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A Cholesterol-independent Membrane Microdomain Serves as a Functional Counter-receptor for E-selectin at the Colo201 Cell Surface and Initiates Signalling on E-selectin Binding

Chikako Suzuki and Naoya Kojima*
The Institute of Glycotechnology, Tokai University, Hiratsuka, Kanagawa 259-1292, Japan

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The present study demonstrates that the functional counter-receptors for E-selectin at the cell surface of Colo201 human colon cancer cells are localized in detergent-insoluble membrane microdomains (DIM). Following isolation of counter-receptors from whole cell lysates using E-selectin-coupled magnetic beads followed by sucrose density gradient separation, both sialyl Lewis a (SLe^a)- and sialyl Lewis x (SLe^x)-carrying glycoproteins which had bound to the E-selectin-beads were distributed in detergent-soluble fractions as well as DIM. In contrast, following isolation of counter-receptors directly from the cell surface, SLe^a-carrying glycoproteins which had bound to E-selectin-beads at the cell surface were localized only in DIM, together with a Src family kinase, Lyn, while SLe^x-carrying glycoproteins were not detected in any fraction. The counter-receptors were distributed in a diffuse pattern on the cell surface but clustered following E-selectin binding, leading to the subsequent phosphorylation of extracellular signal-regulated kinase (ERK). Treatment of the cells with methyl-β-cyclodextrin, a cholesterol-depleting drug, had little effect on either the association of SLe^a-carrying glycoproteins and Lyn with the domain or ERK phosphorylation. Thus, the functional counter-receptors and Lyn are co-localized in a cholesterol-independent microdomain and create a physiological domain ('glycosynapse') at the cell surface that initiates signalling in cancer cells upon binding to E-selectin.

Key words: colon cancer cell, detergent-insoluble microdomain, E-selectin, glycosynapse, sialyl Lewis a, Src family kinase.

Abbreviations: DIM, detergent-insoluble membrane microdomain; FITC, fluoroscein isothiocyanate; MCD, methyl-β-cyclodextrin; Sle^a, sialyl Lewis a; Sle^x, sialyl Lewis x.

Several lines of evidence indicate that cancer cells interact with vascular E-selectin to extravasate and form metastases (*1–3*). Moreover, the binding efficiency of colon cancer cells to E-selectin is directly proportional to their metastatic potential (*4*, *5*), indicating that E-selectin-mediated adhesion of colon cancer cells to endothelial cells is a key event in metastasis. The carbohydrate determinants sialyl Lewis a (SLe^a) and sialyl Lewis x (SLe^x) are known to be expressed on cancer cells and are representative oligosaccharides involved in E-selectin binding (*1*, *2*, *6*, *7*). Furthermore, the expression of SLe^a- or SLe^x-carrying glycoproteins correlates with tumour progression and a poor prognosis (*1*, *8*). To date, several glycoproteins such as MUC-1, LAMP-1, CD43 and CD44, which carry sialylated and fucosylated carbohydrates, have been demonstrated to be potential counter-receptors for E-selectin through their isolation by affinity chromatography using recombinant E-selectin from colon cancer cell lysates (*9–13*). Additionally, several studies have shown that adhesion to E-selectin initiates signalling in both cancer cells (*2*, *14–17*) and immune cells (*18*), suggesting that signalling through E-selectin binding leads to phenotypic changes and modulates the metastatic potential of cancer cells. However, the functional counter-receptors on the surface of colon cancer cells that are involved in signalling have remained unidentified. Recently, death receptor-3 (DR-3) has been identified as a new E-selectin counter-receptor and shown to trigger mitogen-activated protein kinase (MAPK) activation through binding to E-selectin (*19*).

Lipid rafts, also called detergent-insoluble microdomains (DIM), detergent-resistant membranes, caveolae, or glycosphingolipid-enriched microdomains (GEM), are specialized areas in the plasma membrane which are thought to function as membrane signalling platforms that facilitate association with signalling molecules such as GPI-anchored proteins, Src family kinases and small G-proteins (*20–23*). Interestingly, recent studies have shown that E-selectin is localized in cholesterol-enriched microdomains at endothelial cell surfaces, and that the clustering of E-selectin in these domains leads to the activation of several transducer molecules (*24*, *25*). Furthermore, P-selectin glycoprotein ligand-1 (PSGL-1), a functional counter-receptor for P-selectin on stimulated T cells, is localized in cholesterol-enriched microdomains (*26*), and the localization of PSGL-1 in the microdomain

*To whom correspondence should be addressed. Tel: 0463-58-1211 (Ex. 4645), Fax: 0463-50-2012, E-mail: naoyaki@keyaki.cc.u-tokai.ac.jp

has recently been shown to regulate selectin-dependent rolling (*27*). Therefore, the glycosynapse created between selectin and its counter-receptors in cholesterol-enriched microdomains of both types of cell might play an essential role in cell adhesion and signalling (*23*). However, the involvement of lipid rafts/microdomains in cancer cell adhesion to E-selectins has not yet been considered. Since Src family tyrosine kinases and MAPK are modulated in several types of cells, including colon cancer cells, through E-selectin binding (*14–17*, *19*), the functional counter-receptor for E-selectin may be localized in microdomains and associated with specific signal transducers.

In the present study, we examined the cell surface location of functional E-selectin counter-receptors on the cell surface of a colon cancer cell line, Colo201, before and after binding to E-selectin. Isolation of counter-receptors from the cell surface followed by sucrose density gradient separation demonstrated that only SLe^a-carrying glycoproteins localized in detergent (Triton X-100)-insoluble membrane microdomains with Lyn can serve as functional counter-receptors on the cell surface. The ligation of cell surface counter-receptors induced the clustering of microdomains into larger membrane domains, with the subsequent activation of extracellular signal-regulated kinase (ERK). Our results demonstrate that a particular type of membrane microdomain serves as a functional counter-receptor at the surface of Colo201 cells and triggers signaling on binding to E-selectin.

