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J Immunol 2003; 171:3216-3224; doi: 10.4049/jimmunol.171.6.3216
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Lipid Raft Localization of Cell Surface E-Selectin Is Required for Ligation-Induced Activation of Phospholipase Cγ1

Jeanne-Marie Kiely, Yenya Hu, Guillermo García-Cardenea, and Michael A. Gimbrone, Jr.2

E-selectin, an endothelial cell surface adhesion receptor for leukocytes, also acts as a signaling receptor. Upon multivalent ligation, E-selectin transduces outside-in signals into the endothelium leading to changes in intracellular Ca2+ concentration and activation of the mitogen-activated protein kinase signaling pathway. In addition, following leukocyte engagement, E-selectin associates via its cytoplasmic domain with components of the actin cytoskeleton and undergoes alterations in phosphorylation state that result in changes in gene expression. In this study, we show that E-selectin is localized in cholesterol-rich lipid rafts at the cell surface, and that upon ligation E-selectin clusters and redistributes in the plasma membrane colocalizing with a fraction of caveolin-1-containing rafts. In addition, we demonstrate that leukocyte adhesion via E-selectin results in association with and activation of phospholipase Cγ (PLCγ). Moreover, we show that disruption of lipid rafts with the cholesterol-depleting drug methyl-β-cyclodextrin disrupts the raft localization of E-selectin as well as the ligation-induced association of E-selectin with PLCγ, and subsequent tyrosine phosphorylation of PLCγ. In contrast, cholesterol depletion has no effect on E-selectin-dependent mitogen-activated protein kinase activation. Thus, these findings demonstrate that the presence of E-selectin in lipid rafts is necessary for its association with, and activation of, PLCγ, and suggest that this subcellular localization of E-selectin is related to its signaling function(s) during leukocyte-endothelial interactions. The Journal of Immunology, 2003, 171: 3216–3224.
transmembrane molecules, including platelet-derived growth factor (PDGF) receptor, epidermal growth factor (EGF) receptor, endothelin receptor, ICAM-1, and some integrins (11–13). Lipid rafts also are enriched in signaling molecules such as heterotrimeric G proteins, endothelial NO synthase, Ras, nonreceptor tyrosine kinases including src kinases, adaptor molecules such as Shc, Sos, and Grb2, as well as phosphoinositides, phosphatidylinositol-3 kinase, and diacylglycerol (11, 12, 20, 21). The microenvironment of lipid rafts seems to encourage compartmentalization of signaling molecules into large multimolecular complexes. Upon receptor ligation, lipid rafts cluster into large units up to hundreds of nanometers in diameter (22). The clustered rafts often become associated with the cytoskeleton and recruit additional signaling molecules (11). Thus, localization of E-selectin in lipid rafts would be an attractive mechanism for supporting E-selectin signaling, potentially with or through both known and as yet unidentified molecules.

In the present study, we demonstrate that in its unligated state, E-selectin is compartmentalized in cholesterol-enriched membrane microdomains, and following ligation, E-selectin colocalizes with a fraction of plasma membrane associated caveolin-1, a marker of caveolae. Biochemical fractionation of HUVEC lysates using sucrose density gradients demonstrates that E-selectin comigrates with caveolin-1 in the buoyant, “raft” fraction. Moreover, when E-selectin is ligated, it associates with phospholipase Cγ (PLCγ) and PLCγ becomes activated. Treatment of the HUVEC with the cholesterol-depleting drug methyl-β-cyclodextrin delocalizes E-selectin from the lipid raft, and prevents the ligation-induced association of E-selectin with PLCγ and subsequent activation of PLCγ. Thus, these data demonstrate that E-selectin exists in lipid rafts, and that this membrane localization is required for specific E-selectin-dependent signaling.

