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## Abstract

The selectin adhesion molecules and chemoattractant receptors synergistically regulate leukocyte migration into lymphoid tissues and sites of inflammation, but little is known about how these families of receptors modulate each other's function. In this study, L-selectin was found to be phosphorylated in lymphoblastoid cell lines, and phosphorylation was enhanced by phorbol ester (phorbol 12-myristate 13-acetate (PMA)) treatment. Interactions between L-selectin and chemoattractant receptors were therefore examined using transfected rat basophilic leukemia cell lines (RBL-2H3) that expressed human L-selectin along with human leukocyte chemoattractant receptors. L-selectin was rapidly phosphorylated in cells treated with chemoattractants, thrombin, IgE receptor agonists, or PMA. Pertussis toxin or the protein kinase C inhibitor, staurosporine, completely blocked chemoattractant receptor-induced phosphorylation of L-selectin. PMA-induced phosphorylation was on serine residues within the cytoplasmic tail of L- selectin that have been well conserved during recent evolution. Although L- selectin phosphorylation was not essential for basal levels of adhesion through L-selectin in transformed cell lines, the rapid increase in ligand binding activity of L-selectin that occurs following leukocyte activation was blocked by staurosporine. These results demonstrate that L-selectin can be phosphorylated following engagement of chemoattractant receptors and suggest that this may be a physiologically relevant mechanism for the synergistic regulation of these receptors during leukocyte migration.



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# Chemoattractant Receptor-induced Phosphorylation of L-selectin*

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**The selectin adhesion molecules and chemoattractant receptors synergistically regulate leukocyte migration into lymphoid tissues and sites of inflammation, but little is known about how these families of receptors modulate each other's function. In this study, L-selectin was found to be phosphorylated in lymphoblastoid cell lines, and phosphorylation was enhanced by phorbol ester (phorbol 12-myristate 13-acetate (PMA)) treatment. Interactions between L-selectin and chemoattractant receptors were therefore examined using transfected rat basophilic leukemia cell lines (RBL-2H3) that expressed human L-selectin along with human leukocyte chemoattractant receptors. L-selectin was rapidly phosphorylated in cells treated with chemoattractants, thrombin, IgE receptor agonists, or PMA. Pertussis toxin or the protein kinase C inhibitor, staurosporine, completely blocked chemoattractant receptor-induced phosphorylation of L-selectin. PMA-induced phosphorylation was on serine residues within the cytoplasmic tail of L-selectin that have been well conserved during recent evolution. Although L-selectin phosphorylation was not essential for basal levels of adhesion through L-selectin in transformed cell lines, the rapid increase in ligand binding activity of L-selectin that occurs following leukocyte activation was blocked by staurosporine. These results demonstrate that L-selectin can be phosphorylated following engagement of chemoattractant receptors and suggest that this may be a physiologically relevant mechanism for the synergistic regulation of these receptors during leukocyte migration.**

The selectin and integrin families of adhesion molecules regulate leukocyte migration into lymphoid tissues and sites of inflammation (1–4). L-, P-, and E-selectin mediate the initial interactions of leukocytes with endothelium that result in leukocytes rolling along the venular wall (2). During their initial interactions with endothelial cells, leukocytes encounter chemoattractants that bind to cell surface receptors (5–9). Signal transduction through chemoattractant receptors results in increased binding activity for L-selectin, $\beta_2$ integrins, and $\beta_1$ integrins (10), which stabilizes leukocyte interactions with endothelial cells (3, 4).

While much is known regarding the independent functions of adhesion molecules and chemoattractant receptors, little is known about how these receptors modulate the function of each other. In one example, the ligand binding activity of L-selectin can be rapidly up-regulated by exposing leukocytes to a variety of pro-inflammatory agents including chemoattractants (10). Therefore, potential mechanisms by which chemoattractant receptor signaling may modulate L-selectin function were examined using the rat basophilic leukemia cell line, RBL-2H3 (RBL[1] cells), as an *in vivo* model (11–13). Phosphorylation of L-selectin is a potential site for receptor regulation since the cytoplasmic domain of L-selectin contains numerous basic residues surrounding 2 serine residues that have been highly conserved during recent mammalian evolution (2, 14, 15). RBL cells stably transfected to co-express functional human chemoattractant receptors and L-selectin provide direct evidence that activation of chemoattractant receptors induces immediate phosphorylation of L-selectin through a protein kinase C (PKC)-dependent pathway.

## EXPERIMENTAL PROCEDURES

*Immunofluorescence Reagents and Analysis*—Antibodies used in these studies included: mouse LAM1–116 ($IgG_{2a}$) and LAM1–110 ($IgG_1$) mAbs that react with human, mouse, and rat L-selectin (34); anti-CD83 mAb (HB15A $IgG_{2b}$); and anti-human CD3 mAb (RW2–8C8). Antibodies were purified from ascites fluid by sodium sulfate precipitation and DEAE-Sepharose anion exchange column chromatography (Pharmacia Biotech Inc.). The 12CA5 mAb reactive with a 9-amino acid epitope tag was from Boehringer Mannheim.

