Phosphorylation of the Cytoplasmic Domain of E-Selectin Is Regulated During Leukocyte-Endothelial Adhesion[1]

Masayuki Yoshida,[2,3]* Brian E. Szente,[2]* Jeanne-Marie Kiely,* Anthony Rosenzweig,† and Michael A. Gimbrone, Jr.*[4]

E-selectin, a selectin expressed on activated vascular endothelium, supports rolling and stable adhesion of leukocytes at sites of inflammation. Previously, we have reported that leukocyte adhesion to cultured endothelial cells induces association of the cytoplasmic domain of E-selectin with cytoskeletal elements, suggesting that outside-in signaling may occur during E-selectin-mediated adhesion. To investigate this potential signaling function of E-selectin, HUVEC activated with recombinant human IL-1β (10 U/ml, 4 h) were labeled with [^{32}P]orthophosphate, and E-selectin was immunoprecipitated using mAb H18/7. Autoradiography revealed constitutive phosphorylation of E-selectin in these cells and time-dependent dephosphorylation following adhesion of HL-60 cells. Cross-linking of cell surface E-selectin using H18/7 and a polyclonal secondary Ab induced E-selectin dephosphorylation, as did adhesion of beads coated with recombinant P-selectin glycoprotein ligand-1 (PSGL-1), an E-selectin ligand. Using adenoviral vector-mediated transfection in HUVEC of a tail-less E-selectin and phosphoamino acid analysis, we documented phosphorylation occurring exclusively within the cytoplasmic domain and involving serine residues. Additional experiments using a series of cytoplasmic domain mutants of E-selectin expressed in COS-7 cells localized the regions that were constitutively phosphorylated. Preincubation with okadaic acid and sodium vanadate abrogated adhesion-induced dephosphorylation of E-selectin. Thus, E-selectin, which is constitutively phosphorylated in cytokine-activated human endothelial cells, undergoes an enzymatically regulated dephosphorylation following leukocyte adhesion. This process appears to be triggered by multivalent ligand binding and/or cross-linking of cell surface E-selectin. Ligand-dependent regulation of the phosphorylation of E-selectin's cytoplasmic domain provides additional evidence for a transmembrane signaling function of this molecule during leukocyte-endothelial interactions. ***The Journal of Immunology,*** **1998, 161: 933–941.**

Leukocyte-endothelial interactions contribute to a variety of vascular disease processes, such as acute and chronic inflammation, ischemia-reperfusion injury, and atherosclerosis (1). A number of soluble factors (e.g., chemokines and arachidonate products) and cell surface adhesion molecules, expressed by both the endothelial cell and the leukocyte, interact in a complex fashion to efficiently mediate leukocyte recruitment (2–4). E-selectin, one of three members of the selectin family of adhesion molecules, has been shown to support the rolling of leukocytes on activated endothelial cells (1) and may also participate in the transition to stable adhesion that precedes transmigration (5). This family shares a unique mosaic structure consisting of an amino-terminal lectin-like domain, followed by an epidermal growth factor-like domain, a variable number of complement regulatory repeats, a transmembrane domain, and a short cytoplasmic domain (1). Much attention has been directed to the function of the extracellular portions of these molecules, in particular the lectin-like domains that bind complex carbohydrate ligands on the surface of leukocytes and other cell types during their adhesive interactions (6, 7). In contrast, considerably less is known regarding the function(s) of the cytoplasmic domains of the selectins. These domains share little in the way of amino acid sequence homology among E-, L-, and P-selectin (8–10), but are generally well conserved across species for a given selectin family member. Such an evolutionary conservation of sequence is often indicative of the importance of a particular protein domain in processes such as protein-protein interactions, regulation of protein turnover, and signal transduction, that have broader functional significance in a particular cell or tissue.

The cytoplasmic domains of a number of other adhesion molecules have been either implicated in or directly shown to activate intracellular signal transduction. Perhaps the best characterized of all cell adhesion molecules in this regard have been the integrins. The various members of the integrin superfamily have been demonstrated to activate tyrosine and serine/threonine kinase cascades, to induce cytoskeletal reorganization via interaction with cytoskeletal proteins, and to modulate their own affinities and adhesive functions by interaction with tail binding proteins such as cytohesin-1 and β_3-endonexin (11, 12). Members of the cadherin family also appear to support a signaling function. The cadherins are localized to adherens junctions at regions of cell-cell contact, and their cytoplasmic tails associate with a number of putative signaling proteins, including β-catenin, plakoglobin, and p120 (13). A third example of cell adhesion molecules thought to be important signal transducers is the Ig superfamily, which includes ICAM-1

*Vascular Research Division, Department of Pathology, Brigham and Women's Hospital, †Cardiovascular Research Center, Massachusetts General Hospital-East, Harvard Medical School, Boston, MA

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[2] These two authors contributed equally to this work.

[3] Current address: Department of Molecular Genetics, Medical Research Institute, Tokyo Medical and Dental University, 1–5–45 Yushima, Bunkyo-ku, Tokyo, Japan.

[4] Address correspondence and reprint requests to Dr. Michael A. Gimbrone, Vascular Research Division, Department of Pathology, Brigham and Women's Hospital, 221 Longwood Ave., LMRC-401, Boston, MA 02115. E-mail address: gimbrone@bustoff.bwh.harvard.edu

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and platelet/endothelial cell adhesion molecule-1 (PECAM-1).[5] In brain microvessel endothelial cells, ICAM-1 stimulates Src activity and thereby induces the tyrosine phosphorylation of a number of Src substrates, including the actin binding protein cortactin (14). The cytoplasmic domain of PECAM-1 serves as a substrate for Src and, when tyrosine-phosphorylated, can bind the protein tyrosine phosphatase SH2-containing phosphatase-2 (SHP-2) (15, 16). Recent data suggest that the selectins are also active participants in the process of signal transduction (6). To date, the best characterized family member in this respect has been L-selectin. The cytoplasmic domain of L-selectin has been shown to be tyrosine phosphorylated (17), and ligation of L-selectin can lead to mitogen-activated protein (MAP) kinase activation (17), association of Grb2 and Sos (17), and actin polymerization dependent upon Ras and Rac2 (18, 19) within leukocytes.

We have recently shown that leukocyte adhesion to vascular endothelium can induce a biochemical and biophysical association of the cytoplasmic domain of E-selectin with components of the endothelial actin cytoskeleton, such as α-actinin, vinculin, and filamin, as well as certain regulatory elements, such as focal adhesion kinase (FAK) and its substrate paxillin (20), thus suggesting a previously unrecognized signaling role for E-selectin. In the current study we have investigated the signaling function of E-selectin during leukocyte adhesion. We report here that E-selectin in activated HUVEC in culture is constitutively phosphorylated on serine residues within its cytoplasmic domain, and that leukocyte adhesion can induce an enzymatically regulated dephosphorylation of E-selectin. Interestingly, a similar pattern of dephosphorylation of E-selectin is observed when cell surface E-selectin is cross-linked using a mAb directed to the extracellular ligand binding domain followed by a polyclonal IgG. Moreover, the binding of beads coated with recombinant PSGL-1, a glycoprotein ligand for E-selectin, to the endothelial cell surface can induce a similar pattern of dephosphorylation of E-selectin. Thus, the interaction of leukocytes, cross-linking Abs, or complex carbohydrate ligands with the extracellular domain(s) of E-selectin can regulate the phosphorylation state of the cytoplasmic domain of this molecule. These data suggest a novel transmembrane outside-in signaling role for E-selectin that may have implications for the orchestration of endothelial cell responses in the leukocyte-endothelial adhesion cascade.

