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Glycosylation Affects Translocation of Integrin, Src, and Caveolin into or out of GEM[1]

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Endogenous GM3 synthesis and full N-glycosylation in membrane receptors occurred in "4-epimerase-less" ldlD (Krieger's CHO mutant) cells cultured in Gal-containing medium, whereby components of detergent-insoluble, low-density, buoyant membrane fraction, termed "glycolipid-enriched microdomain (GEM)," varied significantly by translocation into or out of GEM. Integrins α3 and α5 were translocated into GEM in the presence of 0.5 or 0.25% Triton X-100, particularly in the absence of Gal, whereby integrins are underglycosylated and GlcCer is the major glycolipid component in GEM. Src family kinase was translocated into and enriched in GEM fractions when prepared in 0.5 or 0.25% Triton X-100 from cells grown in Gal-containing medium, whereby GM3 synthesis is induced. In contrast, caveolin is highly enriched in GEM when GM3 synthesis does not occur, and is translocated into high-density membrane fraction when GM3 synthesis occurs. The results suggest that levels of key molecules controlling cell adhesion and signaling are defined by translocation into or out of GEM, which depends on glycosylation state. © 2000 Academic Press

Key Words: **glycosphingolipid-enriched microdomain (GEM); N-glycosylation; GM3 ganglioside; integrin α3; integrin α5; caveolin; Src family kinase; 4-epimerase-less mutant.**

Functional compartmentalization of cell surface membrane is a subject of considerable interest in current cell biology, for understanding the molecular mechanisms of cell adhesion, cell growth, and signal transduction (1–4), in particular, the role of glycosphingolipids (GSLs) and glycosylation of various surface receptors (4, 5). Growth factor receptors, in activated state, were found to be associated with caveolar membrane, which is enriched in cholesterol and caveolin. Caveolae are involved in endocytosis and signal transduction (for review see 3). Other membrane compartments showing physical and chemical properties similar to those of caveolar membrane have been termed "detergent-insoluble membrane (DIM)" (6) or "detergent-resistant membrane (DRM)" (2). From such mixtures of membranes, "GSL-enriched microdomain (GEM)" (4), separable from caveolar membrane, was found to be involved in GSL-dependent cell adhesion coupled with signal transduction, and the term "glycosignaling domain (GSD)" was applied (5, 7, 8).

In order to clarify the effect of glycosylation on levels of GEM components, we employed the "4-epimerase-less" mutant ldlD (from Chinese hamster ovary) cells, established by Krieger and colleagues (9, 10). ldlD cells grown in the absence of galactose (Gal) synthesize only GlcCer (not LacCer or GM3), and undergo incomplete N-glycosylation without terminal sialyl-Gal unit. Thus, cell glycosylation status affects GEM components, whose levels are defined by translocation into or out of GEM and can be characterized unambiguously in these cells.

MATERIALS AND METHODS

ldlD-14 cell culture. Cell line ldlD-14, a CHO mutant deficient in UDP-Glc 4-epimerase, was maintained in Ham's F12 medium supplemented with 5% fetal bovine serum. Cells were seeded in the medium on day 0, and glycosylation pattern was altered by change of medium on day 1 to serum-free Ham's F12 containing insulin-transferrin-selenium medium (ITS, Collaborative Biomedical Products, Bedford, MA) with or without Gal (9 μM) (10). The cells were used on day 3.

Antibodies. Antibody for cSrc and Src family kinases was rabbit polyclonal IgG, "SRC2," which reacts with cSrc (p60) but cross-reacts with Yes (p62), Fyn (p59), and c-Fgr (p55) (Santa Cruz Biotechnol-

Abbreviations used: DIM, detergent-insoluble membrane; Gal, galactose; GEM, glycolipid-enriched microdomain; GlcCer, Glcβ1→1Cer; GM3, NeuAcα2 → 3Galβ1 → 4Glcβ1 → 1Cer; GSD, glycosignaling domain; GSL, glycosphingolipid; HRP, horseradish peroxidase; ITS, insulin-transferrin-selenium medium; PNF, postnuclear fraction; SRC2, commercial name of polyclonal antibodies with cSrc as immunogen (see text).