Immunofluorescence staining of cells and cell lines was as described previously (16) using mAbs optimally diluted for immunostaining: FITC-conjugated LAM1–116 mAb or unconjugated LAM1–116 mAb detected with FITC-conjugated goat anti-mouse IgG antibodies (Caltag, South San Francisco, CA). Single color immunofluorescence analysis of 10,000 cells was performed on a FACScan flow cytometer (Becton Dickinson) with fluorescence intensity analyzed on a 4-decade log scale. The lectin activity of L-selectin was assessed by incubating transfected RBL cells with biotinylated polyphosphomonoester core polysaccharide (5 mg/ml) from yeast and FITC-conjugated streptavidin using methods similar to those previously described (10).

*Cells and Cell Lines*—RBL cells or RBL cells expressing epitope-tagged chemoattractant receptors were cultured as described (11). RBL or 300.19 cells were co-transfected with L-selectin or LΔM-N cDNA by electroporation, and clones were isolated as described (13, 15). 300.19 cells transfected with human cDNA for either L-selectin or LΔcyto cDNA were as described (16, 17). Human blood lymphocytes were isolated from heparin-anticoagulated venous blood from healthy adult volunteers by centrifugation over Ficoll density gradient medium

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[1] The abbreviations used are: RBL, rat basophilic leukemia; PKC, protein kinase C; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; C5a, cleavage product of complement activation; fMLP, formylmethionylleucylphenylalanine; PAF, platelet-activating factor; HEV, high endothelial venule; IL, interleukin; PMA, phorbol 12-myristate 13-acetate; R, receptor; LΔcyto, a form of L-selectin lacking the cytoplasmic domain; LΔG-S, a form of L-selectin lacking the GKKSKRS peptide within the cytoplasmic domain; LΔM-N, a form of L-selectin lacking the membrane-proximal endoproteolytic cleavage site; L-SS/AA, a form of L-selectin with the 2 cytoplasmic serine residues replaced by alanine residues.

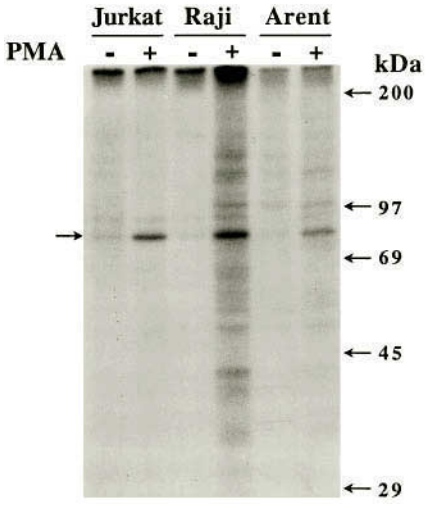


FIG. 1. **Phosphorylation of L-selectin in human lymphoblastoid cell lines.** Cell lines expressing L-selectin were metabolically labeled with [$^{32}$P]orthophosphate and stimulated with PMA (0.5 $\mu$M) during the last 3 min of culture as indicated (+). Cell lysates were immunoprecipitated with the LAM1–116 mAb and analyzed by SDS-PAGE and autoradiography. Molecular weight marker (*right*) and L-selectin (*left*) positions are indicated.

(Nycomed, Oslo, Norway).

*Construction of L-selectin cDNA and Chemoattractant Receptors*—The cytoplasmic domain of human L-selectin is composed of 12 amino acid residues (G$^{323}$KKSKRSMNDPY$^{334}$) (14). The L-selectin cDNA that encodes a protein with the 2 cytoplasmic serine residues replaced by alanine residues (L-SS/AA) and the cDNA that deletes the GKKSKRS sequence (LΔG-S) of the cytoplasmic domain were generated by a two-step polymerase chain reaction and were verified by sequence analysis. The LΔcyto and LΔM-N cDNAs were as described (15, 17). Epitope-tagged chemoattractant receptors were as described (11–13).