Materials and Methods

Reagents

Medium 199, RPMI 1640, Dulbecco’s PBS (DPBS), penicillin, streptomycin, and 1-glutamine were purchased from MA Bioproducts (Walkersville, MD). FBS was obtained from Life Technologies (Grand Island, NY). Endothelial cell growth factor was purchased from Biomedical Technologies (Stoughton, MA). Triton X-100 was purchased from Fisher Scientific (Pittsburgh, PA). Gelatin was purchased from Biomedia (Foster City, CA). Heparin, paraformaldehyde, leupeptin, aprotinin, PMSF, sodium fluoride, sodium orthovanadate, Tween 20, trichloroacetic acid, and methyl-β-cyclodextrin were obtained from Sigma-Aldrich (St. Louis, MO). Soluble recombiant human E-selectin was purchased from Calbiochem (San Diego, CA). The protein A-coated microspheres were from Bangs Laboratories (Fishers, IN). The polyvinylidene fluoride (PVDF) transfer membrane was obtained from Millipore (Bedford, MA). The anti-E-selectin Abs, H18/7, and H4/18 were developed in our laboratory (23). The anti-phosphotyrosine Ab clone 4G10 was from Upstate Biotechnology (Lake Placid, NY). The anti-caveolin-1 polyclonal Ab was purchased from BD Transduction Laboratories (San Diego, CA). The anti-transferrin receptor Ab was from Zymed Laboratories (San Francisco, CA). Protein A/G agarose, anti-PLCγ, anti-paxillin, and HRP-coupled secondary Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The ECL Western blotting detection kit was purchased from Amersham Life Sciences (Buckinghamshire, U.K.). Fluorescent anti-mouse and anti-goat Abs were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cultured cells

HUVEC were isolated from segments of normal term umbilical cords and established in culture as previously described (6). Primary cultures were serially passaged in Medium 199 buffered with 25 mM HEPES buffer, supplemented with endothelial cell growth factor (25 μg/ml), porcine intestinal heparin (50 μg/ml), and 20% FBS. Cells were plated on gelatin-coated 10-cm or 15-cm tissue culture dishes (Corning, Corning NY) for experimental use at subculture 2, with the exception of the immunofluorescence experiments where subculture 1 cells were used. HL.60, a human promyelocytic leukocyte cell line obtained from the American Type Culture Collection (Manassas, VA), was maintained in RPMI 1640 medium supplemented with 10% FBS, 100 μM penicillin, 100 μg/ml streptomycin, and 20 mM L-glutamine.

Immunofluorescence

HUVEC were plated on 0.1% gelatin-coated culture grade polystyrene plastic coverslips (Modern Plastics, Peabody, MA). Following treatment, cells were rinsed in DPBS at room temperature, fixed in 2% paraformaldehyde, and permeabilized with 0.1% Triton X-100 for 1 min. Cells were then incubated in 5% FBS for 2 h to block nonspecific binding, followed by incubation with primary Ab H18/7 (10 μg/ml), or rabbit anti-caveolin-1 (1/1000) in DPBS 1% FBS at 4°C overnight. Coverslips were washed, and incubated with 2% serum followed by secondary Ab (1/50 in DPBS with 0.1% FBS) for 1 h at 4°C. Finally, the coverslips were rinsed with DPBS and mounted in GelMount. The cells were visualized using a Nikon Eclipse TS 300 inverted microscope (Melville, NY) with a ×40 oil immersion objective. Images were acquired using ImagePro software. For confocal images, the cells were visualized using a Leica TCSNT confocal laser-scanning microscope (Deerfield, IL). Images were taken with a ×40 oil immersion lens, and serial sections were taken along the z-axis at 0.4-μm increments from the apical to the basal region of the cell.

Raft gradient separation

HUVEC were activated with IL-1β (10U/ml, 4 h, 37°C) and then lysed as previously described (15, 16). Briefly, cells were washed, and lysed on ice with 50 mM Na–CO3 (pH 11), 10 mM NaF, 1 mM orthovanadate, 10 μg/ml aprotinin, 1 mM PMSF, and 10 μg/ml leupeptin. Lysates were homogenized, then sonicated using an ultrasonic processor (Sonics and Materials, Newtown, CT). The lysates were adjusted to 42.5% sucrose by adding an equal volume of 85% sucrose in MES-buffered saline (MBS) (125 mM NaCl, with 25 mM MES, pH 6.5). The samples were placed in ultracentrifuge tubes then overlaid with a gradient of 6 ml of 30% sucrose (in MBS) followed by 3.5 ml 5% sucrose (in MBS). The gradients were centrifuged at 35,000 rpm for 18 h in an SW41 rotor (Beckman Coulter, Palo Alto, CA). Ten 1.1-ml fractions were harvested from the top of the gradients. For electrophoresis, fractions were precipitated in 10% trichloroacetic acid, then boiled in sample buffer.