MATERIALS AND METHODS

Cells, Reagents and Antibodies—The human colon carcinoma cell line Colo201 was obtained from the American Type Culture Collection. Cells were grown in RPMI 1640 medium (GIBCO) supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Recombinant human E-selectin-Fc and recombinant human Fc were obtained from R&D Systems (Minneapolis, MN). Protein G-Sepharose and Dynabeads Protein G were obtained from GE Healthcare Bio-Science (Uppsala, Sweden) and Dynal Biotech (Oslo, Norway), respectively. An anti-SLe^a monoclonal antibody (KM231, mouse IgG) and an anti-SLe^x monoclonal antibody (KM93, mouse IgM) were purchased from Calbiochem (San Diego, CA). An anti-$DSLe^a$ monoclonal antibody (FH7, mouse IgG3) was kindly provided by Dr Reiji Kannagi (Aichi Cancer Center Research Institute). Rabbit polyclonal anti-phosphoprotein associated with glycosphingolipid-enriched microdomain (PAG) was purchased from Abcam (Cambridge, UK). Rabbit polyclonal anti-caveolin-1, anti-Lyn, anti-Fyn, anti-Yes, anti-CD43, anti-ERK and anti-phospho-ERK antibodies were obtained from SantaCruz Biotechnology (Santa Cruz, CA). Glycopeptidase F (*N*-glycanase) and *O*-sialoglycoprotease were purchased from Takara (Otsu, Japan) and Cosmo Bio (Tokyo, Japan), respectively. Protease inhibitor cocktail and octylglucoside were from Sigma-Aldrich (St Louis, MO)

Sucrose Density Gradient Separation of Cell Lysates—DIM were separated from cell lysates as described previously (*28*). Cultured cells were harvested, washed with phosphate-buffered saline (PBS), and homogenized on ice with 4 volumes of lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 200 μM $NaVO_4$, 1% Triton X-100, containing a protease inhibitor cocktail) using a Dounce homogenizer with 10–15 strokes. The cell lysates were centrifuged at 3,000 rpm for 5 min. The supernatants were adjusted to 42.5% sucrose by adding an equal volume of 85% sucrose. Samples (4 ml) were placed in an ultracentrifuge tube then overlaid with a gradient of 4 ml of 30% sucrose followed by 4 ml of 5% sucrose. The gradients were centrifuged at 40,000 rpm for 18 h in an SW41 rotor (Beckman Coulter). Twelve fractions (each 1 ml) were harvested from the top of the gradient and numbered 1 to 12 from top to bottom. With this procedure, DIM were located mainly in fractions four and five. Aliquots (10 μl) were analysed by Western blotting after SDS–PAGE on 4–20% gradient gels and transfered to polyvinylidene difluoride (PVDF) membranes, using antibodies directed against SLe^a, SLe^x, $DSLe^a$ and various other molecules. An enhanced chemiluminescence detection (ECL) system (GE Healthcare Bio-Science) was used to detect the specific protein bands. For treatment with methyl-β-cyclodextrin (MCD), cells (1×10^7) were suspended in serum-free RPMI 1640 medium (15 ml), incubated for 1 h at 37°C, and then 5 ml of 20 mM MCD were added to the suspension. After a further 1 h incubation at 37°C, cells were harvested, lysed, and then fractionated on a sucrose density gradient as above. To clarify depletion of cellular cholesterol by MCD treatment, cells treated with or without 5mM MCD were harvested, 2 ml of chloroform/methanol (1:1 v/v) were added to 1×10^7 cells, the samples were sonicated for 5 min, and centrifuged at 3,000 rpm at 4°C. The supernatants were collected and dried under N_2 gas. The amounts of cholesterol in the lipid extracts were determined by method of Richmond (*29*) using a cholesterol determination kit (Cholesterol E-test Wako, Wako Pure Chemical, Osaka, Japan). The cholesterol content in non-treated cells was 105.5 μg in 1×10^7 cells, while that in MCD-treated cells was 35.55 μg in 1×10^7 cells, indicating that the MCD treatment depletes cellular cholesterol. To deplete cellular cholesterol alternatively, aliquots of cell suspension were mixed with dimethylsulfoxide (DMSO) containing 2.0 μg/ml of filipin (Sigma), and incubated for 30 min at 37°C (*28*). After incubation cells were harvested, lysed, and then fractionated on a sucrose density gradient as above.

Separation of E-selectin Ligand Microdomains by E-selectin-Fc-coupled Column—DIM (fractions four and five) containing SLe^a-carrying glycoproteins were diluted with five volumes of coupling buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM $CaCl_2$ and 0.1% Triton X-100), centrifuged at 30,000 rpm for 1 h, and suspended in a small volume of coupling buffer. Recombinant human E-selectin-Fc (100 μg) was coupled to protein G-Sepharose beads (1 ml) in coupling buffer for 1 h at room temperature, and the beads were washed twice with coupling buffer. The DIM were mixed with the E-selectin-Fc-coupled protein G-Sepharose beads, and then incubated for 1 h at room temperature with gentle rotation. The beads were packed in a column (0.5 × 5 cm),

and washed with 3 ml of coupling buffer. The column was eluted with 3 ml of elution buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA and 0.1% Triton X-100). Fractions (0.5 ml) were collected and 10 μl of each were processed for Western blot analysis.

Immunoprecipitation and Pull-down Assay—Pull-down assays using recombinant E-selectin-Fc were performed as follows. DIM suspended in coupling buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM $CaCl_2$ and 0.1% Triton X-100) were pre-cleared with recombinant human Fc-coupled protein G-Sepharose (20 μl) for 1 h at 4°C. After centrifugation, the supernatant was incubated with recombinant human E-selectin-Fc-coupled protein G-Sepharose (20 μl) for 1 h at 25°C. The resin was recovered by centrifugation and washed with coupling buffer, after which the molecular complexes were released from the protein G-Sepharose by boiling for 5 min with 15 μl of 2× concentrated sample buffer. Proteins were separated by SDS–PAGE, transferred to a PVDF membrane, and analysed by Western blotting using anti-SLe^a and anti-Lyn antibodies. For immunoprecipitation using anti-Lyn, antibodies samples were pre-cleared with protein G-Sepharose (20 μl) for 1 h at 4°C. After centrifugation, supernatants were incubated overnight with anti-Lyn antibodies (5 μg) at 4°C. Immune complexes were recovered following the addition of protein G-Sepharose (20 μl) after 3 h at 4°C. For controls, control rabbit IgG was used instead of an anti-Lyn antibody.