Materials and Methods

Reagents

Medium 199 and Dulbecco's PBS were obtained from M. A. Bioproducts (Walkersville, MD). FBS and DMEM were purchased from Life Technologies (Grand Island, NY). Fifteen- and thirty-five-millimeter diameter culture plates and 96-well microtiter culture plates were purchased from Costar (Cambridge, MA). Endothelial cell growth factor was obtained from Biomedical Technologies (Stoughton, MA). Recombinant human IL-1β was a gift from Biogen (Cambridge, MA). mAbs H18/7 and W6/32 are both of isotype IgG2a and were prepared as described previously (21, 22). The recombinant PSGL-1-human IgG Fc chimera was provided by Dr. Dale Cummings (Genetics Institute, Cambridge, MA). Biscarboxyethylcarboxyfluorescein acetoxymethyl ester was purchased from Molecular Probes (Eugene, OR). [^{32}P]orthophosphate was obtained from New England Nuclear (Boston, MA). Okadaic acid was purchased from Calbiochem (La Jolla, CA). Fluorescein-conjugated goat anti-mouse IgG was purchased from Caltag (Burlingame, CA).

[5] Abbreviations used in this paper: PECAM-1, platelet/endothelial cell adhesion molecule-1; MAP, mitogen-activated protein; FAK, focal adhesion kinase; PSGL-1, P-selectin glycoprotein ligand-1; DPBS, Dulbecco's phosphate-buffered saline; RSV, Rous sarcoma virus; wt, wild type; PP1, protein phosphatase-1.

Cell culture

The human kidney fibroblast cell line 293 was obtained from the American Type Culture Collection (Rockville, MD) and grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. HL-60, a promyelocytic human leukocyte cell line, was obtained from American Type Culture Collection and grown in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. HUVEC were isolated and established in culture as previously described (23). Primary cultures were serially passaged (<1:3 split ratio) and maintained in medium 199 buffered with 25 mmol/l HEPES and supplemented with 20% FBS, endothelial cell growth factor (25 μg/ml), 100 U/ml penicillin, 100 μg/ml streptomycin, and porcine intestinal heparin (50 μg/ml). For experimental purposes, HUVEC subcultured to passage 2 or 3 were used.

[^{32}P]Orthophosphate labeling of HUVEC

Confluent monolayers (consisting of approximately $3.0-6.0 \times 10^6$ cells) of cytokine activated or adenoviral vector-transduced HUVEC in 35-mm culture plates were preincubated in phosphate-free RPMI 1640 with 1% dialyzed FBS for 1 to 2 h, and then metabolically labeled in the same medium supplemented with 333 μCi/ml of [^{32}P]orthophosphate for 90 min. The labeled monolayers were washed three times with regular RPMI 1640 with 1% FBS and incubated with various reagents, as described below. Cells were then rapidly lysed in 0.25 ml ice-cold lysis buffer (0.1 M Tris-HCl, 0.15 M NaCl, and 5 mM EDTA, pH 7.4) containing 1% Triton X-100, 10 μg/ml leupeptin, 60 U/ml aprotinin, and 1 mM PMSF. In some experiments, HL-60 cells (2×10^6/35-mm dish) or H18/7 mAb to E-selectin (10 μg/35-mm dish) were added to the labeled monolayers before lysis. In other experiments, the effects of phosphatase inhibitors were investigated by preincubation of the labeled monolayers with sodium vanadate (100 μM) and/or okadaic acid (100 nM) for 10 min before addition of HL-60.

Recombinant PSGL-1 beads

Recombinant PSGL-1 beads were prepared as follows. Recombinant PSGL-1-human IgG Fc chimera was provided by Dr. Dale Cummings (Genetics Institute). Tosyl-activated Dynabeads M-450 (5×10^6; diameter, 4.5 μm) were coated overnight with 100 μg of protein A (Zymed, South San Francisco, CA) in 0.1 M carbonate buffer, pH 9.4. After washing twice with DPBS, 0.25 μg of recombinant PSGL-1-human IgG Fc chimera was added to the beads and incubated for 1 h. The beads were washed twice with DPBS containing 1% BSA (DPBS/1% BSA) and then blocked with 200 μg/ml of human IgG1 κ in DPBS/1% BSA. After washing once with DPBS/1% BSA, the beads were kept in this buffer. For binding to IL-1-activated HUVEC monolayers, 10^6 beads were used/35-mm dish.

Immunoprecipitation and cell surface biotinylation

Total cell lysates of labeled HUVEC monolayers were centrifuged at $12,000 \times g$ for 20 min, and the supernatants were precleared with 50 μl of cyanogen bromide-activated Sepharose beads for 1 h at 4°C. Mixtures were centrifuged at $12,000 \times g$ for 5 min, and the supernatants were incubated with 2 or 3 μg of mAb H18/7, directed against human E-selectin for 1 h at 4°C. Fifty micrograms of goat anti-mouse IgG-coupled Sepharose beads, preincubated with unlabeled HUVEC lysates, were then added to the H18/7-lysate mixture and incubated for 1 h at 4°C. The beads were collected by centrifugation at $12,000 \times g$ for 5 min and washed twice each with three different washing buffers (600 mM NaCl, 0.1 M Tris-HCl, and 5 mM EDTA, pH 7.4 containing 1% Triton X-100; 300 mM NaCl, 0.1 M Tris-HCl, and 5 mM EDTA, pH 7.4, containing 1% Triton X-100; 150 mM NaCl, 0.1 M Tris-HCl, and 5 mM EDTA, pH 7.4). Proteins were eluted from beads by boiling for 5 min in 30 μl of 0.5 M Tris-HCl (pH 6.8) containing 0.4% SDS and subjected to SDS-PAGE followed by autoradiography.

The amount of cell surface E-selectin present during the various experimental manipulations was monitored by biotinylation of HUVEC surface proteins, followed by immunoprecipitation of E-selectin from whole cell lysates as described above. Briefly, confluent HUVEC monolayers were washed three times with ice-cold DPBS before being incubated for 30 min with a solution of 0.5 mg/ml of sulfo-*N*-hydroxysulfosuccinimidyl biotin (Pierce, Rockford, IL) on ice. This reaction was quenched by washing the cells twice with cold DPBS containing 1 mg/ml lysine, followed by a single wash with cold DPBS. The cells were lysed, and E-selectin was immunoprecipitated and subjected to SDS-PAGE. Following SDS-PAGE, proteins were transferred to polyvinylidene difluoride membranes. Membranes were incubated for 1 h in blocking buffer (DPBS/3% BSA/0.1% Tween-20) and then incubated with a streptavidin-horseradish-peroxidase conjugate (Amersham, Arlington Heights, IL) diluted 1/1500 in blocking buffer for 1 h.

