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.

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 (RBL1 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 (PKC)-dependent pathway.

**EXPERIMENTAL PROCEDURES**

*Immunofluorescence Regants and Analysis—Antibodies used in these studies included: mouse LAM1–116 (IgG2a) and LAM1–110 (IgG1) mAbs that react with human, mouse, and rat L-selectin (34); anti-CD83 mAb (HB15A IgG2b); 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 polychromosomenoester 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 LAM-N cDNA by electroporation, and clones were isolated as described (13, 15). 300.19 cells transfected with human cDNA for either L-selectin or Lαcyt o 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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Phosphorylation of L-selectin

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 (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.

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 (LAM-N) (15) that is fully functional but not endoproteolytically released from the cell surface (Fig. 2A). RBL cells did not express endogenous L-selectin as determined using two mAbs reactive with rat L-selectin (LAM1–116 and LAM1–110, Fig. 2A). Human L-selectin expressed in RBL cells was functionally and structurally intact since it retained the ability to specifically bind polyphosphonomoester core polysaccharide, which mimics the natural ligand of L-selectin (Fig. 2B) (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. 2C). 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. 3A, lanes 1 and 2). In contrast, PMA treatment of untransfected RBL cells did not induce phosphorylation of proteins in this size range (Fig. 3A, lane 4).

Chemoattractant Receptors Induce L-selectin Phosphorylation—RBL cells that expressed the LAM-N form of L-selectin, as well as native thrombin and IgE receptors, were also transfected with cDNA encoding epitope-tagged human receptors for...
formyl peptides (fMLP), a peptide component of complement activation (C5aR), interleukin-8 (IL-8R), or platelet-activating factor (PAF). 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. 4A). 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. 4A). 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 LAM-N form of L-selectin, while phosphorylated L-selectin was not immunoprecipitated from cells expressing the LAM-N form of L-selectin with the 2 serine residues in the cytoplasmic tail replaced with alanine residues (LAMN-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. 4B, lanes 3 and 5). Pertussis toxin, an inhibitor of signaling through Gi 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. 4B, lane 6). L-selectin phosphorylation was detected at the earliest measurable time point of 7 s following fMLPR-induced activation of RBL cells (Fig. 4C). 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 LAM-N form of L-selectin was retained (data not shown). To determine whether phosphorylation of L-selectin
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 (L-DG-S) deleted, or the entire cytoplasmic tail (L Acetyl) 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 (Fig. 6), and replacement of the serine residues in L-selectin did not inhibit its binding activity 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

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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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Chemoattractant Receptor-induced Phosphorylation of L-selectin
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