Cross-linking of Cell Surface Counter-receptors and Fluorescence Microscopy—Cells were first incubated with E-selectin-Fc (5 μg/ml) for 5 min in RPMI 1640 medium supplemented with 10% FCS at room temperature, washed with the same medium, and then incubated for 30 min on ice with Cy3-conjugated anti-human Fc goat antibodies (1/50 in medium). The cells were washed with medium and fixed in 2% paraformaldehyde. The fixed cells were then incubated with anti-SLe^a monoclonal antibodies (mouse IgG, 5 μg/ml) for 1 h on ice, and then for an additional 30 min with fluoroscein isothiocyanate (FITC)-conjugated anti-mouse IgG goat antibodies (1/50 in medium). Stained cells were mounted on glass slides with Vectashield Hard Set Mounting Medium (Vector Laboratories, Burlingame, CA). For confocal images, cells were visualized using a confocal laser microscope (Bio-Rad). Images were taken with an X100 oil immersion lens, and serial sections were taken along the *z*-axis in 0.5-μm increments from the top to the bottom of the cells.

To crosslink cell surface counter-receptors, cells were incubated with E-selectin-Fc (5 μg/ml) for 5 min on ice in RPMI 1640 medium without serum, and washed once with the same medium, and then anti-human Fc goat antibodies (10 μg/ml) or Cy3-conjugated anti-human Fc goat antibodies (1/50 dilution) in pre-warmed RPMI 1640 medium were added to the cells. The cells were incubated at 37°C for various times. For detection of ERK phosphorylation, cells were lysed with lysis buffer and lysates were analysed by Western blotting after SDS–PAGE on 4–20% gradient gels and transfered to a PVDF membrane, using antibodies directed against ERK and phospho-ERK. For fluorescence microscopy, cells were washed with medium and then fixed in 2% paraformaldehyde. For the detection of non-ligated counter-receptors on the cell surface, cells were first fixed in paraformaldehyde and then stained with E-selectin-Fc and Cy3-conjugated anti-human Fc goat antibodies.

Cell Surface Trapping of Functional Counter-receptors for E-selectin—Recombinant human E-selectin-Fc (10 μg) was added to protein G-conjugated magnetic beads (Dynabeads Protein G, 100 μl) in 20 mM citrate-phosphate buffer (pH 5.7) for 40 min at room temperature to generate E-selectin-Fc-coupled magnetic beads. Cells (5×10^7) were harvested, washed with RPMI 1640 medium without serum and suspended in 2 ml of cold RPMI 1640 medium without serum. The E-selectin-Fc-coupled magnetic beads were added to the cell suspension with gentle mixing. Immediately after mixing, the tube was placed on a magnet (Dynal MPC-S) for 1 min to recover cells that had bound to E-selectin-Fc-coupled magnetic beads. Then the cells were lysed with 20 mM HEPES, pH 6.8, 150 mM NaCl, 5 mM $CaCl_2$ and 1% Triton X-100 for 2 min. The tube was then replaced on the magnet to recover the functional cell surface counter-receptor complexes. The magnetic beads were washed with 20 mM HEPES, pH 6.8, 150 mM NaCl, 5 mM $CaCl_2$ and 0.1% Triton X-100, and the counter-receptors were eluted with 500 μl (total) of 20 mM HEPES, pH 6.8, 150 mM NaCl, 10 mM EDTA and 0.1% Triton X-100. The eluted samples were then subjected to sucrose density gradient separation as described earlier. Twelve fractions (each 1 ml) were harvested from the top of the gradient and numbered 1 to 12 from top to bottom; 10 μl aliquots of each were analysed by Western blotting. Alternatively, counter-receptors for E-selectin were isolated from whole cell lysates. For this, cells were first lysed with 20 mM HEPES, pH 6.8, 150 mM NaCl, 5 mM $CaCl_2$ and 1% Triton X-100, then E-selectin-Fc coupled magnetic beads were added to the cell lysates with gentle mixing. After 2 min, the cell lysates containing magnetic beads were placed on the magnet to recover the counter-receptor complexes. The receptors were eluted and separated as described earlier.

RESULTS

SLe^a-carrying Glycoproteins are Localized in a Low-density Membrane Fraction—To evaluate the subcellular localization of SLe^a epitopes and counter-receptors for E-selectin, Colo201 cells were analysed for the surface expression of these molecules by fluorescence microscopy. As shown in Fig. 1, E-selectin counter-receptors, which appear as E-selectin-Fc-binding sites (*red*), showed a punctate staining pattern. In contrast, SLe^a (*green*) displayed a more diffuse and only slightly punctate staining pattern. When merged, only partial colocalization of SLe^a and cell surface counter-receptors was observed, as evidenced by the *orange* and *yellow* staining. These results indicate that functional counter-receptors for E-selectin with SLe^a epitopes are localized in microdomains or lipid rafts on the cell surface.

To determine whether the potential counter-receptors exist in membrane microdomains, cells were lysed with

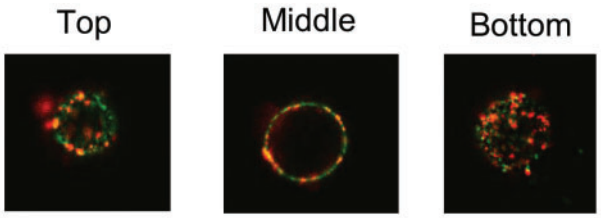

Fig. 1. **Distribution of counter-receptors for E-selectin and SLe^a-epitopes on the cell surface.** Colo201 cells were first incubated with E-selectin-Fc for 5 min at room temperature, and then incubated for 30 min with Cy3-conjugated goat anti-human Fc antibodies. The cells were then fixed and incubated with an anti-SLe^a monoclonal antibody for 1 h on ice, and subsequently for an additional 30 min with FITC-conjugated goat anti-mouse IgG antibody. The cells were visualized using confocal laser microscopy. Images were taken with an X100 oil immersion lens, and serial sections were taken along the *z*-axis at 0.5 μm increments from top to bottom of the cells. Merged images from the top, middle and bottom of the *z*-axis are shown. *Red* indicates E-selectin-Fc binding sites and *green* indicates SLe^a distribution. *Orange* to *yellow* indicates sites where E-selectin counter-receptors and SLe^a are co-localized.