Surface biotinylation

IL-1-activated HUVEC monolayers were surface biotinylated following manufacturer’s instructions using the ECL Protein Biotinylation Module (Amersham Life Sciences). The cells were incubated in 40 mM bicarbonate buffer (pH 8.6) containing biontinamidocaproate N-hydroxysuccinimide ester (manufacturer’s stock diluted 1/25) for 30 min at 4°C on a rocking platform. Following biotinylation, the monolayers were washed three times in DPBS, then lysed and processed as for raft isolation. Following gradient fractionation, samples from the buoyant (raft) fraction, from an intermediate fraction, and from the dense (pellet) fraction of the gradient were selected for immunoprecipitation. Fractions were diluted in 10-fold excess MBS, then centrifuged (35,000 rpm, 45 min) and finally resuspended in 200 μl of radioimmunoprecipitation analysis buffer with 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF. The samples were then centrifuged at 14,000 rpm for 10 min. The supernatant was transferred to a new tube and incubated with 2 μg/ml anti-E-selectin Ab H18/7 for 1 h at 4°C. Following addition of 30 μl of protein A/G agarose, the samples were processed for Western blot analysis.

Immunoprecipitation

After experimental treatment, cells were rinsed with ice-cold DPBS and lysed in lysis buffer (50 mM NaCl, 10 mM Tris, 0.5% Nonidet P-40, 50 mM NaF, 5 mM ZnCl2, 1 mM PMSF, 200 μM Na2VO3, 2 mM C,H4IO4Na, and NaPO3·H2O, pH 7.3) (10) for 10 min on ice. Lysates were scraped off the dishes and placed in microcentrifuge tubes. The lysates were centrifuged at 14,000 rpm for 10 min at 4°C. Lysates were incubated with Ab (1.5 μg/ml) overnight at 4°C. Thirty microliters of protein A/G-agarose were added to the lysate and incubated for an additional hour at 4°C. The immunocomplex was washed first with 600 mM NaCl followed by 150 mM NaCl. The immunocomplex was resuspended in 20 μl of DPBS.

Western blot analysis

The immunoprecipitates were resuspended in 20 μl of 3× sample buffer (187.5 mM Tris-HCl (pH 6.8), 6% SDS, 30% glycerol, 0.3% bromophenol blue) and the tubes were vortexed, then boiled for 5 min. The samples were
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Results

Ab-mediated cross-linking induces clustering and redistribution of cell surface E-selectin

To evaluate E-selectin distribution at the cell surface, we used immunofluorescence microscopy. HUVEC were activated with IL-1β. For cross-linking, the cells were incubated with anti-E-selectin mAb H4/18 followed by incubation with goat anti-mouse IgG. The cells were then fixed, permeabilized, and stained with a second mAb (H18/7) which recognizes a different epitope on the E-selectin molecule. E-selectin was then visualized by staining with a FITC-labeled goat anti-mouse Ab. As seen in Fig. 1A, E-selectin is distributed in a random punctate staining pattern with additional diffuse staining in the perinuclear area. Immunostaining of fixed, nonpermeabilized cells demonstrated that the punctate staining is at the cell surface (data not shown). Following Ab-mediated cross-linking, there was a marked change in distribution of E-selectin with an increase in the number and size of the punctate E-selectin staining foci (Fig. 1B; see also Fig. 3, left panels). Image-Pro software (Media Cybernetics, Silver Spring, MD) was used to quantify this increase in focal E-selectin staining (Fig. 1C). In the absence of cross-linking, there were 13.8 ± 7.9 E-selectin staining foci per cell (n = 7), and following cross-linking there were 60 ± 16.6 foci per cell (n = 7) (p < 0.01). The redistribution of E-selectin at the cell surface is reminiscent of the receptor clustering typically seen with molecules contained in lipid rafts (22).