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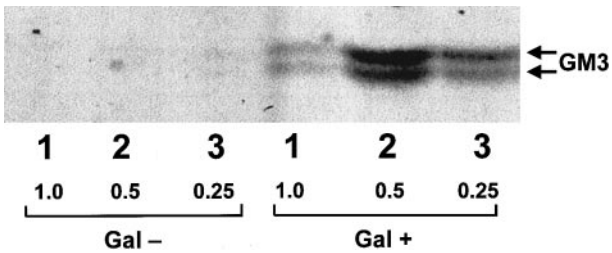

FIG. 1. GM3 ganglioside level in Fr. 5 (GEM) from PNF prepared in 1.0, 0.5, and 0.25% Triton X-100, from ldlD cells grown in the absence (Gal −) or presence (Gal +) of galactose in ITS medium. Bands on TLC are revealed by orcinol-sulfuric acid reaction.

ogy, Santa Cruz, CA). We therefore use the term "SRC2-defined components" rather than cSrc. Antibody for caveolin was rabbit polyclonal IgG, "N20" (Santa Cruz). Antibodies for integrin receptors α3 and α5 were rabbit polyclonals AB1948 and AB1949, respectively (Chemicon International Inc., Temecula, CA), which cross-react with α3 and α5 from human, mouse, rat, and pig. We found that these antibodies also react with hamster CHO cell integrins.

Preparation of GSL-enriched microdomain (GEM). GEM fractions were prepared by a modification of methods described previously (4, 11). Briefly, 2×10^7 cells were suspended in 1.2 ml lysis buffer (10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 75 U aprotinin) containing various concentrations (1, 0.5, or 0.25%) of Triton X-100, and left standing for 20 min in ice bath. The cell suspension was then Dounce homogenized (10 strokes), and the lysate was centrifuged for 5 min at 1300*g* to remove nuclei and large cellular debris. One ml supernatant (postnuclear fraction; PNF) was subjected to sucrose density gradient centrifugation, *i.e.*, the supernatant was mixed an equal volume of 85% sucrose (w/v) in 10 mM Tris buffer (pH 7.5), 150 mM NaCl, and 5 mM EDTA (TNE). The resulting diluent was placed at the bottom of a tube, and overlayed with 5.5 ml of 35% sucrose in TNE, and then 3.5 ml of 5% sucrose in TNE. After centrifugation for 16 h at 39K rpm at 4°C in a Beckman SW41 rotor, samples (0.9 ml each) were collected sequentially from top to bottom, and termed fraction (Fr.) 1, 2, 3, etc. The bottom fraction was Fr. 12. Fr. 5 appeared as a white, light-scattering band under illumination located at ~5–7% sucrose. The entire procedure was performed at <4°C.

Determination of SRC2-defined components, caveolin, and integrins. Fractions from sucrose gradient centrifugation as above were subjected to SDS–PAGE under reducing conditions, transferred electrophoretically to Immobilon-P (Millipore, Bedford, MA), and subjected to Western blotting using the antibodies, the relevant secondary antibodies conjugated with HRP, and chemiluminescence system (Super-Signal-CL-HRP; Pierce, Rockford, IL) (4). Protein content was determined with a MicroBCA kit (Pierce), using bovine serum albumin as standard.

GM3 determination in GEM. GM3 in GEM fraction was adsorbed on C18-silica gel column (Bond-Elut C18 column, Analytichem International, Harbor City, CA), washed with water, eluted with chloroform-methanol 2:1, and analyzed by thin-layer chromatography. GM3 band was detected by orcinol-sulfuric acid or immunostaining with anti-GM3 mAb DH2 (4).