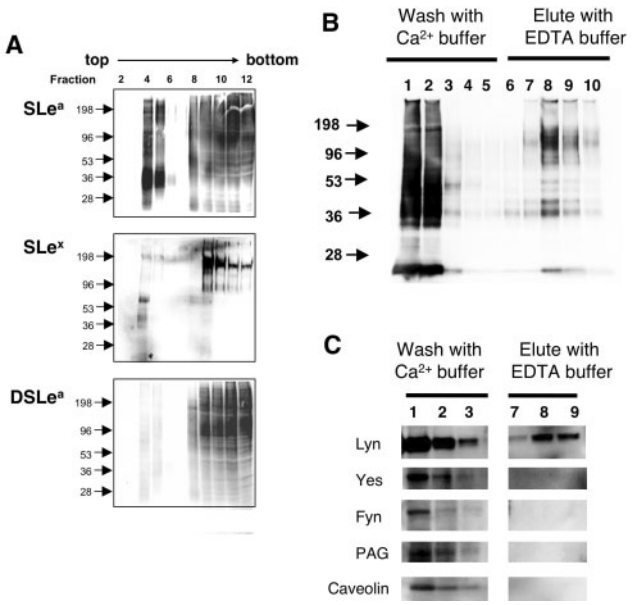

Fig. 2. **Detergent-insoluble microdomains can serve as counter-receptors for E-selectin.** Cells were harvested and homogenized on ice with 1% Triton X-100-containing lysis buffer. The lysates were fractionated on a sucrose density gradient. Twelve fractions were harvested from the top of the gradient and numbered 1 to 12 from top to bottom. By this procedure, low-density membrane fractions were located mainly in fractions 4 and 5. Aliquots (10 μl) of each fraction were analysed by Western blotting using antibodies directed against SLe^a, SLe^x and DSLe^a, as described in 'MATERIALS AND METHODS'. The distribution of carbohydrate epitopes that may serve as possible counter-receptors is shown in panel A. The low-density membrane fractions (Fractions 4 and 5 in Panel A) containing SLe^a-carrying glycoproteins were suspended in Ca^{2+}-containing buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM $CaCl_2$ and 0.1% Triton X-100), mixed with E-selectin-Fc-coupled protein G-Sepharose beads, and incubated for 1 h at room temperature with gentle rotation. The beads were packed in a column (0.5 × 5 cm) and washed with 3 ml of the Ca^{2+}-containing buffer. The column was eluted with 3 ml of EDTA-containing buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA and 0.1% Triton X-100). Elution profiles of SLe^a-epitopes (panel B) and other microdomain-associated molecules (panel C) are shown.

1% Triton X-100-containing buffer, and the resulting cell lysates were separated on sucrose density gradients prior to analysis of carbohydrate epitopes involving E-selectin binding in the different fractions. As shown in Fig. 2A, SLe^a were mainly detected in high-density, detergent-soluble fractions, but a proportion was clearly detectable in detergent-insoluble, low-density fractions. In contrast to SLe^a distribution, majority of SLe^x and DSLe^a epitopes were detected only in high-density fractions, although some bands were non-specifically detected in low-density fraction. CD43, which has been suggested as a potential E-selectin counter-receptor in colon cancer cells (*11*, *12*), was observed in high-density fractions (data not shown). The Src family protein kinases c-Src, Yes, Lyn and Fyn were enriched in DIM. In addition, caveolin-1 and phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), known to exist in cholesterol-rich microdomains and GEM, respectively, were also enriched in DIM (data not shown). Muc-1 and DR3 were not detected by immunoblotting in this cell line.

Low-density Membrane Fraction Containing SLe^a-carrying Glycoproteins Serves as a Potential Counter-receptor Domain for E-selectin—Next, we examined whether the membrane microdomain containing SLe^a-carrying glycoconjugates could serve as a counter-receptor domain for E-selectin. The low-density membrane fractions containing SLe^a-carrying glycoproteins (Fractions 4 and 5 in Fig. 2A) were applied to a recombinant E-selectin-Fc-coupled protein G-Sepharose column (E-selectin column), washed with Ca^{2+}-containing buffer and then eluted with EDTA-containing buffer. As shown in Fig. 2B, a proportion of SLe^a-carrying glycoconjugates was bound to the E-selectin column and eluted with EDTA. Among the several Src family kinases enriched in the DIM, only Lyn bound to an E-selectin column and was eluted with EDTA together with the SLe^a-carrying glycoconjugates (Fig. 2C). It should be noted that caveolin-1 and PAG, both of which are located in the DIM, did not bind to the E-selectin column (Fig. 2C). When the fractions eluted with EDTA were subjected to sucrose density gradient separation, SLe^a-carrying glycoconjugates and Lyn were again distributed in low-density fractions (data not shown). Therefore, Lyn and SLe^a-carrying glycoconjugates are localized in the same microdomain which may serve as a potential counter-receptor domain for E-selectin on the cell surface.