E-selectin migrates in the buoyant fraction upon sucrose density gradient fractionation

To determine whether E-selectin exists in lipid-enriched membrane microdomains, we fractionated lysates from IL-1-activated HUVEC using sucrose density gradients. To preserve associations of molecules in lipid rafts, we used a well-established detergent-free system (15, 16). Gradient fractions were harvested, then separated by SDS-PAGE. Following electrophoresis and transfer to PVDF membranes, the samples were immunoblotted to detect E-selectin, caveolin-1, transferrin receptor, or paxillin. As seen in Fig. 2A, most of the E-selectin was detected in the buoyant raft fractions. Caveolin-1, known to exist in cholesterol-enriched membrane microdomains in endothelial cells, was enriched in the same low density raft fractions. Caveolin-1 is lost from the cholesterol-enriched regions in passaged HUVEC (G. Garcia-Cardena and W. C. Sessa, unpublished observations), thus explaining the presence of caveolin-1 in the more dense fractions of the gradient. Gradient separation of the transferrin receptor demonstrates that a transmembrane molecule is not enriched in the raft fractions, but in the dense pellet fractions. The cytoskeletal molecule paxillin, a nonmembrane molecule, also is not enriched in raft fractions using this detergent-free lysis system. The transferrin receptor and paxillin have been previously reported to not fractionate to lipid raft fractions (15, 24). To determine whether the E-selectin present in the buoyant fractions of the gradients actually represents the E-selectin at the cell surface or at intracellular compartments, IL-1-activated HUVEC were surface biotinylated, then lysed and separated on sucrose density gradients. Surface biotinylation of HUVEC before lysis allowed us to label only the surface-expressed E-selectin. Following gradient fractionation, samples representing the buoyant fraction (raft fractions 2–4), an intermediate fraction (fractions 5–7), or the dense fraction (pellet, fractions 8–10) were selected and subjected to immunoprecipitation (see Materials and Methods) with an anti-E-selectin Ab. The immunoprecipitates were resolved by SDS-PAGE, transferred to nylon membrane, and immunoblotted with streptavidin-conjugated with HRP. There was strong E-selectin staining in the raft or buoyant fraction, no detectable staining in the intermediate fraction, and faint staining in the pellet fraction (Fig. 2B). Thus, cell surface E-selectin resides in lipid rafts.

E-selectin colocalizes with caveolin-1, a marker of caveolae

To determine whether E-selectin localizes to a particular class of lipid raft, we costained IL-1-activated HUVEC for E-selectin and for the caveolae marker caveolin-1, and determined their colocalization using confocal immunofluorescence microscopy. Fig. 3A demonstrates a single x-y section taken at the apical aspect of the cell. E-selectin stained in green (left panel), Fig. 3B shows caveolin-1 stained in red. In the right panel, the merged images demonstrate colocalization of E-selectin and caveolin-1 (yellow). Cells in Fig. 3A were treated with only mAb H4/18 before fixation and staining and demonstrate normal E-selectin distribution with only limited colocalization of E-selectin and caveolin-1 in the absence of (-) or following (+) Ab-mediated cross-linking. These data are representative of at least three separate experiments.