Cholesterol determination. PNF and Fr. 5 were prepared from ldlD cells cultured with or without Gal (20 μM) (10) as described above, and 2.7 ml Fr. 5 or 270 μl PNF were centrifuged at 55,000 rpm for 1 h at 4°C. Supernatant was removed, and precipitate was extracted with 1 ml chloroform-methanol 2:1. The extract was evaporated under nitrogen stream, and dried samples were added with appropriate volume (5–20 μl) of diluent buffer (0.15 M Tris buffer pH 7.0, 7.5 mmol p-chlorophenol) and coloration reagent based on cholesterol oxidase, peroxidase, and aminoantipyrin (Cholesterol CII kit, Wako, Osaka, Japan) according to manufacturer's instructions.

RESULTS

GM3 Level in GEM

The sole GSL in ldlD cells grown in ITS medium is GlcCer. The major GSL becomes GM3 when cells are grown in Gal-added medium, as described originally by Krieger *et al.* (9, 10). The level of GM3 in GEM from ldlD cells grown in Gal-added medium was much higher when PNF was prepared in 0.5 or 0.25% as compared to 1% Triton X-100. No GM3 was detectable in corresponding GEM fraction from cells grown in ITS without Gal (Fig. 1).

Integrin Receptor Levels in GEM

Integrins α3 and α5 were undetectable or detectable at extremely low level in low-density, buoyant fractions (Fr. 4, 5, 6), and were found almost exclusively in high-density Fr. 12, when ldlD cells were Dounce homogenized in Tris buffer containing 1% Triton X-100 and soluble PNF was subjected to sucrose density gradient centrifugation (lane 1 in Figs. 2A and 2B; Figs. 5A and 5B). Similar results were obtained using 2% Empigin BB instead of 1% Triton X-100 (data not shown). Levels of α3 and α5 in low-density fractions increased greatly when cells were homogenized in the same medium containing 0.5 or 0.25% Triton X-100 (lanes 2 and 3 in Figs. 2A and 2B). Levels of α3 and α5 in Fr. 5 were significantly higher in cell extract grown in the absence (compared to presence) of Gal (lanes 2 and 3 in Figs. 2A and 2B; Figs. 5C and 5D). These findings suggest the presence of a specific membrane domain associated with integrin, and that complete N-glycosylation of integrin receptors inhibits while incomplete N-glycosylation promotes its translocation from high to

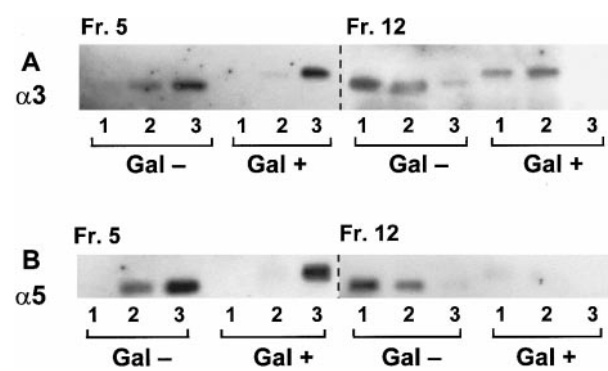

FIG. 2. Association pattern of α3 (A) and α5 (B) integrin receptors in low-density, buoyant Fr. 5 and high-density Fr. 12 prepared from ldlD cells grown in the absence (Gal −) or presence (Gal +) of galactose. Lanes 1, 2, 3: Fr. 5 or 12 prepared in 1%, 0.5%, and 0.25% Triton X-100, respectively. Note that, for Fr. 5, α3 and α5 are absent or very low in lane 1 (1% Triton X-100 extract), but present at significant levels in lanes 2 and 3 (0.5%, and 0.25% Triton X-100) in the absence of Gal.