Two major SLe^a-carrying glycoproteins, which migrated at 120 kDa and 37 kDa, respectively, were found in the E-selectin-binding membrane microdomain, while SLe^a-carrying glycoconjugates which migrated at 200 kDa and 70 kDa were found as E-selectin counter-receptors in high-density soluble fractions (Fig. 3A). *N*-Glycanase treatment slightly reduced the molecular mass of the 120 kDa glycoprotein without altering their reactivity toward anti-SLe^a antibodies, while the same

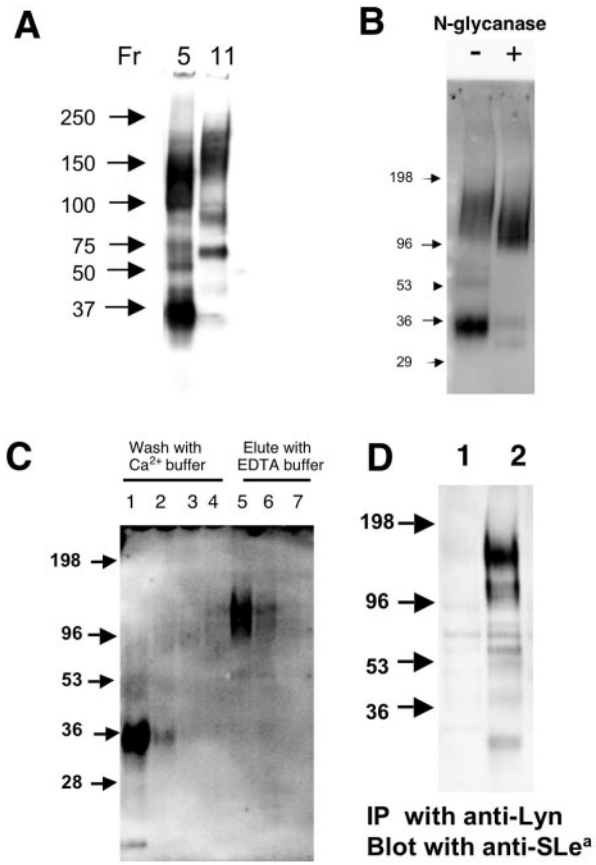

Fig. 3. **Biochemical analysis of counter-receptors.** (A) SLe^a-carrying glycoproteins in counter receptors localized in low- and high-density fractions. Low- and high-density membrane fractions (Fraction 5 and Fraction 11, respectively, in Fig. 2, panel A) were incubated with recombinant human E-selectin-Fc-coupled protein G-Sepharose for 1 h at 25°C. The resin was recovered by centrifugation and washed with buffer, and the bound molecular complexes were released by boiling for 5 min with 15 μl of 2× concentrated sample buffer. Proteins were separated by SDS–PAGE and analysed by Western blotting using anti-SLe^a antibodies. (B) *N*-Glycanase treatment of microdomain fraction that bound to E-selectin-Fc-coupled protein G-Sepharose. (C) The low-density membrane fraction was treated with 1% octylglycoside-containing buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM $CaCl_2$, 1% Triton X-100 and 1% octylglucoside) overnight and then centrifuged; the resultant supernatant was subjected to chromatography on an E-selectin-Fc-coupled protein G-Sepharose column equilibrated with the same buffer. The column was washed with the same buffer and then eluted with EDTA-containing buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100 and 1% octylglucoside). Fractions (0.5 ml) were collected and aliquots analysed by Western blotting using antibodies directed against SLe^a. (D) Glycoconjugates bound to an E-selectin-Fc-coupled protein G-Sepharose column (Fraction 5 in Panel C) were incubated with an anti-Lyn antibody (lane 2) or control rabbit IgG (lane 1), after which immune complexes were recovered by the addition of protein G-Sepharose, as described in 'MATERIALS AND METHODS'.

treatment led to a reduction in SLe^a epitopes in the 37 kDa glycoprotein (Fig. 3B). Binding of the domain to the E-selectin column was not affected by this treatment. When the counter-receptor domain was disrupted by treatment with 1% octyl-glucoside followed by separation on an E-selectin column, the 120 kDa glycoprotein clearly bound to the column and eluted with EDTA, while the 37 kDa glycoprotein completely passed through the column (Fig. 3C). SLe^a epitopes in 120 kDa glycoprotein disappeared on treatment with *O*-sialoglycoprotease. The 120 kDa glycoprotein is likely to be closely associated with Lyn, since the two were co-immunoprecipitated by an anti-Lyn antibody (Fig. 3D). These sequential biochemical analyses suggested that SLe^a carried on *O*-glycans of 120 kDa mucin-type glycoprotein might be responsible for binding of the domain to E-selectin and its association with Lyn. On the other hand, 75 kDa and 200 kDa SLe^a-carrying glycoproteins were isolated from high-density fractions by E-selectin column chromatography. Therefore, these molecules distributed in non-raft fractions may also be potential counter-receptors.

The SLe^a-containing Microdomain is a Functional Cell Surface Counter-receptor Domain on E-selectin Binding—As described previously, we showed that potential E-selectin counter-receptors were localized in Triton X-100-insoluble membrane microdomains. However, it was not clear whether these membrane microdomains serve as functional counter-receptor domains on the cell surface, since both SLe^a-carrying glycoconjugates and SLe^x-carrying glycoconjugates were abundant in Triton X-100 soluble fractions, and some could bind to E-selectin. Therefore, we next tried to isolate such receptors using E-selectin-Fc coupled with protein G-conjugated magnetic beads. First, potential counter-receptors were isolated from whole cell lysates and subjected to sucrose density gradient separation. As shown in Fig. 4A, SLe^a-carrying glycoproteins were distributed in both DIM and high-density fractions, while SLe^x-carrying glycoproteins and CD43 were distributed only in high-density fractions. The molecular masses of E-selectin-binding SLe^a-carrying glycoproteins in low-density fractions were similar to those shown in Fig. 3A. These results again indicate that multiple glycoproteins localized in various membrane regions can serve as counter-receptors when isolated from whole cell lysates. On the other hand, when the counter-receptors were directly isolated from the cell surface, E-selectin-binding SLe^a-carrying glycoconjugates were most abundant in DIM fractions (Fig. 4A, right). Interestingly, SLe^x-carrying glycoproteins and CD43, which could serve as counter-receptors in whole cell lysates, were not detected in the 'non-raft' high-density fraction, indicating that these molecules do not serve as ligands for E-selectin at the cell surface. Therefore, the DIM containing SLe^a-carrying glycoconjugates acts as a functional counter-receptor or receptor domain for E-selectin at the cell surface.

The molecular masses of E-selectin-binding SLe^a-carrying glycoconjugates in microdomains isolated from the cell surface were different from those of comparable glycoconjugates isolated from whole cell lysates (Fig. 4B). The E-selectin-binding microdomain from whole cell lysates contained 120 kDa and 37 kDa SLe^a-carrying glycoproteins, whereas E-selectin-binding microdomains isolated from the cell surface contained 200 kDa, 120 kDa, 60 kDa and 37 kDa SLe^a-carrying glycoproteins.