FIGURE 1. E-selectin redistributes and clusters following Ab-mediated cross-linking. Immunofluorescence microscopy of IL-1β-activated HUVEC shows E-selectin expression in fixed and permeabilized monolayers. A. Unligated distribution, a random punctate pattern as well as more diffuse perinuclear staining. B. E-selectin was subjected to Ab-mediated cross-linking before fixation and staining. E-selectin appears clustered into larger foci and there has been a marked redistribution with more E-selectin present at the periphery of the cell. C. Quantification of E-selectin containing foci per cell in the absence of (-) or following (+) Ab-mediated cross-linking. These data are representative of at least three separate experiments.
of E-selectin cross-linking. Cells in Fig. 3B were treated with mAb H4/18 followed by goat anti-mouse IgG to cross-link E-selectin before fixation and staining (see Materials and Methods). Following cross-linking, E-selectin is redistributed, resulting in a 3-fold increase in colocalization with caveolin-1 at the cell surface. Image-Pro software calculated a colocalization coefficient of 0.04 (percent colocalized pixels relative to total E-selectin pixels) without cross-linking vs a colocalization coefficient of 0.12 after cross-linking. Moreover, in Fig. 3C, HUVEC were incubated for 30 min with 5-μm protein A-coated microspheres (beads) to which the anti-E-selectin Ab H18/7 was attached following manufacturer’s instructions. These beads do not bind to unactivated HUVEC (data not shown). Confocal microscopy at the point of contact of the bead with the HUVEC shows increased E-selectin staining with some punctate foci, and an increase of caveolin-1 (included are x-z images of these sections.) The merged panel demonstrates colocalization at these sites; additionally, a y-z image of the merged figure was included.

Leukocyte adhesion to IL-1-activated HUVEC induces E-selectin association with PLCγ

E-selectin ligation leads to an increase in endothelial cell intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) (25, 26) and, in other cell types, growth factor receptor ligation is reported to recruit PLCγ to the plasma membrane (27, 28), specifically to lipid rafts (29). We speculated that the E-selectin-dependent signaling events required for the increase in [Ca$^{2+}$] occur in lipid raft microdomains. Therefore, we looked for an association of E-selectin with PLCγ following leukocyte adhesion. Fixed HL60 cells (9) were allowed to settle onto a monolayer of IL-1-activated HUVEC that had been serum-starved and then warmed to 37°C for 1, 5, 8, or 15 min. Monolayers were then rinsed in ice-cold DPBS, lysed, and the lysates analyzed for association of E-selectin with PLCγ by immunoprecipitation and immunoblotting. As seen in Fig. 4A, following HL60 adhesion, E-selectin becomes associated with PLCγ in a time-dependent manner. At 1 min (lane 1), there is a low level of association, which increases at 5 min (lane 2), reaches a maximum by 8 min (lane 3), and declines by 15 min (lane 4). Incubating the HUVEC alone at 37°C for 8 min had little effect on the association (lane 5). Total PLCγ remains the same. An aliquot of the total cell lysate of the fixed HL60 gave no detectable band at the m.w. of E-selectin (data not shown). Because HL60 cells adhere to activated HUVEC via ICAM-1 as well as E-selectin, we preincubated HL60 with soluble E-selectin (5 h, 4°C, in DPBS) before fixation to test whether the observed association with PLCγ results from E-selectin ligation. The recombinant soluble E-selectin species contains the 535 amino acids comprising the extracellular portion of the molecule. In other studies, pretreatment of HL60 with soluble E-selectin has been shown to block HL60 adhesion to immobilized soluble E-selectin and to reduce adhesion of treated HL60 to TNF-treated HUVEC monolayers (30, 31). As seen in Fig. 4B, pretreatment of HL60 cells with soluble E-selectin reduced the E-selectin/PLCγ association (lanes 4 and 5 vs lane 2).

Furthermore, in three separate experiments, we observed 49, 50, and 53% inhibition of adhesion-dependent PLCγ association using this approach as assessed by NIH Image densitometric analysis of scanned blots. These results demonstrate that the PLCγ activation following HL60 adhesion is E-selectin dependent.