The membranes were washed twice in 1× blocking buffer, twice in 0.1× blocking buffer, and twice in DPBS with 0.1% Tween-20 (5 min/wash). Peroxidase was detected using enhanced chemiluminescence (Amersham) according to the manufacturer's protocols.

Phosphoamino acid analysis

HUVEC monolayers in 100-mm dishes were infected with the adenoviral E-selectin expression construct, AdRSV(wt-E). Forty-eight hours after infection, the cells were washed free of virus and preincubated for 1 h in phosphate-free RPMI 1640 containing 1% dialyzed FBS. The HUVEC were then incubated for 4 h (to maximize incorporation) with [^{32}P]orthophosphate at a concentration of 200 μCi/ml in 5 ml of phosphate-free RPMI 1640 containing 1% dialyzed FBS. After incorporation of the radiolabeled phosphate, the cells were washed three times with RPMI 1640 supplemented with 1% FBS and subjected to HL-60 adhesion (1.2×10^7 HL-60/100-mm dish) for 10 min. The cells were then quickly washed with RPMI and lysed in 500 μl of ice-cold lysis buffer (0.1 M Tris-HCl, 0.15 M NaCl, and 5 mM EDTA, pH 7.4) containing 1% Triton X-100, 10 μg/ml leupeptin, 60 U/ml aprotinin, and 1 mM PMSF. The lysates were clarified and subjected to immunoprecipitation using the anti-E-selectin mAb H18/7. The resulting immunoprecipitates were resolved by SDS-PAGE and transferred to nitrocellulose. After a brief exposure to x-ray film, the bands corresponding to E-selectin were identified and excised from the nitrocellulose membrane. Next, the membrane slices were incubated in 200 μl of 5.7 M HCl for 1 h at 100°C to effect partial acid hydrolysis of the radiolabeled E-selectin. The hydrolysis products were then lyophilized, resuspended in 500 μl of dH_2O, and lyophilized for a second time. The lyophilized material was resuspended in 5 μl of a solution containing 15 parts pH 1.9 buffer (2.2% formic acid and 1.4 M acetic acid, in dH_2O) and 1 part nonradioactive phosphoamino acid standards (1.0 mg/ml each of phosphoserine, phosphothreonine, and phosphotyrosine). Finally, the samples were resolved by two-dimensional, high voltage, thin layer electrophoresis at pH 1.9 in the first dimension and at pH 3.5 in the second dimension using a Hunter thin layer electrophoresis system (HTLE-7000, CBS Scientific, Del Mar, CA) (24). The positions of the nonradioactive phosphoamino acid standards were identified by spraying the thin layer electrophoresis plate with ninhydrin followed by baking at 80°C for 10 min. The relative positions of the radiolabeled phosphoamino acids were then localized by autoradiography.

Construction of wild-type and mutant E-selectin recombinant adenoviral vectors

Three replication-defective recombinant type 5 adenoviruses were used in these studies: AdRSV(wt-E), AdRSV(ΔCyto-E), and AdRSVβgal. AdRSVβgal was constructed as described previously (29). Both AdRSV(wt-E) and AdRSV(ΔCyto-E) use the pJM17 backbone, contain E1/E3 deletions, and were generated as follows. The cDNAs encoding either a full-length human E-selectin (wt-E-selectin) or a cytoplasmic deletion mutant of E-selectin (ΔCyto-E-selectin) (20) were ligated into pAdRSV4 (provided by Dr. Beverly Davidson, University of Iowa, Ames, IA) (25) between the RSV long terminal repeat and the SV40 polyadenylation signal. The resulting plasmids were cotransfected with the plasmid pJM17 in 293 cells (26). Plaques were isolated and characterized for protein expression by a fluorescent immunobinding assay as described previously (27), using a saturating concentration of H18/7 (anti-E-selectin mAb) and FITC-conjugated F(ab′)$_2$ goat anti-murine IgG (Caltag, San Francisco, CA). Viruses were propagated in 293 cells, purified by double cesium gradient centrifugation, and titrated by plaque assay in 293 cells as previously described (28). Stock titers ranged from 10^{10} to 10^{12} plaque-forming units/ml, with a particle to plaque-forming unit ratio of approximately 10. HUVEC were infected as described previously (29).

Construction of serial cytoplasmic deletion mutants of E-selectin

To generate serial deletion mutants of E-selectin cDNA, a PCR-directed mutagenesis approach was used. A cytoplasmic deletion mutant (ΔCyto) E-selectin construct was constructed as previously described (20). Three new mutants (E1953, E1925, and E1899) were generated as follows (see Fig. 4*A*). In the E1953 mutant, a stop codon was substituted in place of Lys^{605}, resulting in deletion of the cytoplasmic domain after Lys^{605}. In the E1925 mutant, a stop codon was introduced at Gln^{595}. In the E1899 mutant, a stop codon was substituted for Lys^{587}. A forward primer F2 (5-GGTT TGGTGAGGTGTGCTCATTC-3) and three different reverse primers, R4 (5-GCGTTAACTTACTTTGCTTTCCGTAA-3), R5 (5-GCGTTAACT TAGCAGCTGCTGGCAGG-3), and R6 (5-GCGTTACTATTGGTAGCT TCCATCT-3), were used to amplify three fragments from E-selectin cDNA. All three reverse primers (R4, R5, and R6) contained both a stop codon and an *Hpa*I site at their 3′ ends to make mutated E-selectin cytoplasmic domain fragments. In all cases, the amplified fragments were ligated into a TA cloning vector (Invitrogen, San Diego, CA), and the mutated sequences were confirmed by the Sanger dideoxynucleotide chain termination method of sequencing. These fragments were prepared by digestion with *Hpa*I/*Eco*RI and were used to replace the corresponding *Hpa*I/*Eco*RI fragments of the wild-type E-selectin cDNA in the expression vector pCDM8 (Invitrogen).

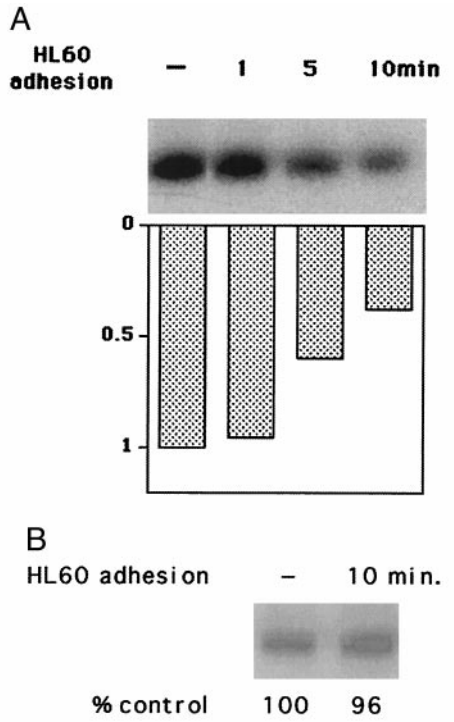

FIGURE 1. *A*, E-selectin is constitutively phosphorylated in activated HUVEC, and leukocyte adhesion induces its dephosphorylation. Cytokine-activated HUVEC were metabolically labeled with [^{32}P]orthophosphate and incubated for various times with HL-60, a human promyelocytic cell line that demonstrates E-selectin-dependent adhesion. Following adhesion, the monolayers were lysed and subjected to immunoprecipitation using anti-E-selectin mAb, H18/7. The relative amount of [^{32}P]orthophosphate incorporated into E-selectin was quantified by densitometric analysis of the autoradiograms and is displayed in the lower bar graph. These data are representative of two separate experiments. *B*, Western blotting of total cellular E-selectin during the time course of leukocyte adhesion. Cytokine-activated HUVEC were treated identically to those in *A*, except for [^{32}P]orthophosphate metabolic labeling. Following adhesion, the monolayers were lysed, and the lysates were immunoprecipitated with H18/7. The precipitates were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes, and E-selectin was detected by Western blotting with H18/7 followed by chemiluminescent detection.