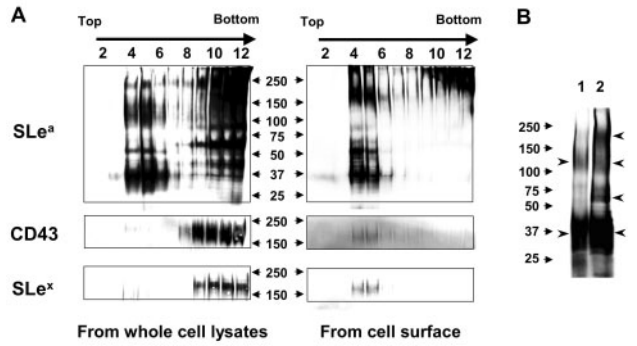

Fig. 4. **Direct isolation of E-selectin counter-receptors from the cell surface.** (A) Cells (5×10^7) were suspended in 2 ml of cold RPMI 1640 medium without serum, and E-selectin-Fc-coupled magnetic beads were added to the cell suspension with gentle mixing. Immediately after mixing, the tube was placed on a magnet for 1 min. To recover bound cells the tube was removed from the magnet, and the cells were lysed with 20 mM HEPES, pH 6.8, 150 mM NaCl, 5 mM $CaCl_2$ and 1% Triton X-100 for 2 min. The tube was then placed on the magnet to recover functional cell-surface counter-receptor complexes. The magnetic beads were washed with 20 mM HEPES, pH 6.8, 150 mM NaCl and 5 mM $CaCl_2$, and the counter-receptors were eluted from the beads with 500 µl (total) of 20 mM HEPES, pH 6.8, 150 mM NaCl and 10 mM EDTA. Alternatively, cells were first lysed with 20 mM HEPES, pH 6.8, 150 mM NaCl, 5 mM $CaCl_2$ and 1% Triton X-100, after which E-selectin-Fc-coupled magnetic beads were added to the whole cell lysates. After 2 min the tube was placed on the magnet to recover counter-receptors which were subjected to sucrose density gradient separation as described. (B) Low-density membrane fractions (fractions 4 and 5 in Panel A) of counter-receptors isolated from the cell surface (lane 2) or whole cell lysates (lane 1) were separated on a 4–20% gradient gel and analysed by Western blotting using antibodies directed against SLe^a.

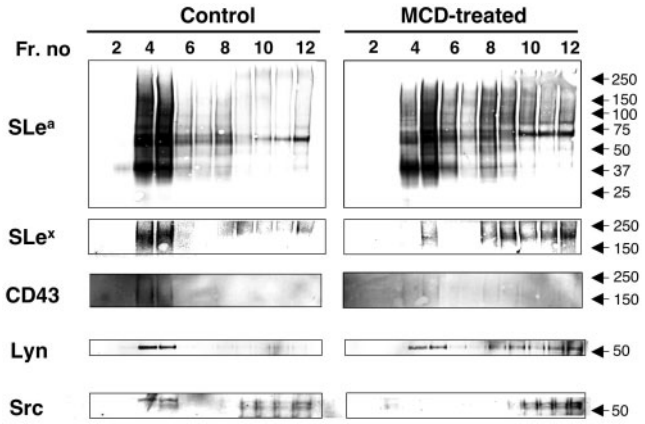

Fig. 5. **Effect of methyl-β-cyclodextrin on the distribution of molecules associated with counter-receptor domains.** Cells were suspended in serum-free medium and then treated with 5 mM (final concentration) MCD for 1 h at 37°C to deplete cell surface cholesterol. The cells were harvested and cell surface counter-receptors were isolated as described in the legend to Fig. 4, followed by sucrose density gradient separation. Aliquots of each fraction were analysed by Western blotting using antibodies directed against SLe^a, SLe^x, CD43, Lyn and Src.

It should be noted that small amounts of SLe^x-carrying glycoproteins with molecular masses of about 200 kDa and CD43 were found in this domain. These results suggest that the additional receptors involving E-selectin binding are accumulated in the counter-receptor domain upon E-selectin binding to cells as a result of clustering of receptors.

Methyl-β-cyclodextrin Treatment has no Effect on Cell Surface Counter-receptor Distribution, Cell Adhesion, or Signalling—Since PSGL-1 has been shown to be localized in cholesterol-enriched microdomains, and leucocyte rolling on P-selectin has been demonstrated to depend on the localization of PSGL-1 in such microdomains (*25*, *26*), we examined the involvement of cholesterol-enriched microdomains in the adhesion of colon cancer cells to E-selectin. Functional counter-receptors were isolated from cells cultured in serum-free medium with or without MCD (final concentration 5 mM) for 1 h followed by sucrose density gradient separation. This treatment depletes cellular cholesterol (35.55 µg and 105.5 µg in 1×10^7 of MCD-treated and non-treated cells, respectively) and causes a loss of compartmentalization of cholesterol-enriched microdomain-associated molecules. As shown in Fig. 5, functional counter-receptors (SLe^a-carrying glycoproteins) were localized in microdomain fractions and Lyn and Src were clearly associated with the functional counter-receptor domains in non-treated cells. In addition, small amounts of CD43 and SLe^x-carrying glycoproteins were co-localized in the functional counter-receptor domains. On treating cells with MCD, most SLe^x-carrying glycoproteins, CD43, and Src were translocated to high-density fractions, suggesting that they were distributed in cholesterol-enriched microdomains. In contrast, SLe^a-carrying glycoproteins and Lyn were still abundant in microdomain fractions even after the treatment of cells with MCD, although a proportion of each was translocated to high-density fractions, indicating that most of these molecules were localized in a particular type of microdomain distinct from cholesterol-enriched microdomains. In additon, distribution of SLe^a-carrying glycoproteins and Lyn was not affected by the treatment of cells with filipin, another cholesterol-depleting drug (data not shown).