PLCγ becomes tyrosine phosphorylated following ligation of E-selectin by leukocyte adhesion

To efficiently hydrolyze phosphoinositol 4,5-bisphosphate (PIP2), PLCγ must be activated by tyrosine phosphorylation (27, 29, 32). We assessed tyrosine phosphorylation of PLCγ following HL60 adhesion to serum-starved IL-1β activated (4 h, 37°C) HUVEC. Following HL60 adhesion (see above), PLCγ was immunoprecipitated from cell lysates and the tyrosine phosphorylation state was determined by immunoblotting (Fig. 5A). PLCγ is normally tyrosine phosphorylated at a low level in HUVEC (lane 1). IL-1 treatment per se did not change this basal phosphorylation (lane 2). However, following HL60 adhesion (Fig. 5A, lanes 3 and 4),
tyrosine phosphorylation is markedly increased. To demonstrate that this activation of PLC\(_{H9253}\) depends on the ligation of E-selectin, we pretreated HL60 cells with soluble E-selectin before fixation. The immunoblot in Fig. 5B shows that blocking HL60-mediated E-selectin ligation results in a decrease in tyrosine phosphorylation of PLC\(_{H9253}\) (lanes 4, 5, and 6 vs lane 3) supporting a role for E-selectin in this activation.

Methyl-\(H9252\)-cyclodextrin treatment disrupts E-selectin raft localization and E-selectin association with PLC\(_{H9253}\) as well as subsequent PLC\(_{H9253}\) tyrosine phosphorylation

To determine whether E-selectin-mediated PLC\(_{\gamma}\) signaling requires E-selectin localization in cholesterol-enriched membrane microdomains, we treated serum-starved HUVEC with the cholesterol binding drug methyl-\(\beta\)-cyclodextrin. This treatment depletes cellular cholesterol and causes the loss of compartmentalization of raft-associated molecules (22, 33, 34). Serum-starved HUVEC were activated with IL-1 and during the final hour of IL-1 incubation, methyl-\(\beta\)-cyclodextrin (5 mM, final concentration) was added to the cultures. The cells were lysed and fractionated on sucrose density gradients. As seen in Fig. 6A, treatment with methyl-\(\beta\)-cyclodextrin disrupts E-selectin location in the buoyant fraction of sucrose gradients, in comparison to the normally restricted distribution (Fig. 2A). To investigate whether depletion of cholesterol has an effect on the HL60 adhesion-induced association of E-selectin with PLC\(_{\gamma}\), serum starved, IL-1-activated HUVEC were treated with 5 mM methyl-\(\beta\)-cyclodextrin for 1 h. Following the adhesion of fixed HL60 cells, HUVEC were warmed to 37°C for 7 min, then lysed. The lysates were immunoprecipitated with an anti-PLC\(_{\gamma}\) Ab. The immunoprecipitates were separated by SDS-PAGE, transferred to a nylon membrane, and immunoblotted for E-selectin. Cholesterol depletion resulted in a decrease in the amount of E-selectin associated with PLC\(_{\gamma}\) (Fig. 6B, lane 2 vs lane 4). Treatment of HUVEC with methyl-\(\beta\)-cyclodextrin also inhibits the E-selectin ligation-induced tyrosine phosphorylation of PLC\(_{\gamma}\) (Fig. 6C). In experiments similar to those in Fig. 6B, the immunoblots were incubated with an anti-phosphotyrosine Ab (4G10, 1 \(\mu\)g/ml). Although methyl-\(\beta\)-cyclodextrin treatment caused an increase in PLC\(_{\gamma}\) tyrosine phosphorylation (lane 1 vs lane 3), the HL60 adhesion-induced tyrosine phosphorylation of PLC\(_{\gamma}\) was blocked (lane 2 vs lane 4). Interestingly, cholesterol depletion with methyl-\(\beta\)-cyclodextrin had no effect on E-selectin-mediated MAPK activation (method as described in Ref. 9) (Fig. 6D). These data demonstrate that E-selectin localization in cholesterol-enriched membrane microdomains is essential for its association with PLC\(_{\gamma}\), and for the subsequent tyrosine phosphorylation of PLC\(_{\gamma}\).