*Immunoprecipitations*—Cells (5–10 × 10$^6$) were surface labeled with $^{125}$I, lysed, immunoprecipitated using the indicated mAbs, resolved by SDS-PAGE, and visualized by autoradiography as described (11–13). Metabolic labeling of cells with [$^{32}$P]orthophosphate was as described (11–13). Labeled cells were incubated with or without agonists (thrombin receptor peptide, SFLLRN, 100 $\mu$M, Peninsula Laboratories, Belmont, CA; thrombin, 1 unit/ml; fMLP, 1 $\mu$M; PAF, 100 nM; PMA, 0.5 $\mu$M, all from Calbiochem; IL-8, 100 nM, Genzyme, Cambridge, MA; C5a, 100 nM, Sigma) for 3 min at 37 °C. Cells were also activated with IgE (0.2 $\mu$g/ml) plus antigen (dinitrophenyl-conjugated bovine serum albumin, 0.1 $\mu$g/ml) as described (11). In some cases the cells were also pretreated for 5 min with 100 nM staurosporine (Calbiochem) or overnight with 100 ng/ml of pertussis toxin (List Biological Laboratories) before stimulation with agonists. For lymphoblastoid cell lines, cells were incubated in the presence of protease inhibitors as described (18, 19). The cells were lysed after 3 min. Phosphorylated L-selectin or chemoattractant receptors were immunoprecipitated with the LAM1–116 (15 $\mu$g) or 12CA5 (10 $\mu$g) mAbs, respectively, analyzed by SDS-PAGE, and visualized by autoradiography.

*Cell Binding Assay*—Binding of transfected 300.19 cells expressing native or modified forms of L-selectin to high endothelial venules (HEVs) was assessed as described (16, 20). Human blood lymphocytes were stimulated through the CD3 receptor as described (10) in the presence of 100 nM staurosporine or $Me_2SO$ carrier.

## RESULTS

*L-selectin Phosphorylation*—Previous attempts to demonstrate phosphorylation of L-selectin by us and others have been unsuccessful probably because of the rapid endoproteolytic release of L-selectin from the cell surface following cellular activation (21–24). Therefore, metabolically labeled human lymphoblastoid cell lines that express L-selectin were cultured for 3 min with either medium or PMA, a known activator of PKC, in the presence of a protease inhibitor that blocks the endoproteolytic release of L-selectin (18, 19). These inhibitors do not affect leukocyte activation or adhesive interactions between leukocytes and endothelial cells (18, 19, 25). Following lysis of the cells and immunoprecipitation with an L-selectin-specific mAb, an appropriately sized phosphoprotein band was isolated (Fig. 1). In each case, PMA treatment of the cell lines resulted in increased phosphorylation of this protein. Therefore, RBL cells transfected with L-selectin cDNA were used as a model system to verify that L-selectin was the phosphorylated protein immunoprecipitated from lymphoblastoid cell lines.

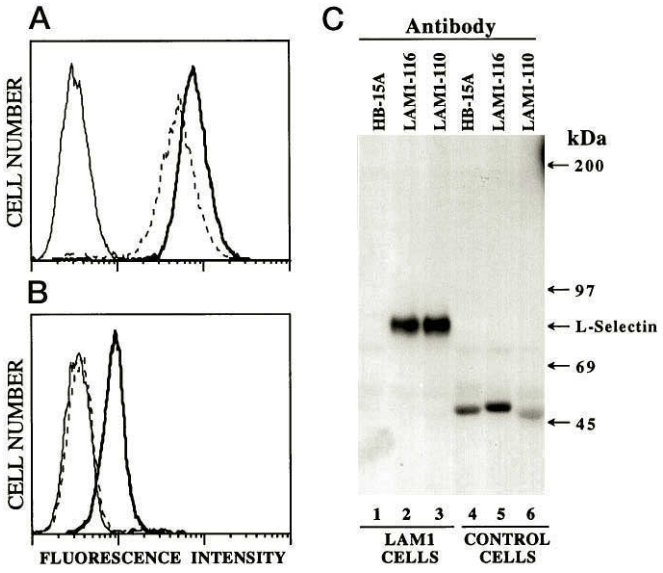


FIG. 2. **L-selectin expression by cDNA-transfected RBL cells.** *A*, direct immunofluorescence staining of native RBL cells (*thin line*), RBL cells stably expressing human L-selectin (*broken line*), or the LΔM-N form of L-selectin (*bold line*) with FITC-conjugated LAM1–116 mAb. *B*, binding of polyphosphomonoester core polysaccharide by native RBL cells in the presence of calcium (*thin line*) and LΔM-N cDNA-transfected cells in the presence (*bold line*) or absence (*broken line*) of calcium. *C*, SDS-PAGE analysis of immunoprecipitated L-selectin. Cells expressing the LΔM-N form of L-selectin (*LAM1 cells*) untransfected RBL cells (*control cells*) were radioiodinated, solubilized, and immunoprecipitated with L-selectin-specific mAbs (*LAM1–116* and *LAM1–110*) or an irrelevant isotype-matched mAb (*HB-15A*). Nonspecific bands of ~45 kDa were variably immunoprecipitated from untransfected and transfected cells. These results are representative of three independent experiments.