To evaluate the extent of clustering of counter-receptors on the cell surface during E-selectin binding and following signalling, we performed a crosslinking experiment. Serum-starved cells were treated with E-selectin-Fc followed by either Cy3-conjugated anti-Fc antibodies or anti-Fc antibodies to crosslink E-selectin counter-receptors. Counter-receptors showed a diffuse or partially punctate staining pattern before binding to E-selectin, whereas after binding there was a marked change in distribution, with an increased number of punctate counter-receptor foci (Fig. 6A). This suggests that counter-receptor-containing microdomains cluster into larger membrane domains through a process of rearrangement. Treatment with MCD did not affect the distribution or clustering of counter-receptors. In addition, cell adhesion to E-selectin coated plates was unchanged after treatment with MCD (data not shown).

Interaction between colon cancer cells and E-selectin activates ERK pathways in cancer cells (*14–17*).

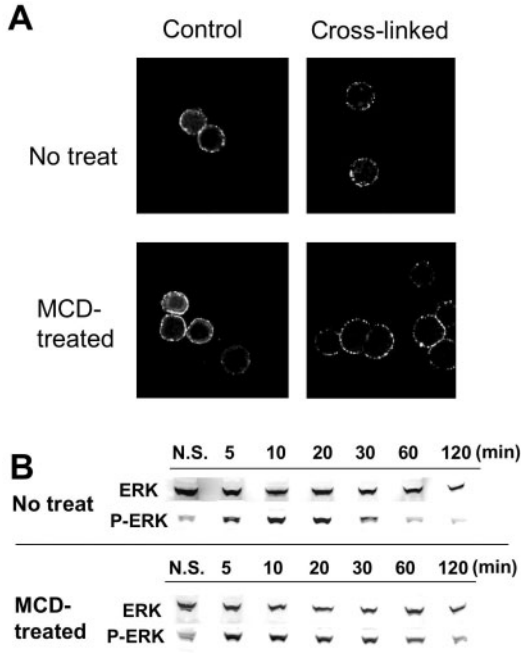

Fig. 6. **Crosslinking of counter-receptors induces clustering and signalling but these were not diminished by treatment with methyl-β-cyclodextrin.** (A) Clustering of cell surface counter-receptors upon crosslinking with E-selectin-Fc. Colo201 cells were treated with 5 mM MCD for 1 h at 37°C, incubated with E-selectin-Fc for 5 min on ice, and then incubated for 10 min at 37°C with Cy3-conjugated goat anti-human Fc antibodies. For the control, the treated cells were fixed and stained with E-selectin-Fc. (B) Phosphorylation of ERK by crosslinking of cell surface counter-receptors. Cells were cultured in serum-free medium for 2 h, and then cultured with or without 5 mM MCD for an additional 1 h. The cells (1×10^7) were incubated with 5 μg/ml of E-selectin-Fc on ice for 5 min, and then 10 μg/ml of anti-human Fc goat antibody was added to the mixture. After incubation at 37°C for the indicated times, the cells were lysed and ERK activation was assessed by immunoblotting. Neither counter-receptor clustering nor ERK activation were diminished by treatment with MCD.

We therefore assessed the activation of ERK pathways by ligation of counter-receptors with E-selectin, and the effect of MCD treatment on E-selectin-mediated activation of this pathway. As shown in Fig. 6B, ERK phosphorylation markedly increased within 10 min of the ligation of cell surface counter-receptors with E-selectin-Fc followed by crosslinking with anti-Fc antibodies, and subsequently gradually decreased, indicating that signal transduction had occurred as a result of E-selectin binding by counter-receptor domains and subsequent ligation of receptors. When cells were treated with MCD followed by ligation of cell surface counter-receptors, ERK phosphorylation was unchanged in comparison with non-treated cells (Fig. 6B).

DISCUSSION

Mucin-type glycoproteins having tandem repeat peptides with multiple *O*-linked glycans are carriers of various glycoepitopes for E-, P- and L-selectins and are involved in selectin-mediated cell adhesion (*30*). In particular, PSGL-1 has been characterized as a dimeric mucin-type glycoprotein with a novel N-terminal epitope and tandem repeat *O*-linked glycans (*31*), the expression of which is essential for P-selectin-dependent adhesion. In addition, MUC-1 and CD43, both of which are mucin-type glycoproteins, carry both SLe^a and SLe^x, and have been shown to serve as potential counter-receptors for E-selectin in colon cancer cells. Although Hakomori and colleagues have demonstrated that PSGL-1 and MUC-1 are highly enriched in a cholesterol-enriched membrane microdomain fraction in a human T-cell lymphoma cell line (*25*), studies of mucin-type glycoprotein-containing microdomains have been limited. Several studies have shown that LAMP-1, CD43, CD44, MUC-1 and DR-3 can serve as potential counter-receptors on colon cancer cells (*9–13*, *19*), and that the adhesion of cancer cells to E-selectin initiates signalling in these cells (*14–17*, *19*). However, little has been known about the presence of these potential counter-receptors in microdomains or their direct or indirect association with signal transducers. In addition, it was not clear whether these molecules serve as functional counter-receptors on the cell surface under physiological conditions, since they had been isolated from cancer cell lysates by E-selectin-coupled column chromatography. The major contribution of our study is to have shown by the direct isolation of counter-receptors from the cell surface that the functional/physiological E-selectin counter-receptors on the cell surface of Colo201 colon cancer cells are mainly localized in cholesterol-independent membrane microdomains together with Src family kinases to create functional counter-receptor domains, and that binding of the functional domains to E-selectin leads to their rearrangement and subsequent ERK activation.