Results

E-selectin is constitutively phosphorylated in cytokine-activated HUVEC, and leukocyte adhesion induces its dephosphorylation

Immunoprecipitation of E-selectin from [^{32}P]orthophosphate-labeled, activated HUVEC revealed constitutive phosphorylation of E-selectin in the absence of additional stimuli (Fig. 1*A*). Adhesion of HL-60 cells induced a time-dependent dephosphorylation of this molecule (Fig. 1*A*). Prolonged adhesion of HL-60 for up to 35 min resulted in dephosphorylation of E-selectin comparable to that demonstrated at 10 min (data not shown). Radiolabeled HUVEC incubated in parallel in the absence of leukocyte adhesion demonstrated no spontaneous loss of the incorporated [^{32}P]phosphate from E-selectin during the same periods of incubation (data not shown).

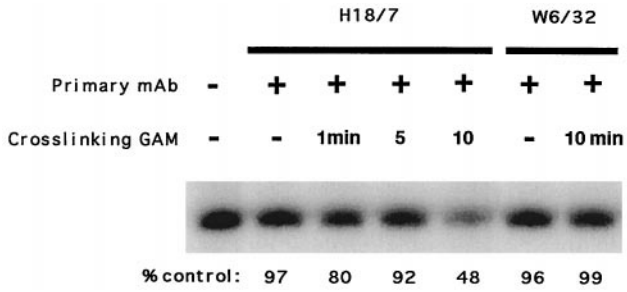

FIGURE 2. Ab cross-linking of cell surface E-selectin results in dephosphorylation of E-selectin in activated HUVEC. Cytokine-activated HUVEC monolayers (10 U/ml IL-1β, 4 h) were metabolically labeled with [^{32}P]orthophosphate, then incubated with saturating amounts of murine mAbs specific for either the extracellular domain of E-selectin (H18/7) or that of HLA-A,B (W6/32), followed by a cross-linking polyclonal goat anti-murine IgG (GAM) for the times indicated. E-selectin was immunoprecipitated from total cell lysates using mAb H18/7 and then subjected to SDS-PAGE and autoradiography. The percentages of control values represent the relative amounts of ^{32}P incorporation into E-selectin quantified by densitometry. These data are representative of two separate experiments.

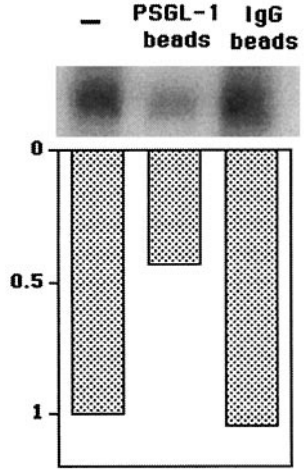

FIGURE 3. Binding of PSGL-1-coated beads to E-selectin induces dephosphorylation of E-selectin in activated HUVEC. Activated HUVEC were prelabeled with [^{32}P]orthophosphate and incubated with PSGL-1-IgG chimera-coated beads or IgG-coated (control) beads for 10 min. The monolayers were lysed and subjected to immunoprecipitation using anti E-selectin mAb, H18/7. The immunoprecipitates were resolved by SDS-PAGE followed by autoradiography. The relative amount of ^{32}P incorporated into E-selectin was quantified by densitometry and is displayed in the lower bar graph. The data are representative of two separate experiments.

The amount of cell surface E-selectin present during this procedure was monitored by surface biotinylation of HUVEC followed by immunoprecipitation of E-selectin from whole cell lysates using mAb H18/7. This labeled cohort of cell surface E-selectin did not significantly change during the time course of these experiments (up to 30 min) and was not influenced by leukocyte adhesion (100% at time zero, 99.7% at 1 min, 130 $\pm$ 17% at 5 min, 83 $\pm$ 8% at 10 min, and 110 $\pm$ 27% at 15 min). In parallel, total cellular E-selectin was examined by Western blotting and also was observed not to change significantly during the course of leukocyte adhesion (Fig. 1*B*). Taken together, these data suggest that the observed reduction of phosphorylation illustrated in Figure 1 reflects changes in the phosphorylation state of cell surface E-selectin induced by HL-60 adhesion rather than either spontaneous loss of phosphate or changes in the amount of E-selectin protein at the cell surface.

Ab cross-linking induces dephosphorylation of cell surface E-selectin in HUVEC

To better understand the nature of the stimulus required to induce dephosphorylation of E-selectin, we next examined the effect of cross-linking cell surface E-selectin with mAb. We incubated [^{32}P]orthophosphate-labeled, IL-1β-activated HUVEC with H18/7 (mAb to E-selectin) in the absence or the presence of a secondary goat anti-mouse polyclonal IgG. H18/7 binds to the extracellular lectin domain of E-selectin and is capable of blocking a significant proportion of leukocyte adhesion (21). As shown in Figure 2, this Ab alone did not induce dephosphorylation of E-selectin (97% of control). However, addition of goat anti-mouse IgG (to physically cross-link E-selectin at the cell surface) for 10 min induced dephosphorylation of E-selectin to a comparable extent (48% of control) as HL-60 adhesion (see Fig. 1*A*). As was observed with leukocyte adhesion, the biotinylated cohort of cell surface E-selectin was also not significantly altered by Ab-mediated cross-linking (100% at time zero, 91.5 $\pm$ 12% at 5 min, 95.0 $\pm$ 13% at 10 min, and 93.0 $\pm$ 10% at 15 min). As a control for the specificity of this stimulus, the effect of W6/32 (a mAb to HLA-A,B), either by itself or cross-linked, was also examined. The binding of this Ab and its cross-linking at the endothelial surface had no effect on the state of E-selectin phosphorylation/dephosphorylation (Fig. 2). These data indicate that Ab-induced cross-linking of cell surface E-selectin on HUVEC is a selective stimulus for its dephosphorylation.