RBL cells transfected with human L-selectin cDNA (LAM1 cells) expressed high levels of either unmodified L-selectin (14) or a modified form of L-selectin (LΔM-N) (15) that is fully functional but not endoproteolytically released from the cell surface (Fig. 2*A*). RBL cells did not express endogenous L-selectin as determined using two mAbs reactive with rat L-selectin (LAM1–116 and LAM1–110, Fig. 2*A*). Human L-selectin expressed in RBL cells was functionally and structurally intact since it retained the ability to specifically bind polyphosphomonoester core polysaccharide, which mimics the natural ligand of L-selectin (Fig. 2*B*) (26) and was bound by seven mAbs directed against distinct epitopes of its extracellular domains (data not shown). In addition, only LAM1 cells displayed an ~80-kDa surface protein that was immunoprecipitated with mAbs specific for L-selectin (Fig. 2*C*). L-selectin immunoprecipitated from unactivated RBL cells was only weakly phosphorylated or not phosphorylated at detectable levels. However, phosphorylation of L-selectin was markedly increased following PMA treatment of LAM1 cells for 3 min (Fig. 3*A*, *lanes 1* and *2*). In contrast, PMA treatment of untransfected RBL cells did not induce phosphorylation of proteins in this size range (Fig. 3*A*, *lane 4*).

*Chemoattractant Receptors Induce L-selectin Phosphorylation*—RBL cells that expressed the LΔM-N form of L-selectin, as well as native thrombin and IgE receptors, were also transfected with cDNA encoding epitope-tagged human receptors for

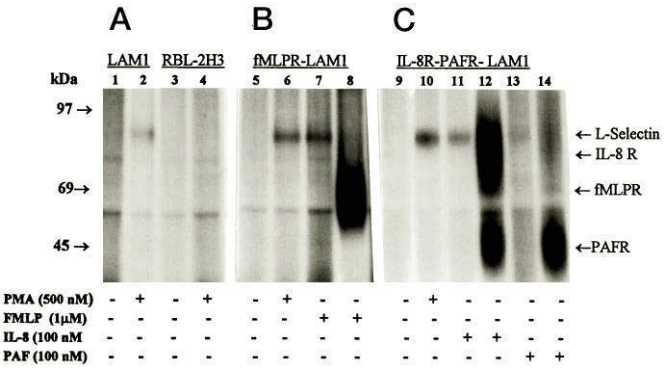


FIG. 3. **Chemoattractant receptor-initiated phosphorylation of L-selectin.** RBL cells co-expressing L-selectin along with different epitope-tagged chemoattractant receptors (all recognized by the 12CA5 mAb) were labeled with [$^{32}$P]orthophosphate and stimulated as indicated. Cells were lysed, immunoprecipitated with either the 12CA5 (*lanes 8, 12*, and *14*) or LAM1–116 mAbs (*all other lanes*), and analyzed by SDS-PAGE and autoradiography. The positions of L-selectin (*lanes 2, 6, 7, 10, 11*, and *13*), IL-8R (*lane 12*), fMLPR (*lane 8*), and PAFR (*lanes 12* and *14*) are indicated. Similar results were obtained in three experiments.

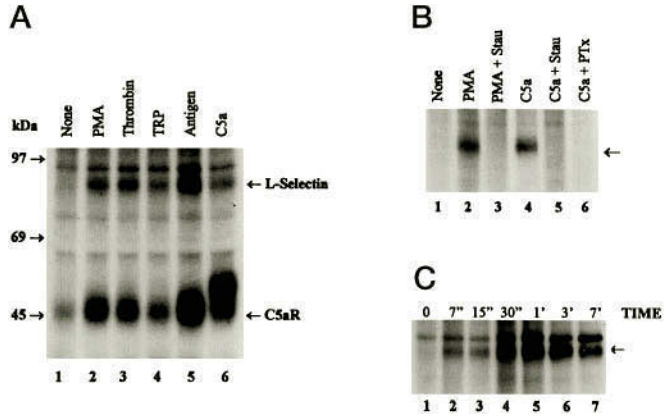


FIG. 4. **Phosphorylation of L-selectin is PKC-dependent.** *A* and *B*, RBL cells co-expressing L-selectin and C5a receptor were $^{32}$P-labeled and stimulated with PMA, thrombin, thrombin receptor peptide (*TRP*), IgE plus autigen, or C5a as indicated. Cell lysates were immunoprecipitated with both LAM1–116 and 12CA5 mAbs and analyzed by SDS-PAGE and autoradiography. *B*, equal numbers of cells were also pretreated for 5 min with 100 nM staurosporine (*Stau, lanes 3* and *5*) or overnight with pertussis toxin (*PTx, lane 6*) before stimulation with agonists. *C*, time course of fMLP-induced phosphorylation of L-selectin. RBL cells co-expressing L-selectin and fMLPR were labeled with [$^{32}$P]orthophosphate and stimulated with fMLP. At the indicated times, cells were lysed, immunoprecipitated with the LAM1–116 mAb, and analyzed by SDS-PAGE and autoradiography. *Arrowheads* indicate the position of L-selectin in both *B* and *C*.