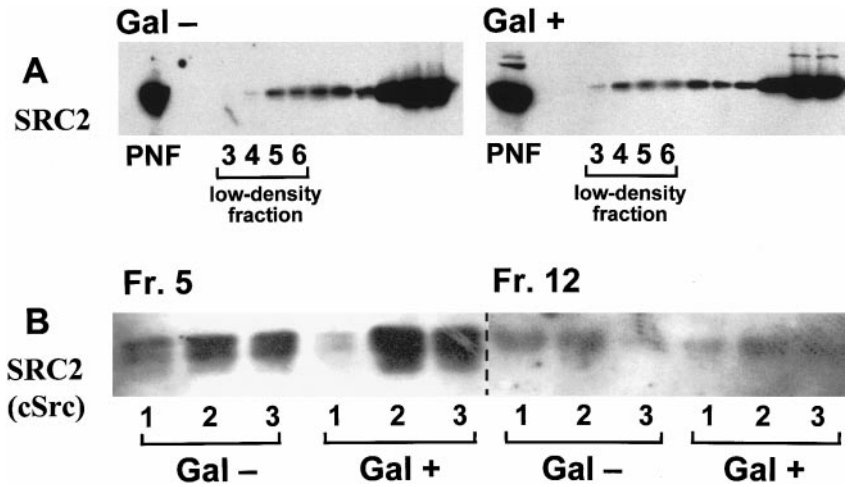

FIG. 3. Levels of SRC2-reactive components associated with various membrane compartments of ldlD cells. (A) Distribution pattern of SRC2-reactive components in various ldlD cell fractions separated by sucrose density gradient centrifugation from PNF prepared with 1% Triton X-100. (B) Association pattern of SRC2-reactive components with Fr. 5 and 12 from PNF prepared with 1% (lane 1), 0.5% (lane 2), and 0.25% (lane 3) Triton X-100, prepared from ldlD cells grown in the absence (Gal −) or presence (Gal +) of galactose.

low density domain, presumably enhancing its association with lipid components in GEM.

Level of SRC2-Reactive Components (cSrc or Src Family Kinase)

When cells were homogenized in Tris containing 1% Triton X-100, the majority of SRC2-reactive components was associated with high-density Fr. 10–12. Only a minor portion was found in low-density, buoyant Fr. 3–6. SRC2-reactive components increased by translocation into Fr. 4 and 5 only from cells grown in the presence of Gal, whereby GM3 synthesis occurred (Fig. 3A). In contrast, when cells were homogenized in Tris containing 0.5 or 0.25% Triton X-100, the majority of SRC2-reactive components was found in Fr. 5, and only a minor portion was found in high-density Fr. 12. In this case, again, the level of SRC2-reactive components was higher in cells grown in the presence of Gal (Figs. 3B; Figs. 5E and 5F). These findings indicate that conversion of GlcCer to GM3 in GEM causes translocation of SRC2-reactive components into low-density GEM fraction (see Discussion).

Caveolin Level

Caveolin level was greatly enriched in Fr. 4–6 (highest in Fr. 5) of cells grown in the absence of Gal, and