Binding of beads coated with an E-selectin ligand (PSGL-1) induces dephosphorylation of E-selectin in activated HUVEC

Finally, we examined the ability of natural ligand binding to induce E-selectin dephosphorylation. The glycoprotein PSGL-1 bears the carbohydrate moiety sialyl-Lewis X (sLe^x) and has been reported to bind both P- and E-selectin (30). A recombinant chimeric form of PSGL-1 was chosen to test whether ligand-dependent interaction with the extracellular domain of E-selectin can induce dephosphorylation of its cytoplasmic tail. The PSGL-1-IgG chimera was coupled to tosyl-activated magnetic beads, and human IgG-coupled beads were used as a negative control. FACS analysis of the PSGL-1-coated beads using an anti-PSGL-1 mAb confirmed that the recombinant protein had uniformly coated the beads at a relatively high density (data not shown), and when examined in a parallel plate flow chamber, the PSGL-1-coated beads rolled on and adhered to activated HUVEC in a fashion similar to leukocytes (31). These beads were added to cytokine-activated HUVEC metabolically labeled with [^{32}P]orthophosphate. After 10 min of static adhesion, the phosphorylation state of E-selectin was examined as described above. As shown in Figure 3, incubation with PSGL-1 beads for a comparable period induced dephosphorylation of E-selectin on activated HUVEC (43% of control). This result is similar in magnitude to the dephosphorylation observed after both leukocyte adhesion (see Fig. 1*A*) and mAb cross-linking (see Fig. 2). As anticipated, the negative control IgG-coated beads failed to induce dephosphorylation of E-selectin.

The regulation of E-selectin phosphorylation occurs in its cytoplasmic domain

To investigate the functional significance of the cytoplasmic domain in dephosphorylation of E-selectin, we generated two adenoviral vectors: one (AdRSV(wt-E)) containing the wt E-selectin and the other (AdRSV(ΔCyto-E)) a deletion mutant cDNA of E-selectin (ΔCyto) lacking a cytoplasmic domain. These constructs were

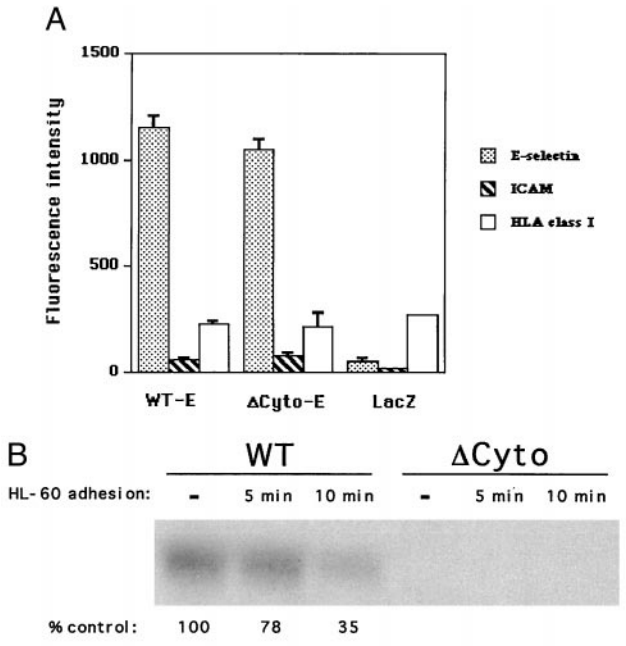

FIGURE 4. *A*, Surface expression of E-selectin on HUVEC transduced with recombinant adenoviral vectors AdRSV(wt-E) and AdRSV(△Cyto-E). HUVEC plated in a 96-well microtiter plate were infected with either AdRSV(wt-E) or AdRSV(ΔCyto-E) (each at 2.5–4.0 × 10^6 plaque-forming units/well), or with the AdRSVβgal construct (also at 2.5–4.0 × 10^6 plaque-forming units/well) as a negative control. To assay E-selectin surface expression, a fluorescence immunobinding assay was performed 48 h postinfection, using three different murine mAbs (H18/7, E-selectin; Hu5/3, ICAM-1; and W6/32, HLA-A,B). Immunobinding data are expressed as the relative fluorescence intensity and are derived from wells plated in triplicate (mean ± SD). These data are representative of three separate experiments. *B*, The cytoplasmic domain of E-selectin expressed by adenoviral transduction is constitutively phosphorylated and is dephosphorylated by HL-60 adhesion. Quiescent HUVEC were infected with either AdRSV(wt-E) (full-length E-selectin) or AdRSV(△Cyto-E) (cytoplasmic domain truncation of E-selectin). Between 48 and 72 h postinfection, these cells were metabolically labeled with [^{32}P]orthophosphate. Following labeling, they were subjected to 5 or 10 min of HL-60 adhesion. The ΔCyto mutant failed to incorporate any radiolabeled phosphate, while the full-length E-selectin not only incorporated phosphate but also was dephosphorylated by HL-60 adhesion in a time-dependent manner.

used to infect cultured HUVEC, which were then used for experimentation 48 to 72 h postinfection. As shown in Figure 4*A*, both these adenovirus constructs (AdRSV(wt-E) and AdRSV(ΔCyto-E)) resulted in the expression of comparable levels of immunoreactive E-selectin protein in infected HUVEC, as confirmed by fluorescence immunoassay, while a third construct, AdRSVβgal, containing the *lacZ* gene (29), did not lead to E-selectin expression. These cell surface levels of E-selectin were similar in magnitude to those observed after standard IL-1 stimulation of HUVEC. ICAM-1 expression was also analyzed to monitor the activation state of the HUVEC during adenoviral vector-mediated transfection. The expression level of ICAM-1 was marginally, but significantly, increased by adenoviral infection (66 ± 14 relative fluorescence units in cells expressing E-selectin constructs vs 18 ± 3 relative fluorescence units in AdRSVβgal-infected cells; $p \leq 0.05$; Fig. 4*A*), suggesting that this method of gene transduction did not significantly activate cultured HUVEC. In addition, expression of VCAM-1 and elaboration of IL-8 were not detected in these E-selectin-transfected HUVEC (data not shown) (29).

The wt- and ΔCyto-E-selectin transduced HUVEC were metabolically labeled with [^{32}P]orthophosphate, and E-selectin was immunoprecipitated from total cell lysates using anti-E-selectin mAb H18/7. As shown in Figure 4*B*, wt-E-selectin, but not ΔCyto-E-selectin, incorporated [^{32}P]phosphate, thus confirming that the cytoplasmic domain of E-selectin was the locus of phosphorylation in cultured HUVEC, and that phosphorylation of E-selectin could occur in the absence of cytokine activation. The adhesion of HL-60 cells to wt-E-selectin-transduced HUVEC monolayers induced a pattern of dephosphorylation similar to that observed in cytokine-activated HUVEC monolayers (see Fig. 1*A*), while adhesion of comparable numbers of HL-60 cells was without effect on the ΔCyto-E-selectin monolayers. These data indicate that both constitutive and regulated phosphorylation/dephosphorylation of E-selectin take place in its cytoplasmic domain.

To better define the region of the E-selectin cytoplasmic domain containing the phosphorylation site(s), we constructed a series of truncation mutant cDNAs of E-selectin (Fig. 5*A*). These mutants were cloned into a mammalian expression vector, pCDM8, and transfected into COS-7 cells. Comparable surface expression levels of the transfected mutants of E-selectin were again confirmed by fluorescence immunoassay. These COS-7 cells transfectants were metabolically labeled with [^{32}P]orthophosphate, and E-selectin was immunoprecipitated using H18/7. As shown in Figure 5*B*, constitutive phosphorylation was observed with wt E-selectin and the E1953 mutant. However, the mutant E-selectins E1925, E1899, and ΔCyto failed to show any phosphorylation, suggesting that the cytoplasmic region from Gln^{595} to Gln^{604} (containing three serines and one tyrosine) contains the E-selectin phosphorylation site(s).