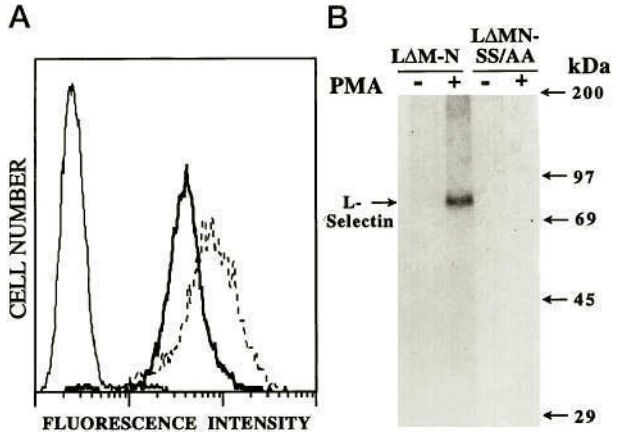


FIG. 5. **L-selectin is phosphorylated on cytoplasmic serine residues.** RBL cells expressing the LΔM-N form of L-selectin with an intact cytoplasmic domain or with the 2 serine residues substituted with alanine residues (LΔMN-SS/AA) were generated. *A*, indirect immunofluorescence staining of native RBL cells (*thin line*), RBL cells expressing the LΔM-N (*broken line*), or LΔMN-SS/AA (*heavy line*) forms of L-selectin with LAM1–116 mAb. *B*, RBL cells expressing LΔM-N or LΔMN-SS/AA were $^{32}$P-labeled and stimulated with PMA as indicated. Cell lysates were immunoprecipitated with the LAM1–116 mAb and analyzed by SDS-PAGE and autoradiography.

formyl peptides (fMLPR), a peptide component of complement activation (C5aR), interleukin-8 (IL-8R), or platelet-activating factor (PAFR). RBL clones expressing these chemoattractant receptors respond to chemoattractants by activating similar signal transduction pathways as do leukocytes including actin polymerization, phosphoinositide hydrolysis, calcium mobilization, phospholipase D activation, and degranulation (11–13). L-selectin expressed together with human chemoattractant receptors (Fig. 3, *B* and *C*) was phosphorylated following PMA treatment (Fig. 3, *lanes 2, 6,* and *10*). Activation for 3 min of chemoattractant receptors for fMLP, IL-8, and PAF with their respective ligands also resulted in phosphorylation of L-selectin in co-transfected cells (*lanes 7, 11*, and *13*). fMLP induced strong phosphorylation of L-selectin, while PAF activation resulted in weaker phosphorylation (*lanes 7* and *13*). IL-8, fMLP, and PAF stimulation also resulted in the homologous phosphorylation of their receptors (*lanes 8, 12*, and *14*) as well as cross-phosphorylation of the PAFR by IL-8R activation as described previously (11–13, 27). Activation of RBL cells through endogenous thrombin, IgE receptors, or ectopic C5a receptors also resulted in phosphorylation of L-selectin (Fig. 4*A*). Activation of RBL cells through these receptors also results in phosphorylation of the C5a receptor (11), which was simultaneously immunoprecipitated with L-selectin to provide an internal control for receptor signaling (Fig. 4*A*). In addition, dose-response studies showed that concentrations (1–3 nM) of C5a analogous to those found at sites of inflammation induced L-selectin phosphorylation (data not shown). Therefore, L-selectin was rapidly phosphorylated following chemoattractant receptor activation.

*L-selectin Is Phosphorylated on Serine Residues following PKC Activation*—L-selectin was phosphorylated specifically on serine residues following PMA treatment. Phosphorylated L-selectin was immunoprecipitated from RBL cells transfected with a cDNA encoding the LΔM-N form of L-selectin, while phosphorylated L-selectin was not immunoprecipitated from cells expressing the LΔM-N form of L-selectin with the 2 serine residues in the cytoplasmic tail replaced with alanine residues (LΔMN-SS/AA) (Fig. 5). Since PKC is activated by all of the agonists used above, its role in L-selectin phosphorylation was examined using staurosporine, a PKC inhibitor (28). Both PMA- and C5a-induced phosphorylation of L-selectin was completely inhibited by treating LAM1 cells with staurosporine (Fig. 4*B*, *lanes 3* and *5*). Pertussis toxin, an inhibitor of signaling through $G_i$ proteins (29), also blocked C5a-induced phosphorylation of L-selectin, indicating that the production of second messengers through G-protein activation is required for chemoattractant receptor-induced phosphorylation of L-selectin (Fig. 4*B*, *lane 6*). L-selectin phosphorylation was detected at the earliest measurable time point of 7 s following fMLPR-induced activation of RBL cells (Fig. 4*C*). These results demonstrate that L-selectin was immediately phosphorylated on serine residues following cellular activation by a wide range of pro-inflammatory mediators that activate PKC.