The localization of E-selectin counter-receptors in microdomains was first achieved by immunostaining using E-selectin-Fc. The punctate staining pattern of E-selectin-Fc binding sites indicated ligand distribution in lipid rafts or microdomains. Subsequently, by separating cell lysates on a sucrose density gradient followed by affinity chromatography, we found that a portion of the low-density buoyant membrane fractions, which contained SLe^a-carrying mucin-type glycoproteins, could bind to an E-selectin column, and that the Src family protein kinase Lyn was characteristically associated with the E-selectin-binding microdomain. Finally, we found that E-selectin-Fc-binding SLe^a-carrying mucin-type glycoproteins directly isolated from the cell surface were concentrated in the DIM together with Lyn and Src. It should be noted that the majority of SLe^a-carrying glycoproteins that served as E-selectin counter-receptors were found only in DIM. In contrast, many SLe^a-carrying glycoproteins were found in high-density soluble fractions when the counter-receptors were isolated from whole cell lysates. This indicates that SLe^a-carrying glycoproteins in microdomains can serve as preferential E-selectin counter-receptors on the surface of Colo201 cells under physiological conditions. Protein-glycan interactions are expected to be typically of low affinity, requiring multivalent interactions to achieve a biological effect (*32*, *33*), and human colon cancer cells express multiple E-selectin counter-receptors (*9*, *10*). Therefore, the multivalent localization of the counter-receptors in

microdomains might restrict which potential counter-receptors (SLe^a-carrying glycoproteins) are functional ligands *in situ* (*29*). Interestingly, SLe^x-carrying glycoproteins and CD43 serving as counter-receptors were barely detectable in membrane microdomains or high-density soluble fractions when counter-receptors were isolated directly from the cell surface, whereas they were clearly detectable in high-density soluble fractions when the counter-receptors were isolated from whole cell lysates. Therefore, most SLe^x-carrying glycoproteins and CD43 do not serve as cell surface receptors, even if they are expressed on the cell surface.

We have shown in this article that Src family kinases are associated with counter-receptor domains and that ERK phosphorylation is transiently increased when the cell surface counter-receptors are ligated with E-selectin-Fc. In addition, we have observed the ligation-induced redistribution and clustering of E-selectin counter-receptors. Some SLe^a-and SLe^x-carrying glycoproteins and CD43, which were found in detergent-soluble high-density fractions in the unbound state, were partially translocated into DIM upon binding to E-selectins. Therefore, as a consequence of the adhesion of colon cancer cells to E-selectin, the counter-receptor domains may be rearranged into larger membrane domains, and may recruit additional counter-receptors into DIM. This would seem possible since Tilghman and Hoover (*34*) have reported that following leucocyte adhesion E-selectin and intercellular adhesion molecule-1 in endothelial cells are incorporated into DIM. The close association of mucin-type counter-receptors with transducers in the same microdomains, and the rearrangement of microdomains upon binding to E-selectin, raise the interesting possibility that E-selectin binding to the epitope carbohydrates (SLe^a) in *O*-linked glycoclusters in the particular membrane domains triggers signal transduction, leading to phenotypic changes in cancer cells.

Caveolin-1 and PAG were not detected in the E-selectin counter-receptor domain. Since caveolin is a known scaffolding protein of caveolae (*35*), and PAG is a ubiquitously expressed adaptor protein present in glycosphingolipid-enriched microdomains (GEM) (*36*), the counter-receptor domains in Colo201 cells might be distinct from currently recognized characteristic membrane microdomains such as caveolae or GEM. Recently, based on the accumulation of evidence concerning the functional roles of carbohydrate epitopes in microdomains (*37–41*), Hakomori has proposed the 'glycosynapse' as a new concept for microdomains (*23*, *42*). The term 'glycosynapse' is applied to the membrane assembly involved in glycosylation-dependent cell adhesion and signaling. According to this definition, the counter-receptor domain for E-selectin can be considered as one type of 'glycosynapse'. To date, three types of glycosynapse have been proposed (*23*): (i) GSL clusters organized with signal transducers (type 1); (ii) *O*-linked mucin-type adhesion epitopes organized with signal transducers in cholesterol-rich lipid microdomains (type 2) and (iii) *N*-glycosylated adhesion receptors complexed with tetraspanin and gangliosides (type 3). Mucin-type glycoproteins such as MUC-1 and PSGL-1 have been clearly shown to exist in cholesterol-enriched lipid microdomains (type 2 glycosynapse) based on their insusceptibility to treatment with MCD, a cholesterol-depleting drug (*26*). Recently, it has been reported that the disruption of lipid rafts with MCD strongly reduces the selectin-dependent rolling of leucocytes (*27*), suggesting that type 2 glycosynapses might play a major role in leucocyte rolling and subsequent signalling. On the other hand, the treatment of Colo201 cells with MCD had only a small effect on the localization of SLe^a-carrying mucin type glycoproteins and Lyn. Src and SLe^x-carrying glycoproteins localized in the domains were clearly dispersed by the same treatment. Furthermore, neither the rearrangement of cell surface counter-receptors nor ERK phosphorylation induced by the ligation of E-selectin counter-receptors, or cell binding to E-selectin, were diminished by this treatment, suggesting that the functional receptors for E-selectin are localized in a particular type of microdomain distinct from the cholesterol-enriched lipid microdomain in which functional PSGL-1 and MUC-1 are located. Thus, the counter-receptor domain for E-selectin on Colo201 cells may be a new type of 'glycosynapse' containing *O*-linked mucin-type adhesion epitopes.

In conclusion, mucin-type counter-receptors for E-selectin carrying SLe^a-epitopes and Lyn are co-localized in DIM distinct from cholesterol-enriched microdomains and caveolae-like microdomains, and create a physiological counter-receptor domain at the cell surface. Upon cell binding to E-selectin, the counter-receptor domains rearrange into large domains in a process involving the recruitment of additional receptors into a functional domain. Although potential E-selectin ligands have been isolated from several colon cancer cells and identified as LAMP-1, LAMP-2, CD44, MUC-1 and DR3, these potential counter-receptors were not detected in the E-selectin ligand microdomain of Colo201 cells by Western blotting using specific antibodies. CD43 expressed in this cell line seems not to serve as a functional receptor on the cell surface. Further experiments are required to elucidate the functional molecules present in the counter-receptor domains. Recent studies have shown that E-selectin is localized in cholesterol-enriched microdomains at the cell surface of endothelial cells, and that clustering of E-selectin in these domains leads to the rearrangement of membrane compartments and the activation of several transducer molecules such as phospholipase Cγ and Src family tyrosine kinases (*24*, *25*). These findings might indicate that interaction between clustered E-selectin in microdomains on the endothelial cell surface and clustered counter-receptors in microdomains on cancer cell surfaces is crucial for the binding/adhesion of colon cancer cells to endothelial cells in physiological conditions, and that consequent changes in signalling lead to phenotypic changes in both types of cell.

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