L-Selectin Shedding Is Independent of Its Subsurface Structures and Topographic Distribution

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L-selectin (CD62L), a lectin-like adhesion molecule, mediates lymphocyte homing and leukocyte accumulation at sites of inflammation. Its transmembrane (TM) and intracellular (IC) domains confer clustering of L-selectin on microvilli of resting leukocytes, which is important for L-selectin function. Following activation of protein kinase C (PKC) or calmodulin inhibition, the wild-type (WT) protein is rapidly cleaved in its membrane-proximal ectodomain. To examine whether L-selectin topography or TM/IC domains are involved in this shedding process, we used stable transfectants expressing WT L-selectin (on microvilli) or chimeric molecules consisting of the L-selectin ectodomain linked to the TM/IC domains of CD44 (excluded from microvilli) or CD31 (randomly distributed). PKC activation by PMA altered the cells’ surface morphology, but did not induce a redistribution of L-selectin ectodomains. All cell lines shed ectodomains upon PMA activation in a dose-dependent fashion and with similar kinetics. Calmodulin inhibition by trifluoperazine induced shedding in both WT and chimera transfectants. At high trifluoperazine concentrations, shedding of WT L-selectin was significantly more pronounced than that of chimeric molecules. Regardless of the activating stimulus, shedding was blocked by a hydroxamate-based metalloprotease inhibitor, suggesting that ectodomain downregulation occurred through proteolytic cleavage by identical protease(s). These results show that the recognition site(s) for PKC-induced L-selectin shedding is exclusively contained within the ectodomain; the nature of subsurface structures and surface topography are irrelevant. Shedding induced by calmodulin inhibition has two components: one requires the L-selectin TM/IC domain, and the other is independent of it. The Journal of Immunology, 2001, 167: 3642–3651.

L-leukocyte recruitment from the blood into tissues is mediated by multistep cascades that require the sequential engagement of several distinct surface receptors on leukocytes and endothelial cells (1–3). Most adhesion cascades are initiated by one or more members of the selectin family, which mediate initial contact (tethering) and rolling of leukocytes in post-capillary venules (4, 5). L-selectin (CD62L), a leukocyte surface adhesion molecule, is essential for leukocyte recruitment to many sites of inflammation and for homing of naive lymphocytes to peripheral lymph nodes (6–9). This glycoprotein has a distinctive ectodomain consisting of an N-terminal C-type lectin domain, an epidermal growth factor-like domain, and two short consensus repeats. The ectodomain is followed by a single transmembrane (TM) domain and a short (17-aa) C-terminal intracellular (IC) domain (10–13).

L-selectin mediates leukocyte tethering and rolling through binding to ligands on vascular endothelial cells in the presence of hydrodynamic shear (6, 7). It is constitutively expressed on most leukocytes (except a subset of effector/memory cells), including granulocytes, monocytes, and lymphocytes (reviewed in Ref. 4). The exquisite ability of L-selectin to initiate cell-cell contact in the presence of strong hydrodynamic shear is facilitated by its surface topography (14, 15); L-selectin is clustered on the tips of microvillus surface protrusions on neutrophils and lymphocytes (16, 17). This topography is maintained even when L-selectin cDNA is expressed in cells that are normally devoid of L-selectin (14, 18, 19).

Previous studies have established the importance of microvillous topography for leukocyte tethering and rolling in vitro and in vivo (14, 15). Transfected lymphoid cells expressing wild-type (WT) L-selectin on microvilli initiated rolling interactions on physiologic ligands in lymph node high endothelial venules much more efficiently than cells that expressed randomly distributed L-selectin ectodomains. In turn, the latter cells rolled more frequently than transfectants expressing L-selectin ectodomains only on the cell body (15).

It has been proposed that the physiologic presentation of L-selectin clusters on the most distal aspects of the cell surface enhances the molecule’s bioavailability during brief collisions between rapidly flowing leukocytes and ligand-bearing endothelial cells (16). Consistent with this concept, other leukocyte adhesion molecules that initiate adhesion to endothelium under flow are similarly concentrated on microvilli. Besides L-selectin, the two α4 integrins, α4β1 and α4β2 (20, 21), the sialomucin P-selectin glycoprotein ligand-1 (22, 23), and E-selectin ligand-1 (24) are all concentrated on microvilli. In contrast, the hemopoietic isoform of CD44 and the β2 integrins LFA-1 (αLβ2) and Mac-1 (αMβ2) are found predominately on the cell body (14, 20, 25). Other leukocyte-expressed molecules, such as the murine T cell marker Thy1 and sialyl-Lewisx, are randomly distributed over the entire cell circumference (16, 26).