*Role of Phosphorylation in L-selectin Function*—Native L-selectin was endoproteolytically released from the cell surface within minutes following activation of RBL cells with PMA, while the LΔM-N form of L-selectin was retained (data not shown). To determine whether phosphorylation of L-selectin

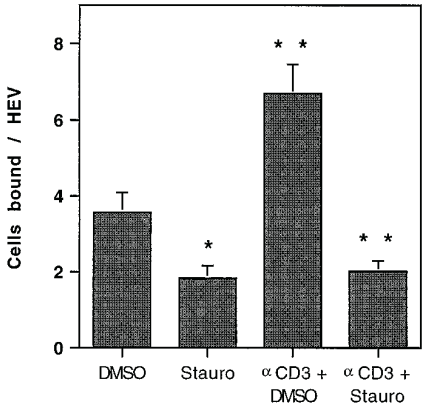


FIG. 6. **Up-regulation of L-selectin binding to HEVs is dependent on protein kinases.** Human blood lymphocytes were stimulated through the CD3 receptor in the presence of staurosporine (*Stauro*) or $Me_2SO$ (*DMSO*) carrier. Their ability to bind HEVs of rat peripheral lymph nodes was then assessed using a frozen section binding assay. Values represent the mean ± S.E. of the average number of lymphocytes bound per HEV in five experiments with >100 HEVs counted per experiment. Differences between control cells and treated cells were significant. $p < 0.01$ (*) and $p < 0.001$ (**) using the Student's $t$ test.

regulates its endoproteolytic release from the cell surface, RBL and 300.19 cells, a mouse pre-B cell line, were transfected with L-selectin cDNAs that encoded native receptors with the 2 serine residues in the cytoplasmic tail replaced with alanine residues (L-SS/AA), the 7-amino acid region containing the serine residues (LΔG-S) deleted, or the entire cytoplasmic tail (LΔcyto) deleted (see "Experimental Procedures"). In all cases, the spontaneous or PMA-induced endoproteolytic release of L-selectin was not measurably affected by these modifications (data not shown). Therefore, endoproteolytic release of L-selectin was not regulated by the cytoplasmic domain.

The cytoplasmic domain of L-selectin is required for receptor-mediated adhesion *in vivo* and *in vitro* (17). Furthermore, the binding activity of L-selectin for ligand increases rapidly following lymphocyte activation through the T cell receptor (CD3) complex or neutrophil activation with cytokines (10). Since cross-linking CD3 activates PKC (30) a role for PKC-mediated phosphorylation in L-selectin-dependent binding is possible. Unfortunately, it is not feasible to transfect normal leukocytes with L-selectin cDNAs lacking the cytoplasmic serine residues to test this directly. Likewise, it has not been possible to demonstrate up-regulated L-selectin binding activity in RBL cells or lymphoblastoid cell lines.[2] Therefore, the effects of blocking receptor phosphorylation on up-regulated L-selectin binding activity were examined indirectly by treating cells with a PKC inhibitor. Treatment of lymphocytes with staurosporine completely inhibited the CD3-mediated increase in L-selectin-dependent binding to HEVs and reduced, but did not eliminate, basal L-selectin binding (Fig. 6). Since leukocyte binding to HEVs, both basal and up-regulated, is completely inhibited by blocking L-selectin function (10) and chemoattractant receptor-induced adhesion through integrins is not PKC-dependent (31), the current results suggest that rapid PKC-mediated phosphorylation of L-selectin may up-regulate its binding activity *in vivo*.

The effects of receptor phosphorylation on basal L-selectin binding activity were examined directly using cDNA-transfected 300.19 cells. The L-SS/AA-modified L-selectin did not significantly affect 300.19 cell binding to HEVs of peripheral lymph nodes (L-selectin, 7.8 ± 0.8 cells/HEV; L-SS/AA, 6.3 ± 1.2 cells/HEV; $p < 0.1$) in four experiments. By contrast, the LΔG-S mutation (0.6 ± 0.3 cells/HEV) was similar to that of LΔcyto (1.2 ± 1.0 cells/HEV; $p < 0.0001$ *versus* L-selectin) and showed very weak if any binding to HEVs. Therefore, although phosphorylation was not necessary for basal adhesion through L-selectin, the region containing the serine residues within the cytoplasmic domain was required.

