992; Bohuslav et al., 1993) and monocytes (Bohuslav et al., 1995). In studies performed on nonlymphoid cells, it was shown that these domains are disrupted in cells depleted of cholesterol, or treated with sterol-binding drugs such as nystatin or filipin, leading to unclustering of GPI-anchored proteins (Rothberg et al., 1990a, 1992). We found here that, in lymphoid cells, nystatin inhibits the antibody-induced internalization of CD59 while the clathrin-
Figure 4. Forskolin inhibits CD59 but not CD3/TCR downmodulation. JE6.1 cells were preincubated for 30 min with forskolin (10 μM) or not and labeled with [125I] CD59 or CD3 mAb followed by secondary antibody for 2 h at 37°C. Results are expressed as the percentage of variation relative to control cells (3,000 cpm and 12,000 cpm, respectively, for CD59 and CD3).

Figure 5. Cytochalasin H inhibits the endocytosis of [125I] CD59 mAb but not those of [125I] CD3 mAb. JE6.1 cells were preincubated for 30 min at 37°C with or without cytochalasin H at 1 μM, and then labeled with [125I]-labeled antibodies to CD59 or CD3, followed by secondary antibody for 60 min at 4°C. Cells were then warmed to 37°C for 60 min. Surface label was removed by incubating cells twice with low pH medium. The fraction of internalized radiolabel is expressed as the percentage of the total amount of cell-bound radioactivity for each condition.

dependent TCR internalization pathway was unaffected. Taken together, our data also suggest that CD59 is not internalized through the clathrin-coated pit pathway. In this respect, a GPI-anchored form of CD4 was shown to be preferentially internalized through uncoated vesicles on transfected CHO cells (Keller et al., 1992). Similar observations were reported about other GPI-anchored proteins such as the folate receptor (Rothberg et al., 1990b) and Thy-1 glycoprotein (Bamezai et al., 1992). Thus, it appears that the glycolipid-based domains from lymphoid cells are also involved in the formation of endocytic vesicles, as it has been reported for glycolipid-based domains in nonlymphoid cells (Rothberg et al., 1990b; Parton et al., 1994b). Finally, we used in our experiments two antibodies in order to cross-link CD59, a phenomenon which in general is considered to induce an artificial organization of the plasma membrane molecules. However, it might be important to note that since CD59 is constitutively clustered on the cell surface (Van den Berg et al., 1995), adding two antibodies may rather stabilize the native distribution of CD59 on cell membrane.

One important question is how mechanisms control the endocytic pathway of GPI-anchored proteins. We found that actin reorganization was a subsequent critical step, since we observed that Cyto H inhibited CD59 internalization, and that patches of polymerized actin colocalized with CD59 clusters before internalization. In addition, we found that Cyto H blocked the internalization of GM1 GSL (data not shown), which are enriched in caveolae (Parton et al., 1994b). The actin cytoskeleton has been shown to be involved in intracellular trafficking (Anderson, 1991), but its role in internalization remains unclear. In mammalian cells, cytochalasin D reduces uptake via a nonclathrin-dependent pathway (Sandvig and van Deurs, 1990), while apical uptake from polarized epithelial cells is also reduced by depolymerization of the actin cytoskeleton (Gottlieb et al., 1993).

Furthermore, cytochalasin D inhibits okadaic acid–induced wash-out of caveolae from epithelial cell membranes (Parton et al., 1994b). However, we observed no effect (or weak effects) of Cyto H on the antibody-induced internalization.
Figure 7. Immunogold localization of CD59 and CD3 on Cyto H-treated cells. JE6.1 cells were preincubated for 30 min at 37°C with cytochalasin H at 1 μM, and then labeled with CD59, or CD3 mAb followed by secondary antibody conjugated with 10 nm gold for 60 min at 4°C. Cells were then warmed to 37°C for 60 min before glutaraldehyde fixation. A–E show semi-thick Epon sections representative of the distribution of gold particles associated with CD59 (A–C), or with CD3 (D and E). Gold label has clustered on plasma membrane (A–C). The arrowhead shows gold label near an uncoated structure at the membrane (C). Arrows show CD3-associated gold label in multivesicular bodies (D and E). Bars, 100 nm.