When leukocytes become activated, L-selectin is rapidly shed from the cell surface by a zinc-based metalloprotease that cleaves the L-selectin ectodomain at a membrane-proximal site (27–33).
Addition of PMA, which activates the protein kinase C (PKC) pathway, triggers the rapid disappearance of L-selectin from the surface of leukocytes to nearly complete loss of expression within a few minutes (28). Several observations have established that activation-induced down-regulation of L-selectin is mediated by proteolytic cleavage of the ectodomain: the supernatant of activated leukocytes contains increased levels of a protein that reacts with Abs to epitopes on the ectodomain, but not the IC domain of L-selectin (30); conversely, activated neutrophils lose ectodomain epitopes, but maintain a 6-kDa protein fragment that is detected by Abs to the IC domain of L-selectin and is not found in resting leukocytes (30); hydroxamate-based inhibitors of zinc metalloproteases block activation-induced L-selectin shedding (34–36); finally, several groups have shown that mutations in the membrane-proximal short consensus repeats can reduce or abolish activation-induced shedding of L-selectin ectodomains (30–33). Indeed, the cleavage site of L-selectin has been located between Lys321 and Ser322, which are predicted to lie within the first 15 amino acids proximal to the cell membrane (30).

To date, the protease(s) responsible for L-selectin cleavage (sheddase) from mature leukocytes remain(s) to be identified. One likely sheddase candidate is TNF-α-converting enzyme (TACE), which has been shown to mediate activation-induced L-selectin shedding from fetal thymocytes (37). Interestingly, a recent study has shown that TACE has a punctate distribution on the surface of human THP-1 and Jurkat cells (38). This staining pattern resembles that seen on human leukocytes stained for L-selectin (25).

Thus, we speculated that the extremely fast kinetics of L-selectin shedding could be facilitated by colocalization of the sheddase and its substrate on the tips of microvilli. However, this possibility has not been explored experimentally. Moreover, the extra- and intracellular factors that control sheddase activity are poorly understood. It has been shown that a partial truncation of L-selectin, called LΔcyto, in which 11 of the 17 predicted cytoplasmic amino acids were deleted, does not affect microvillar presentation or PMA-induced shedding of the ectodomain (19, 31). In contrast, as mentioned above, replacement of the entire TM/IC region alters L-selectin topography (14), indicating that critical L-selectin-specific recognition motifs must be contained within the TM domain or the juxtamembrane cytoplasmic stump of the LΔcyto mutant. Whether these regions are also involved in regulating shedding has been unknown.

One recent study has demonstrated that calmodulin, a calcium regulatory protein, binds directly to the IC domain of L-selectin (39). When leukocytes were treated with trifluoperazine (TFP) or other calmodulin inhibitors, proteolytic shedding of L-selectin was observed. Thus, it was postulated that calmodulin may regulate the expression of L-selectin by interacting with its IC domain (39). Calmodulin binding to L-selectin was abrogated upon mutation of either one of two amino acids within the membrane-proximal cytoplasmic region that was not deleted in the LΔcyto construct (39). Together, these observations indicate that the C-terminal 11 amino acids of the L-selectin IC domain are not required for shedding, but a role for the remainder of the IC domain or the TM region cannot be excluded. Furthermore, it has not been tested whether shedding is altered by mutations in L-selectin that abolish calmodulin binding.

To clarify the cellular mechanisms of L-selectin shedding, we set out to determine whether the TM/IC fragment of L-selectin plays a role in this process. Using transfectants that expressed full-length L-selectin ectodomains with different TM/IC domains, we show that significant differences in the surface topography of chimeric proteins, all transfected cell lines shed L-selectin efficiently upon activation with PMA or inhibition of calmodulin activity. Scanning immunoelectron microscopy of activated cells revealed distinct changes in the appearance of cell surface protrusions, but the distribution of ectodomains was not significantly altered compared with that of resting cells. Loss of L-selectin ectodomains correlated with the presence of soluble L-selectin (sL-selectin) in culture supernatants, and ectodomain cleavage in WT and mutant transfectants was blocked by a hydroxamate-based protease inhibitor, suggesting that a similar protease(s) cleaves L-selectin regardless of ectodomain distribution or subsurface structures. Thus, although a subtle role for intracellular calmodulin binding to L-selectin was evident, subsurface domains are not required for activation-induced L-selectin shedding from leukocytes.