## DISCUSSION

The finding that L-selectin was rapidly phosphorylated in lymphoblastoid cell lines (Fig. 1) and transfected RBL cells (Figs. 3 and 4) immediately following chemoattractant or PMA stimulation suggests a physiologically relevant role for L-selectin phosphorylation in the inflammatory response. Indeed, signaling of RBL cells through endogenous thrombin and IgE receptors or ectopic receptors for fMLP, C5a, IL-8, and PAF all induced L-selectin phosphorylation (Figs. 3 and 4). That all of these receptors activate PKC-dependent pathways, that L-selectin phosphorylation was completely blocked by a PKC inhibitor (Fig. 4*B*), and that L-selectin was phosphorylated on serine residues (Fig. 5) suggest that L-selectin may be phosphorylated directly by PKC. Although phosphorylation of L-selectin in human neutrophils freshly isolated from blood could not be demonstrated, this is likely to result from the fact that L-selectin from neutrophils is heavily glycosylated and runs as a diffuse and broad band when analyzed by SDS-PAGE (22, 32). Also, metabolic labeling of these terminally differentiated cells is inefficient, and endoproteolytic release of L-selectin from neutrophils is not completely inhibited in the presence of protease inhibitors (18). These factors in combination are likely to explain the previous inability to demonstrate L-selectin phosphorylation in native leukocytes by us and others (21–24). Nonetheless, a requirement for L-selectin phosphorylation may explain why the amino acid sequence of the cytoplasmic domain of L-selectin has been highly conserved during recent mammalian evolution (particularly the serine residues) (2).

Phosphorylation of L-selectin occurs within seconds following cell exposure to fMLP (Fig. 4*C*), which is consistent with an *in vivo* role for L-selectin in leukocyte-endothelium interactions. The ligand binding activity of L-selectin is also rapidly up-regulated within seconds following lymphocyte activation or leukocyte stimulation with pro-inflammatory agents that activate PKC (10). The finding that activation-induced up-regulation of L-selectin adhesive function is staurosporine-sensitive (Fig. 5) suggests that these two events are likely to be related. L-selectin phosphorylation was not required for basal adhesion through this receptor since staurosporine does not eliminate basal adhesion of lymphocytes to HEVs (Fig. 6), and replacement of the serine residues in L-selectin did not inhibit its ability to bind to HEVs. However, deletion of the cytoplasmic region containing the serine residues or the entire cytoplasmic domain eliminated all adhesion. This suggests that this region of L-selectin may mediate intermolecular associations critical for L-selectin function. The functional outcome of these intermolecular interactions could be regulated by phosphorylation of L-selectin in native leukocytes but not in transfected cells. Alternatively, PKC-dependent phosphorylation of other proteins could indirectly affect L-selectin-dependent adhesion and lead to its enhanced adhesive function following leukocyte activation.

Phosphorylation and up-regulated binding activity of L-selectin are both induced more rapidly than endoproteolytic release of L-selectin from the cell surface (10). Consistent with this, phosphorylation of L-selectin on serine residues is not required for endoproteolytic release of L-selectin since the elimination of serine residues within the cytoplasmic domain of L-selectin did not affect receptor cleavage. Whether serine

[2] B. Haribabu, D. A. Steeber, H. Ali, R. M. Richardson, R. Snyderman, and T. F. Tedder, unpublished observations.

phosphorylation of L-selectin is required for signal transduction through L-selectin following its ligation remains an open issue. Recent studies have demonstrated rapid tyrosine phosphorylation of L-selectin following cross-linking by antibodies (33). However, ligation of L-selectin through conserved ligand-binding regions within the lectin domain induces rapid and potent intercellular adhesion in human, mouse, and rat leukocytes that is also induced in cell lines expressing L-selectin lacking the cytoplasmic serine residues (L-SS/AA) or the cytoplasmic tyrosine residue (34). Although it is not currently feasible to express mutant L-selectin molecules in primary leukocytes to assess the biological significance of L-selectin phosphorylation *in vivo*, the current studies provide a rationale for further studies examining this issue.

The finding that L-selectin phosphorylation was inhibited by both a PKC inhibitor and pertussis toxin suggests that L-selectin phosphorylation may be one of the rapid G-protein-regulated activation events involved in leukocyte interactions with vascular endothelium under physiologic flow conditions (35, 36). In current models of leukocyte recruitment to inflammatory sites, initial interactions between selectins and their ligands result in rolling followed by chemoattractant-mediated integrin activation leading to firm adhesion (3, 4, 35). Our results suggest that activation of chemoattractant receptors induces L-selectin phosphorylation through PKC-dependent pathways. Phosphorylation of L-selectin may induce transient changes in L-selectin binding activity that may contribute directly to leukocyte interactions with endothelial cells and account in part for lineage-specific differences in leukocyte migration (10).