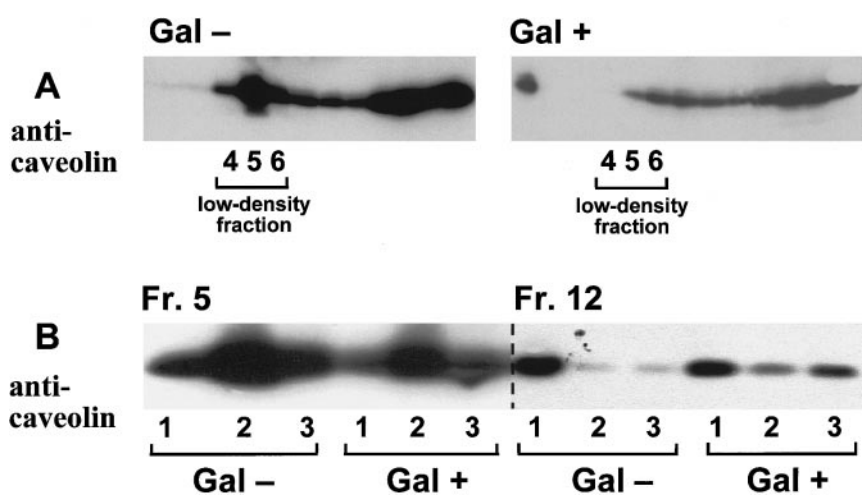

FIG. 4. Levels of caveolin associated with various membrane compartments of ldlD cells grown in the absence (Gal −) or presence (Gal +) of galactose. (A) Distribution pattern of caveolin in various ldlD cell fractions prepared with 1% Triton X-100. Note that caveolin is highly enriched in Fr. 5 only for cells grown in the absence of Gal. (B) Association pattern of caveolin with Fr. 5 and 12 from PNF prepared with 1% (lane 1), 0.5% (lane 2), and 0.25% (lane 3) Triton X-100 as in Figs. 2A and 2B.

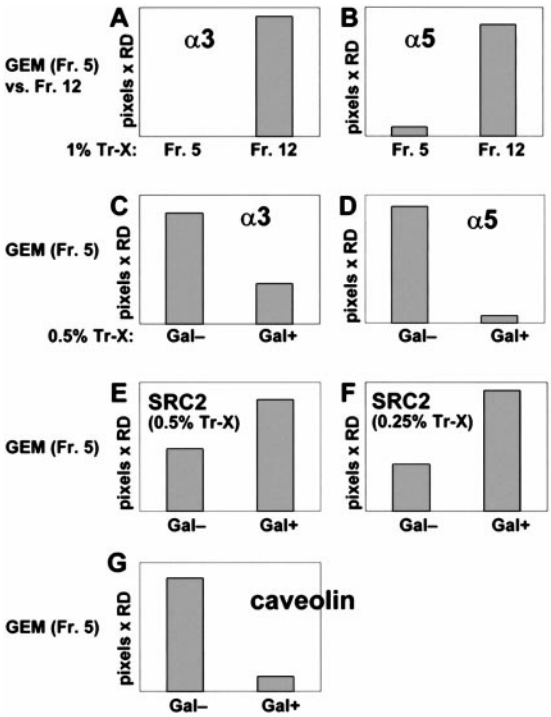

FIG. 5. Relative quantity of integrin, Src family kinase, and caveolin in GEM (or DIM) of ldlD cells prepared under various conditions. Selected bands from Figs. 2, 3, and 4 were scanned and measured using the program Scion Image for Windows (v. 4.0.1, Scion Corp., www.scioncorp.com). The ordinate in each graph represents the quantity (area of band) × (relative darkness (RD) of band), quantified as [band pixels × (band mean − background mean)] as measured by the program. (A and B) Intensities of α3 and α5 bands present in Fr. 5 (GEM) as compared to Fr. 12 (high density fraction) of PNF prepared in 1% Triton X-100. Note that both α3 and α5 are associated almost exclusively with Fr. 12. (C and D) Intensities of α3 and α5 bands present in Fr. 5 (GEM) of PNF prepared in 0.5% Triton X-100 from ldlD cells grown in the absence (Gal −) or presence (Gal +) of galactose. (E and F) Intensities of Src family kinase (defined by antibodies SRC2) in Fr. 5 (GEM) of PNF prepared in 0.5% or 0.25% Triton X-100 from ldlD cells grown in the absence (Gal −) or presence (Gal +) of galactose. (G) Intensities of caveolin in Fr. 5 of PNF prepared in 1% Triton X-100 from ldlD cells grown in the absence (Gal −) or presence (Gal +) of galactose.

greatly decreased in the presence of Gal. This trend was seen for 1% Triton X-100 extract (Fig. 4A; Fig. 5G), and more strongly for 0.5 or 0.25% Triton X-100 extract (Fig. 4B). This finding suggests that conversion of GlcCer to GM3, and completion of N-glycosylation in GEM-associated receptor, cause translocation of caveolin from low-density buoyant fraction to high-density fraction.