E-selectin is phosphorylated exclusively on serine residues

The adenoviral construct, AdRSV(wt-E) was used to infect quiescent HUVEC, resulting in a high level of expression of wt E-selectin. These cells were then radiolabeled and subjected to 10 min of HL-60 adhesion. The E-selectin from these HUVEC was immunoprecipitated using the specific mAb H18/7, and subsequent autoradiography revealed adhesion-dependent dephosphorylation analogous to that demonstrated with endogenous E-selectin. Phosphoamino acid analysis of this immunoprecipitated material revealed that only phosphoserine was present in E-selectin, both preadhesion (Fig. 6*A*) and postadhesion (Fig. 6*B*), even though the cytoplasmic domain of the protein contains two tyrosines that might have served as potential phosphorylation sites.

Effect of phosphatase inhibitors on E-selectin dephosphorylation

The effect of phosphatase inhibitors also was examined to determine whether the observed leukocyte adhesion-induced dephosphorylation of E-selectin was enzymatically regulated. [^{32}P]orthophosphate-labeled, IL-1β-activated HUVEC were incubated for 10 min before HL-60 adhesion with either 100 μM sodium vanadate, a broad spectrum inhibitor of protein tyrosine phosphatases, or 100 nM okadaic acid, a specific inhibitor of the serine/threonine phosphatases PP1 and PP2A. Both phosphatase inhibitors completely blocked the leukocyte adhesion-induced dephosphorylation of E-selectin in activated HUVEC (shown in Fig. 7). This strongly suggests that the observed dephosphorylation of E-selectin was dependent upon the action of an intracellular phosphatase(s).

Discussion

Interestingly, although significant homology exists among the extracellular domains of the selectins, there is little homology among their cytoplasmic domains (8–10). However, the latter are well conserved across species for each selectin family member, suggesting divergent but evolutionarily conserved functions. Such putative functions probably include roles in signal transduction,

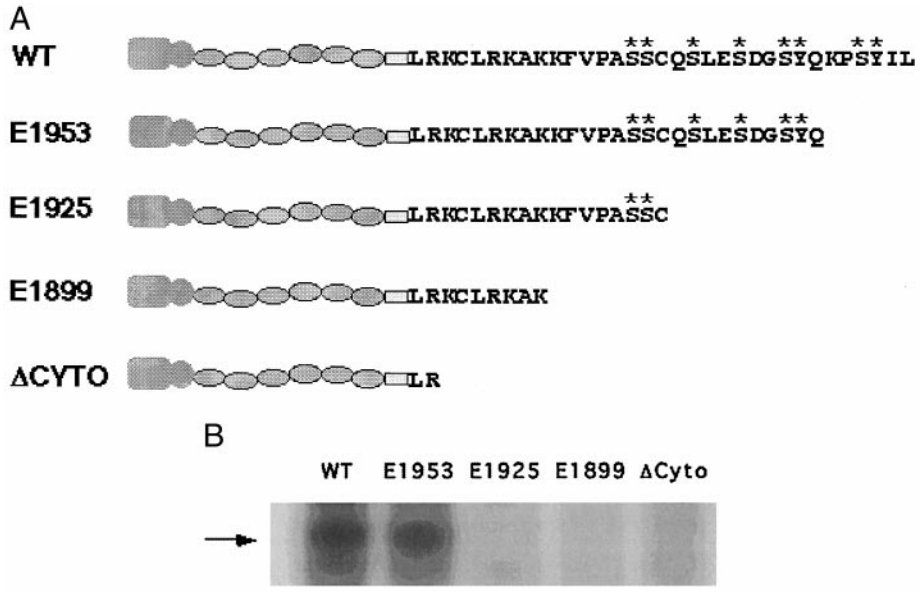

FIGURE 5. *A,* Cytoplasmic deletion mutants of E-selectin. The complete amino acid sequence of the E-selectin cytoplasmic domain (single letter code) and the cytoplasmic sequences of the E-selectin truncation mutants. Asterisks indicate potential phosphorylation sites. *B,* Phosphorylation of cytoplasmic deletion mutant E-selectin in COS-7 transfectants. Cytoplasmic deletion mutants of E-selectin and full length wt E-selectin were transiently transfected into COS-7 cells and metabolically labeled with [^{32}P]orthophosphate as described in *Materials and Methods*. Immunoprecipitation using anti-E-selectin mAb (H18/7) followed by SDS-PAGE and autoradiography revealed that ^{32}P incorporation was observed in wt and E1953 mutant E-selectin, but not in the E1925, E1899, and △Cyto mutant E-selectin molecules.

protein-protein interactions, subcellular localization, post-translational processing, and protein turnover. Of the selectins, the best characterized to date in terms of its cytoplasmic domain function has been L-selectin. Based on domain swapping experiments and deletion analysis, the cytoplasmic domain of L-selectin (consisting of 17 amino acids, with three potential phosphorylation sites: two serines and one tyrosine) (10) appears to be important for the homing of lymphocytes to high endothelial venules in vivo (32) and participates in the selective localization of this molecule on microvilli at the leukocyte surface (33). Ligation (with or without cross-linking) of the extracellular domain of L-selectin by either monoclonal Ab or sulfated glycolipids induces tyrosine phosphorylation of both L-selectin itself and MAP kinases and association of Grb2 and Sos with L-selectin (17, 18), thus indicating propagation of an outside-in signal. Additionally, the cytoplasmic domain of L-selectin appears to be constitutively associated with α-actinin in leukocytes (34). The cytoplasmic domain of P-selectin (consisting of 35 amino acids, including seven potential phosphorylation sites: two serines, two threonines, one tyrosine, and two histidines) (8) appears to be important in trafficking to storage granules (α-granules in platelets, Weibel-Palade bodies in endothelial cells) (35, 36) or to the lysosomal compartment and, ultimately, in degradation (37). During cell activation, phosphorylation of the cytoplasmic domain of P-selectin is rapidly increased on serine, threonine (38), and tyrosine residues (39). Recently, a novel histidine phosphorylation pathway was identified for P-selectin (40), although the significance of this is as yet unclear.

We recently have demonstrated that the cytoplasmic domain of E-selectin (consisting of 32 amino acids, including six serines and two tyrosines) is important for biomechanical linkage to the cytoskeleton during adhesive interactions mediated by its extracellular (in particular, lectin) domain. Certain actin-associated proteins, including α-actinin, filamin, vinculin, FAK, and paxillin, copurified with E-selectin when the latter was selectively immunoextracted using Ab-coated magnetic beads (20). A mutant of E-selectin (ΔCyto) lacking a cytoplasmic tail failed to support these associations. Thus, we concluded that E-selectin interacts with the cytoskeleton directly via its cytoplasmic domain, rather than through an undefined auxiliary protein (or analogous lipid or carbohydrate partner). The association of FAK and paxillin with the cytoplasmic tail of E-selectin also raised the question of an outside-in signaling function for E-selectin during leukocyte-endothelial adhesion.