Figure 8. FACS and scanning confocal analysis of the distribution of F-actin during CD59 clustering. JE6.1 cells were preincubated or not with Cyto H at 1 μM for 30 min at 37°C, and then labeled or not with CD59 mAb followed by secondary antibody for 0–30 min at 37°C. At the indicated times, cells were fixed, permeabilized in 0.05% saponin, and labeled with FITC-phalloidin. F-actin content was determined either by FACS analysis (A) or by confocal analysis (B). The data are expressed as the relative fluorescence index (RFI), relative to control cells, and represents the mean of triplicate determinations ± SD (A). B shows representative confocal images of inactivated cells (a) and cells activated by CD59 mAb for 15 min (b) or 30 min (c) while d represents cells incubated in the presence of Cyto H at 1 μM before activation by CD59 mAb for 15 min.

dothelial cells have been found to be enriched in actin and actin-binding protein, heterotrimeric G-proteins, and small G-proteins from the Rab and Rap families (Chang et al., 1994; Lisanti et al., 1994b). There is compelling evidence that both heterotrimeric and small G proteins regulate assembly/deassembly of actin filaments (Sheffy et al., 1985; Hall, 1994). Thus, although they are not fully characterized, the lymphocyte glycolipid-based domains provide likely sites for initiation of actin nucleation and assembly.

Proteins involved in regulating cytoskeletal organization or in the formation of endocytic vesicles are likely targets...
Figure 9. Scanning confocal analysis of the colocalization of F-actin and CD59 clusters at the cell surface. JE 6.1 cells were labeled with CD59 mAb followed by TRITC-conjugated GAM antibody for 10 min at 37°C. The fixed and permeabilized cells were then incubated with FITC-conjugated phalloidin for 30 min at room temperature and examined by scanning confocal microscopy. A and B show separate labelings while C shows double-labeling superposition. Bar, 10 μm.

for signal transduction elements, and both kinases and phosphatases play a central role in lymphocyte signaling pathways (Mustelin, 1994; Weiss and Littman, 1994). The finding that Src-related tyrosine kinases and GPI-anchored proteins are complexed within glycolipid-based domains is therefore of critical importance, since antibodies against these GPI-anchored proteins have been shown to trigger signals within different cell types (Brown, 1993). Indeed, we recently found that cross-linking of CD59 triggers activation of the associated p56Lck kinase and stimulates both CD3/TCR-dependent and -independent signaling pathways in T cells (Deckert et al., 1995). Serine and threonine kinases are also critical for cell signaling, and stimulation of protein kinase C activity by phorbol esters has been shown to inhibit folate receptor internalization in MA104 epithelial cells (Smart et al., 1994). In addition, a population of PKCx molecules were found to reside in the caveolar membrane, whose activity is required for caveolae invagination (Smart et al., 1995). In the present study we found that staurosporine, a PKC inhibitor, prevented CD59 internalization within human lymphoid cells. In Jurkat variants deficient in TCR/CD3 or CD45 expression, we have observed that CD59 was still internalized (not shown). This establishes that the TCR/CD3 complex and the tyrosine phosphatase CD45 do not transduce biochemical signals required for internalization of GPI-anchored molecules, although these signals are critical for T cell activation (Weiss and Littman, 1994). Moreover, activation of p56Lck induced by cross-linking CD59 (Deckert et al., 1995) was unaffected by actin filament depolymerization (data not shown). It is tempting to propose that internalization is a negative regulatory mechanism of activation signals, which would have an important role during cell activation.

In conclusion, our data provide clues about the functions of glycolipid-based domains and the molecular mechanisms underlying their organization. Keeping in mind that lymphocytes are unpolarized cells in a resting state, but become polarized upon cell-cell or cell-matrix interaction, such domains may provide critical spatial information and sites of integration for signaling pathways. Since many GPI-anchored molecules are involved in cell adhesion and in signal transduction, endocytosis of GPI-anchored molecules is a likely means for reversible adhesion and for fine tuning of cell signaling.

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