DISCUSSION

Lipid and protein composition of GEM depend highly on (i) presence of long-chain saturated alkane (lipids with highly unsaturated alkene are generally excluded from GEM) (for review see 2); (ii) long-chain acylation of protein, *e.g.*, myristoylation, palmitoylation, farnesoylation (12, 13; for review see 14). The concept of GSL signaling domain ("glycosignaling domain;" GSD) organized by clustering of GSLs with various signal transducer molecules was proposed, and GSD was claimed to be involved in cell adhesion coupled with signal transduction (8). Clustering of GSD and sphingolipids in GEM may depend on high interaction between GSLs or between sphingolipids (*cis* interaction) due to presence of hydrogen bond donor as well as acceptor. This is in contrast to glycerolipids, which lack hydrogen bond donor (15). The effect of glycosylation of lipid or protein in GEM has not been critically evaluated, in spite of increasing evidence that GEM may contain not only GSL but also various N-glycosylated protein receptors such as growth factor and integrin receptors, and tetraspan membrane proteins (TMP). The present study is designed to evaluate such effect.

Results of this study, using Krieger's CHO mutant ldlD cells deficient in UDP-Glc 4-epimerase, unambiguously demonstrate the effect of lipid and protein glycosylation on composition of GEM or DIM, presumably based on translocation of components into or out of such domain. There were three major findings of interest: (i) In contrast to previous reports that integrin is absent in GEM or caveolar membrane (3), we found both α3 and α5 in GEM fraction when low concentration (0.5 or 0.25%) of Triton X-100 was applied in preparation of PNF. Levels were higher in medium without Gal, *i.e.*, integrins are translocated from high to low density, particularly when underglycosylated. (ii) cSrc or Src family kinase may translocate into GEM in association with GM3 synthesis. This trend is obvious in GEM from PNF prepared with low concentration (0.25 or 0.5%) of Triton X-100. (iii) Caveolin was greatly enriched due to translocation in GEM when cells were grown in the absence of Gal, and translocated from GEM to high-density fraction when cells were grown in the presence of Gal, whereby GM3 synthesis occurs. Caveolin is known as a cholesterol-binding, scaffold protein of caveolae (16). Cholesterol level in GEM of ldlD cells is unchanged when cells are grown in the presence vs. absence of Gal (data not shown). The increase or decrease of caveolin from GEM by translocation is therefore controlled by GM3 in GEM; *i.e.*, GM3 and caveolin are incompatible in GEM. In fact, GSD isolated from DIM is highly enriched in GM3 and Src but lacking caveolin, whereas caveolar membrane is enriched in cholesterol and caveolin but lacking GM3 or Src (8).

Level of SRC2-reactive components tends to increase by translocation into GEM when GM3 synthesis occurs. Such translocation was clear from PNF prepared with low concentration (0.25 or 0.5%) of Triton X-100. SRC2-reactive components in GEM fraction of melanoma B16 cells are co-immunoprecipitated with anti-GM3 mAb DH2 but not with anti-caveolin antibodies,

indicating association of GM3 with cSrc or Src family kinases (8). SRC2-reactive components in ldlD cells are not identified clearly; they are not enriched in GEM fraction when 1% Triton X-100 is used, but do show enrichment when 0.5 or 0.25% Triton X-100 is used. This suggests that SRC2-reactive components may have relatively weak interaction with GSLs, as compared to Yes, Fyn, or Lyn which are more enriched in GEM fraction prepared with 1% Triton X-100.

Enrichment of integrins α3 and α5 in GEM parallels enrichment of caveolin in GEM when ldlD cells are grown in the absence of Gal. This suggests that integrin, particularly in underglycosylated state, may interact with caveolin. The presence of integrin-associated microdomains was indicated by recent demonstration of a complex of $\alpha v\beta 3$ integrin with CD47 and heterotrimeric G-protein in GEM prepared from human melanoma cells, which was shown to be involved in signal transduction (17). Multiple microdomains at the cell surface membrane may be coordinated for control of cell adhesion coupled with signal transduction. The present study suggests that such coordination is regulated by lipid and protein glycosylation status. This view is somewhat conflicting with the observation that properties and protein profile of "DRM" are essentially the same between GSL-deficient mutant GM-95 and its parental cell line MEB-4 (18). Further studies may resolve this discrepancy.

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