**FIGURE 3.** Redistribution of E-selectin leads to increased colocalization with caveolin-1. Confocal images taken at the apical surface of IL-1β HUVEC show E-selectin staining (green) and caveolin-1 staining (red) with limited colocalization (yellow) in the absence of E-selectin cross-linking (A). Following Ab-mediated cross-linking of E-selectin, redistribution of E-selectin leads to a 3-fold increase in E-selectin and caveolin-1 colocalization (B). Ligation of E-selectin by a bead coated with an anti-E-selectin Ab shows a focus of colocalization of E-selectin and caveolin-1 at the site of bead contact with the endothelial cell (C). These data are representative of three separate experiments.
Discussion

Leukocyte tethering via E-selectin is one of the earliest steps in the recruitment of blood-borne leukocytes to sites of inflammation. This tethering event initiates an orchestrated response by the endothelial cell that includes both outside-in and inside-out signaling. Some of the consequences of E-selectin ligation at the cell surface have been identified, for example, an increase in \([Ca^{2+}]_i\) (25, 26). Following ligation, E-selectin becomes associated with actin cytoskeletal elements (6), and we have demonstrated ligation-induced signaling including activation of MAPK that is dependent on the phosphorylation of a specific tyrosine residue in the cytoplasmic domain of E-selectin (9). In this study, we demonstrate that following Ab-mediated cross-linking, cell surface E-selectin dramatically redistributes in the membrane, that this redistribution leads to E-selectin colocalization with some caveolin-1, and the association of PLCγ with this E-selectin-containing complex. Furthermore, following HL60 adhesion to the surface of IL-1-activated HUVEC monolayers, PLCγ becomes associated with E-selectin and is itself tyrosine phosphorylated. To determine that ICAM-1 ligation was not responsible for these responses, we pre-treated the HL60 with soluble E-selectin as a selective adhesion-blocking reagent. Both E-selectin/PLCγ association and PLCγ tyrosine phosphorylation were inhibited by this pretreatment, thus demonstrating a specific requirement for E-selectin ligation in these responses.

Interestingly, PLCγ association with E-selectin and subsequent PLCγ activation require intact lipid rafts. Translocation of PLCγ to the plasma membrane occurs following ligation of receptors for growth factors such as PDGF and EGF (28, 35). Additionally, TCR ligation targets PLCγ specifically to lipid rafts (24). Falasca et al. (29) have proposed a mechanism by which this translocation can occur. They have shown that PLCγ targets to the plasma membrane by binding via its pleckstrin homology domain to the phosphoinositide PI3,4,5-P3, and that phosphatidylinositol-3 kinase may have a role in targeting PLCγ to the membrane and in the subsequent tyrosine phosphorylation of PLCγ. In endothelial cells, PLCγ-mediated signaling consequences include increased monolayer permeability (36) and binding of PLCγ to the cytoskeletal component vinculin (37). Ligation of ICAM-1 in microvascular endothelial cells leads to the phosphorylation of PLCγ (38). Thus, our data suggest that E-selectin may be involved in some other functions, similar to those seen in the receptors for PDGF and EGF, that include cell survival and proliferation.

The demonstration of ligation-induced association of PLCγ with E-selectin-containing lipid rafts provides a potential mechanism both for the reported E-selectin ligation-induced \([Ca^{2+}]_i\), and E-selectin-dependent cytoskeletal changes (Fig. 7C). Phosphoinositides, including PIP2, are enriched in lipid rafts (39), and Pike et al. (40, 41) have demonstrated that PIP2 hydrolysis preferentially occurs in intact lipid rafts. In addition to its role as a substrate for PLCγ in the mobilization of \([Ca^{2+}]_i\), and activation of protein kinase C, PIP2 is involved in cytoskeletal modulation (32, 39). PIP2 can bind to α-actinin, and vinculin, cytoskeletal molecules known to associate with the cytoplasmic domain of E-selectin following...
the phosphorylation of the src kinase substrate cortactin, another cytoskeletal binding protein, and consequently to cytoskeletal reorganization (46). Cortactin is reported to associate with the cytoplasmic domain of E-selectin, and inhibition of cortactin phosphorylation blocks E-selectin clustering at the site of leukocyte attachment (47). Taken together, these data suggest that upon ligation, various adhesion molecules including E-selectin link to the cytoskeleton via cytoskeletal linkers such as ERM molecules and/or cortactin, and this association is required for the subsequent cytoskeletal changes associated with leukocyte binding.