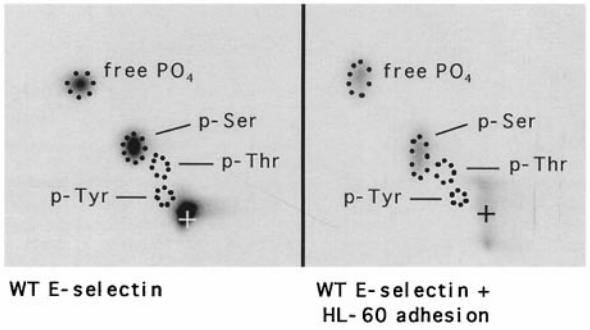

FIGURE 6. Phosphoamino acid analysis of E-selectin. Quiescent HUVEC monolayers were infected with the adenoviral construct AdRS-V(wt-E) to obtain abundant expression of E-selectin, then labeled with [^{32}P]orthophosphate, and some were bound with HL-60 for 10 min. E-selectin was immunoprecipitated, subjected to partial acid hydrolysis, and then analyzed by two-dimensional thin layer electrophoresis. *Left panel,* wt-E-selectin expressed in untreated (i.e., no cytokine activation) HUVEC. *Right panel,* wt-E-selectin after 10 min of HL-60 adhesion. The positions of the origin (+), free phosphate (PO_4), phosphoserine (p-Ser), phosphothreonine (p-Thr), and phosphotyrosine (p-Tyr) are indicated.

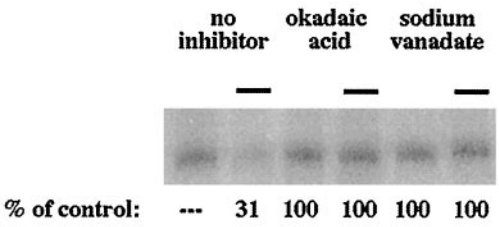

FIGURE 7. Effects of phosphatase inhibitors on E-selectin dephosphorylation. Cytokine-activated HUVEC were metabolically labeled with [^{32}P]orthophosphate and incubated with either okadaic acid (100 nM) or sodium vanadate (100 μM) for 10 min before addition of HL-60. The cells were lysed and subjected to immunoprecipitation using H18/7, followed by SDS-PAGE and autoradiography. Bars above lanes indicate 10 min of HL-60 adhesion.

In this study, we have demonstrated that E-selectin is constitutively phosphorylated in its cytoplasmic domain both when induced by cytokine activation in cultured HUVEC and when expressed as a recombinant protein by adenovirally mediated transduction in unactivated HUVEC. Furthermore, we have shown that this phosphorylation occurs exclusively on the amino acid serine (Fig. 6). The constitutive phosphorylation of E-selectin is rapidly and significantly decreased in response to stimuli that interact with the extracellular domain of this molecule. These stimuli, which may result in E-selectin clustering in the plane of the cell membrane, include 1) leukocyte adhesion, 2) mAb-mediated cross-linking of cell surface E-selectin, and 3) the binding of beads coated with an E-selectin glycoprotein ligand, namely PSGL-1. In conjunction with our previously reported data regarding the association of E-selectin with cytoskeletal proteins (20), these observations provide the first experimental evidence for an outside-in signaling function for E-selectin. Such a function may have important implications for the orchestration of endothelial cell responses during leukocyte-endothelial interactions involving E-selectin-dependent adhesion.

Smeets et al. have previously reported that in cultured HUVEC, TNF-α-induced E-selectin was constitutively phosphorylated (41). To investigate the effects of leukocyte adhesion mediated via E-selectin on its phosphorylation state, we conducted experiments using HL-60, a promyelocytic leukocyte cell line that expresses E-selectin ligands (30). As shown in Figure 1*A*, adhesion of HL-60 induced a time-dependent dephosphorylation of E-selectin in cytokine-activated HUVEC. This is the first demonstration that leukocyte adhesion can modulate the phosphorylation of E-selectin expressed on endothelial cells. Dephosphorylation of E-selectin was again observed when cell surface E-selectin was cross-linked by binding of a specific mAb (H18/7) and polyclonal anti-mouse IgG (Fig. 2). We also demonstrated that dephosphorylation of E-selectin can be induced by the binding of PSGL-1-coated microspheres (Fig. 3), suggesting that a physiologically relevant ligand could also trigger this process. In parallel, we observed that a biotinylated cohort of cell surface E-selectin did not change during the time course of either adhesion or cross-linking, nor was the total cellular E-selectin content altered by these stimuli. The observed decrease in the phosphate content of E-selectin therefore presumably reflects a biochemical modification of this protein in response to ligation and/or cross-linking, as opposed to an increased rate of protein turnover and/or cell surface shedding. Additionally, we found it interesting that the dephosphorylation of E-selectin induced by our three distinct experimental stimuli reached a maximum of approximately 50 to 60% of control values (Figs. 1, 2, 3, and 4*B*). There are at least two potential explanations for this. First, certain of the phosphorylated amino acid residues of E-selectin may be regulated by dephosphorylation, while others may not. Second, while cell surface E-selectin is subject to adhesion/cross-linking and subsequent dephosphorylation, the intracellular cohort of E-selectin molecules (i.e., nascent molecules, etc.) is not directly exposed to these input stimuli. Either of these mechanisms alone or in conjunction could account for the results that we observed.

Using adenoviral vectors to transduce E-selectin into cultured HUVEC, we determined that phosphorylation of E-selectin is occurring within the cytoplasmic domain of the protein (Fig. 4*B*). As shown in Figure 4*A*, adenoviral gene transduction per se did not significantly activate endothelial cells, as judged by expression of the activation-responsive adhesion molecule ICAM-1. Previously we have also shown that adenoviral gene transduction in HUVEC does not up-regulate expression of either E-selectin or VCAM-1 (29). HUVEC transduced with wild-type E-selectin exhibited a pattern of phosphorylation in the absence of cytokine activation similar to that observed in cytokine-activated HUVEC. Conversely, HUVEC transduced with an E-selectin mutant lacking a cytoplasmic domain (ΔCyto) failed to show any phosphorylation after metabolic labeling with [^{32}P]orthophosphate (Fig. 4*B*). Given that the ΔCyto mutant was expressed at levels comparable to those of the full-length E-selectin, we conclude that the cytoplasmic domain of E-selectin is not required for targeting of the protein to the cell surface.

Adhesion of HL-60 induced dephosphorylation of the adenovirally transduced wt E-selectin in a time-dependent fashion (Fig. 4*B*). The truncated E-selectin mutant, ΔCyto, which was not phosphorylated, did not appear to be affected by HL-60 adhesion. As we have previously reported, both wt and ΔCyto-E-selectin-transfected HUVEC support HL-60 adhesion to the same extent (20). Since the HUVEC were not activated by adenovirus infection and yet dephosphorylation of the wt E-selectin did occur, it is likely that this adhesion-dependent dephosphorylation does not require the milieu of a cytokine-activated endothelial cell. Thus, the phosphatase responsible for dephosphorylation of the serine(s) of E-selectin appears to be constitutively expressed in endothelial cells.