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REFERENCES

1. Ley, K., and Tedder, T. F. (1995) *J. Immunol.* **155,** 525–528
2. Tedder, T. F., Steeber, D. A., Chen, A., and Engel, P. (1995) *FASEB J.* **9,** 866–873
3. Butcher, E. C. (1993) *Res. Immunol.* **144,** 695–698
4. Springer, T. A. (1995) *Annu. Rev. Physiol.* **57,** 827–872
5. Murphy, P. M. (1994) *Annu. Rev. Immunol.* **12,** 593–633
6. Snyderman, R., and Uhing, R. J. (1992) in *Inflammation: Basic Principles and Clinical Correlates* (Gallin, J. I., Goldstein, I. M., and Snyderman, R., eds) pp. 421–439, Raven Press Ltd., New York
7. Ben-Baruch, A., Michiel, D. F., and Oppenheim, J. J. (1995) *J. Biol. Chem.* **270,** 11703–11706
8. Gerard, G., and Gerard, N. P. (1994) *Curr. Opin. Immunol.* **6,** 140–145
9. Bokoch, G. M. (1995) *Blood* **86,** 1649–1660
10. Spertini, O., Kansas, G. S., Munro, J. M., Griffin, J. D., and Tedder, T. F. (1991) *Nature* **349,** 691–694
11. Ali, H., Richardson, R. M., Tomhave, E. D., Didsbury, J. R., and Snyderman, R. (1993) *J. Biol. Chem.* **268,** 24247–24254
12. Ali, H., Richardson, R. M., Tomhave, E. D., DuBose, R. A., Haribabu, B., and Snyderman, R. (1994) *J. Biol. Chem.* **269,** 24557–24563
13. Richardson, R. M., DuBose, R. A., Ali, H., Tomhave, E. D., Haribabu, B., and Snyderman, R. (1995) *Biochemistry* **34,** 14193–14201
14. Tedder, T. F., Ernst, T. J., Demetri, G. D., Isaacs, C. M., Adler, D. A., and Disteche, C. M. (1989) *J. Exp. Med.* **170,** 123–133
15. Chen, A., Engel, P., and Tedder, T. F. (1995) *J. Exp. Med.* **182,** 519–530
16. Tedder, T. F., Penta, A. C., Levine, H. B., and Freedman, A. S. (1990) *J. Immunol.* **144,** 532–540
17. Kansas, G. S., Ley, K., Munro, J. M., and Tedder, T. F. (1993) *J. Exp. Med.* **177,** 833–838
18. Allport, J. R., Ding, H. T., Ager, A., Steeber, D. A., Tedder, T. F., and Luscinskas, F. W. (1997) *J. Immunol.*, in press
19. Preece, G., Murphy, G., and Ager, A. (1996) *J. Biol. Chem.* **271,** 11634–11640
20. Butcher, E. C., Scollay, R. G., and Weissman, I. L. (1979) *J. Immunol.* **123,** 1996–2003
21. Kishimoto, T. K., Julita, M. A., Berg, E. L., and Butcher, E. C. (1989) *Science* **245,** 1238–1241
22. Griffin, J. D., Spertini, O., Ernst, T. J., Belvin, M. P., Levine, H. B., Kanakura, Y., and Tedder, T. F. (1990) *J. Immunol.* **145,** 576–584
23. Jung, T. M., and Dailey, M. O. (1990) *J. Immunol.* **144,** 3130–3136
24. Spertini, O., Freedman, A. S., Belvin, M. P., Penta, A. C., Griffin, J. D., and Tedder, T. F. (1991) *Leukemia* (*Baltimore*) **5,** 300–308
25. Feehan, C., Darlak, K., Kahn, J., Walcheck, B., Spatola, A. F., and Kishimoto, T. K. (1996) *J. Biol. Chem.* **271,** 7019–7024
26. Yednock, T. A., Butcher, E. C., Stoolman, L. M., and Rosen, S. D. (1987) *J. Cell Biol.* **104,** 725–731
27. Richardson, R. M., Haribabu, B., Ali, H., and Snyderman, R. (1996) *J. Biol. Chem.* **271,** 28717–28724
28. Watson, S. P., McNally, J., Shipman, L. J., and Godfrey, P. P. (1988) *Biochem. J.* **249,** 345–350
29. Gilman, A. G. (1989) *J. Am. Med. Assoc.* **262,** 1819–1825
30. Manger, B., Weiss, A., Imboden, J., Laing, T., and Stobo, J. D. (1987) *J. Immunol.* **139,** 2755–2760
31. Laudanna, C., Campbell, J. J., and Butcher, E. C. (1996) *Science* **271,** 981–983
32. Tedder, T. F., Matsuyama, T., Rothstein, D. M., Schlossman, S. F., and Morimoto, C. (1990) *Eur. J. Immunol.* **20,** 1351–1355
33. Brenner, B., Gulbins, E., Schlottmann, K., Koppenhoefer, U., Busch, G. L., Walzog, B., Steinhausen, M., Coggeshall, K. M., Linderkamp, O., and Lang, F. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 15376–15381
34. Steeber, D. A., Engel, P., Miller, A. S., Sheetz, M. P., and Tedder, T. F. (1997) *J. Immunol.*, in press
35. Bargatze, R. F., and Butcher, E. C. (1993) *J. Exp. Med.* **178,** 367–372
36. Bjerknes, M., Cheng, H., and Ottaway, C. A. (1986) *Science* **231,** 402–405