Our data demonstrate that E-selectin is localized to lipid rafts. Interestingly, Tilghman and Hoover (48) recently reported that upon ligation, E-selectin is incorporated into detergent insoluble membrane domains. In the experiments reported here, we have used a combination of biochemical approaches and cholesterol depletion to demonstrate E-selectin raft localization in the absence of ligation. We have previously demonstrated that ligation induces E-selectin association with the detergent insoluble cytoskeleton, specifically with the actin binding proteins α-actinin, vinculin, filamin, and with focal adhesion kinase and its substrate paxillin (6). Additionally, as a consequence of ligation, E-selectin associates with signaling intermediates leading to MAPK activation. These associations may influence the detergent (Triton X-100) solubility change observed by Tilghman and Hoover (48).

The localization of E-selectin in lipid rafts provides a mechanism for temporal and spatial integration of the endothelial cell response to transiting leukocytes. Molecules in lipid rafts diffuse more slowly in the plasma membrane, supporting more sustained interactions between signaling partners. Many transmembrane molecules that support signaling functions associate with their molecular signaling partners in multimolecular complexes in lipid rafts. T cell receptors (24, 34, 49), B cell receptors (50), some integrins (33), and other molecules (51, 52) have been reported to localize cholesterol-rich microdomains. We have observed that ligation-induced redistribution of E-selectin leads to an increased colocalization with caveolin-1. At this time, we do not know the relative distribution of E-selectin in lipid rafts vs caveolae. However, the localization and redistribution of E-selectin suggest potential distinct signaling functions of E-selectin in endothelial cells. In caveolae, several different types of signaling molecules, including src kinases, heterotrimeric G proteins, and endothelial NO synthase, bind directly to caveolin-1 and are regulated by this association (12, 19, 53). However, we note that not all E-selectin-dependent signaling requires the raft environment. E-selectin-dependent activation of MAPK occurs in cholesterol-depleted cells, when E-selectin is delocalized from the raft.

Thus, a temporal and spatial model is emerging of the consequences of E-selectin ligation at the cell surface. As seen in Fig. 7, E-selectin expression is an early event in the activation of endothelial cells at sites of inflammation. Its presence on the surface of the activated endothelial lining serves as a “sign-post” for blood-borne leukocytes. Leukocyte tethering via E-selectin leads to outside-in signaling events (e.g., cytoskeletal association, MAPK activation, PLCγ translocation). Some of these events subsequently lead to changes in gene expression. The ability of E-selectin, as a transmembrane receptor, to physically tether interacting leukocytes and simultaneously mediate outside-in signaling events in the endothelial cell may be important for the efficient orchestration of leukocyte-endothelial transmigration, and for the regulation of the endothelial cell phenotype (e.g., cell survival, resistance to oxidative stress) at a developing inflammatory site.
FIGURE 7. Proposed model for the downstream signaling consequences of E-selectin ligation in inflammation. A. The plasma membrane of unactivated endothelial cells contains lipid rafts and caveolae. B. Following inflammatory activation, E-selectin is expressed and is localized in cholesterol-enriched membrane microdomains that do not contain caveolin-1. E-selectin may associate with PIP2 via a consensus binding sequence in the E-selectin cytoplasmic domain. C. Upon leukocyte adhesion via E-selectin, E-selectin-containing lipid rafts cluster into larger membrane microdomains that may contain hydrolyzable PIP2, (40) and also may contain caveolin-1. PLCγ associates with E-selectin in these larger rafts and can hydrolyze PIP2 resulting in an increase in [Ca2+]i. E-selectin associates with elements of the actin cytoskeleton leading to cytoskeletal reorganization, potentially including junctional remodeling. Concomitantly, phosphorylation changes occur in the cytoplasmic domain of E-selectin leading to MAPK activation and subsequent changes in gene transcription.

Acknowledgments

We gratefully acknowledge the expert assistance of Kay Case and Deanna Lamont in cell culture. We thank Dr. Brett Blackman for helpful discussions and assistance with microscopy, and Dr. GuoHao Dai and Jason Comander for valuable help with image analysis.

References