Based upon our results using adenoviral transduction as a method of gene transfer, it is clear that this is a particularly powerful technique for analysis of E-selectin function. The infection of HUVEC is highly efficient, resulting in up to 90% of cells expressing E-selectin or the E-selectin mutants (data not shown). This is a stark contrast to the difficulties encountered when using more traditional methods of gene transfection (e.g., calcium phosphate coprecipitation, lipofectin, or electroporation) with HUVEC. Additionally, infection with these adenovirus constructs does not appear to significantly perturb the basal state of cultured HUVEC, as judged by the lack of induction of the endogenous E-selectin gene (a sensitive indicator of activation in HUVEC) as well as other inducible genes, including VCAM-1 and IL-8 (29) (Fig. 4*A*) (J.-M. Kiely, unpublished observations), thus allowing us to dissociate activation-dependent and activation-independent phenomena.

Using transient transfection in COS-7 cells, we also tested a series of truncation mutants to establish the importance of a particular region as a potential phosphorylation site in the E-selectin cytoplasmic domain. As shown in Figure 5*B*, only the E1953 mutant was phosphorylated, following [^{32}P]orthophosphate labeling, to an extent similar to full-length E-selectin. The E1953 mutant contains six potential phosphorylation sites: five serines and one tyrosine (Fig. 5*A*). None of the remaining truncation mutants, including E1925 with its two serines, incorporated any appreciable amount of [^{32}P]orthophosphate. Thus, a site(s) of E-selectin phosphorylation must lie between Gln^{595} and Gln^{604}. Candidate residues in this region are Ser^{596}, Ser^{599}, and Ser^{602}. Based upon our existing data we cannot determine what role, if any, Ser^{607} plays in E-selectin phosphorylation. Currently, we are exploring the relative importance of the phosphorylation state of these candidate residues for E-selectin association with the endothelial cytoskeleton and other leukocyte adhesion-mediated processes.

The modulation of protein phosphorylation states plays a central role in the regulation of numerous cellular processes, including cell growth, differentiation, cell cycle progression, and cytoskeletal reorganization (42). Protein kinases and protein phosphatases are the enzymes that mediate this process of phosphorylation/dephosphorylation. The constitutive serine phosphorylation of E-selectin (Fig. 6) and its ligation-induced dephosphorylation led us to investigate the potential effect of protein serine/threonine phosphatases. As shown in Figure 7, the phosphatase inhibitors okadaic acid and

sodium vanadate both prevented the dephosphorylation of E-selectin. Okadaic acid is a specific inhibitor of both protein phosphatase-1 (PP1) and protein phosphatase-2A (PP2A), although the concentration of okadaic acid that we used (100 nM) was in the effective range for inhibition of PP1 (IC_{50} of 10–15 nM) (43). On the other hand, sodium vanadate is a broad spectrum inhibitor of protein tyrosine phosphatases. Little information is available regarding the action of sodium vanadate on PP1 or PP2A, although PP2A is known to be activated by tyrosine dephosphorylation (44). The results of sodium vanadate inhibition of dephosphorylation would thus favor PP1 as a candidate instead of PP2A. Another possibility is that the dephosphorylation of E-selectin is accomplished via the action of a dual specificity protein tyrosine phosphatase, such as either MAP kinase phosphatase-1 (MKP-1) or the vaccinia H1-related human dual specificity phosphatase (VHR) (45, 46). These enzymes are capable of hydrolyzing not only the phosphate groups of tyrosines but also those of serines and threonines. This might, then, account for the effectiveness of both okadaic acid and sodium vanadate as inhibitors of E-selectin dephosphorylation. Our results using phosphatase inhibitors thus favor a phosphatase with a specificity similar to that of PP1. Its activity may be secondarily regulated by tyrosine phosphorylation, or it may be a dual specificity enzyme. Currently, efforts are in progress to determine the identity of the phosphatase that is responsible for dephosphorylation of E-selectin and to define the events required for its activation.

The signaling functions of adhesion molecules have drawn a great deal of attention in recent years. Of particular interest have been the integrins, the cadherins, and the Ig superfamily of adhesion proteins. Integrin receptors have been extensively characterized with respect to their outside-in and inside-out signaling functions in various cell types (including endothelial cells) as a response to binding to counter-receptors on interacting cells or to extracellular matrix ligands (11, 12). Signaling via the integrins can activate kinase cascades, induce cytoskeletal reorganization, and modulate integrin affinities and adhesive functions (11, 12). The cadherins, expressed at cell borders and cell-cell junctions, regulate both junctional permeability and cellular transmigration. The cytoplasmic domain of VE-cadherin, for example, has been shown to be an active participant in signal transduction via its interaction with β-catenin, plakoglobin, and p120 (13). PECAM-1, a member of the Ig superfamily of adhesion molecules, has been recently reported to play a role in transmitting signals via modulation of the phosphorylation state of its cytoplasmic domain. In fact, PECAM-1 is a substrate of the tyrosine kinase, Src, and when tyrosine phosphorylated can bind the protein tyrosine phosphatase, SH2-containing phosphatase-2 (SHP-2) (15, 16).

It is becoming apparent that cell surface E-selectin is capable of transducing a transmembrane signal in the endothelial cell. Our previous data indicated that E-selectin can establish a direct molecular linkage to the cytoskeleton (20). Among the cytoskeletal proteins found to interact with E-selectin were the tyrosine kinase, FAK, and its substrate paxillin, which themselves have been shown to participate in signaling cascades involving Cas, Grb2, Crk, Ras, and MAP kinase (47). The present study extends this concept of a signaling function for E-selectin by demonstrating constitutive serine phosphorylation of the E-selectin cytoplasmic tail. Enzymatic dephosphorylation, as demonstrated by our phosphatase inhibitor data, provides a potential explanation for the regulation of E-selectin's association with cytoskeletal components. In platelets, for example, PP1 and PP2A are differentially associated with the cytoskeleton depending upon whether the platelets have been activated by thrombin (48). As a second example, inhibition of PP1 in unactivated HUVEC results in marked changes in intracellular architecture, including perinuclear condensation of microtubules and F-actin (49). The extent to which E-selectin phosphorylation/dephosphorylation impacts upon the behavior of endothelial cell cytoskeletal components is the subject of ongoing investigation.

Our current observations suggest that biochemical and structural changes induced in and by E-selectin may propagate signals important for co-ordinating endothelial responses in the leukocyte-endothelial adhesion cascade. For example, initial leukocyte adhesion-induced E-selectin clustering in the membrane (reflecting localized association with the cortical cytoskeleton) could dynamically regulate the avidity of subsequent leukocyte adhesion events. Alternatively, ligation of E-selectin at the apical endothelial surface could trigger events that might influence lateral cell-cell junctional permeability, perhaps by altering the phosphorylation state of protein components of adherens junctions (i.e., VE-cadherin, β-catenin, plakoglobin, and p120) or of tight junctions (i.e., occludin, cingulin, Zonula occludens-1 (ZO-1), and Zonula occludens-2 (ZO-2). Finally, an E-selectin-transduced signal might lead ultimately to gene activation, perhaps through MAP kinase signaling intermediates or via downstream interactions in the FAK-paxillin cascade. These putative E-selectin-mediated functions would have important implications for the role of this vascular selectin in the integrated pathophysiologic response of endothelial cells at sites of inflammation.

Acknowledgments

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