Materials and Methods

Abs and reagents

The mAb DREG-200 (mouse IgG1), which recognizes an epitope in the ectodomain of human L-selectin (29), was purified from culture supernatants following standard procedures. CFSE-labeled DREG-200 monovalent Fab were prepared as previously described (6) and used for flow cytometry. Whole IgG was used for immunoelectron microscopy. Anti-L-selectin mAb DREG-55, biotinylated DREG-200, and the hydroxamic acid-based metalloprotease inhibitor, KD-IX-73-4, were provided by Dr. T. K. Kishimoto (Boehringer Ingelheim, Ridgefield, CT). Colloidal gold (12 nm)-conjugated goat anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Streptavidin-HRP and 2,2′-azino-di-3-ethylbenzthiazolesulfonic acid (ABTS) were purchased from Zymed (San Francisco, CA). TFP was obtained from Calbiochem (San Diego, CA), and PMA was obtained from Sigma (St. Louis, MO).

Preparation of cells

We used murine L1-2 pre-B lymphoma cell lines that were stably transfected with human WT L-selectin (40) or with chimeric molecules consisting of the human L-selectin ectodomain linked to the TM/IC segment of CD44 (L/CDCD44) (14) or the TM/IC segment of CD31 (L/CDCD31) (15). In the two chimeric cell lines, the last amino acid (Pro173) of the predicted ectodomain of mature L-selectin was linked to the first amino acid of the predicted TM domains of human CD44H (Trp238) and human CD31 (Gly235), respectively. A parental cell line transfected with the pMRB101 expression vector alone (L1-2vector) served as a negative control. All L1-2 transfectants were cultured in six-well tissue culture plates (BD Biosciences, Franklin Lakes, NJ) in RPMI 1640 medium (BioWhittaker, Walkersville, MD) containing standard supplements, 2.5 μg/ml mycophenolic acid (Sigma), 125 μg/ml xanthine (Sigma), 1× hypoxanthine/thymidine (Life Technologies, Grand Island, NY), and 1.5 mM HEPES (pH 6.5). For experiments, cells were washed and counted, and dead cells were removed, if necessary, by centrifugation over Histopaque 1077 (Sigma). Subsequently, cells were resuspended to 1×10⁷ cells/ml in the same medium and used within 1 h for experiments.

FACS analysis

Expression of WT and chimeric L-selectin ectodomains on L1-2 cells was assessed before and after activation with PMA. Cell samples (1×10⁶/ml in RPMI 1640 (BioWhittaker) containing 10% FBS (Gemini Bioproducts, Calabasas, CA) and 20 μg/ml CFSE-DREG-200 Fab were incubated for 20 min at room temperature or 4°C, washed twice, and analyzed on a FACScan flow cytometer (BD Biosciences). Control experiments showed that incubation with CFSE-DREG-200 Fab (up to 1 h at 37°C) did not alter ectodomain expression on any transfectant used in this study (data not shown). FACS results were expressed as specific mean fluorescence channel number (MFI) of 5000 cells after subtracting the background MFI, which was determined by parallel staining of L1-2vector cells. For some experiments, specific MFI was converted to mean copy number of WT and chimeric ectodomains on transfectants using a calibrated microbead system (Quantum Simply Cellular, Flow Cytometry Standards, San Juan, Puerto Rico) following the manufacturer’s instructions.

Low voltage scanning electron microscopy (LVSEM)

Transfectants were stained with anti-L-selectin ectodomain mAb followed by colloidal gold-conjugated goat anti-mouse IgG and prepared for analysis of surface features and colloidal gold distribution by LVSEM as previously described (14, 17). WT L-selectin on L1-2 cells has been shown.

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Previously to be clustered on the tips of microvilli (14, 17, 18), whereas L-CD44 is preferentially targeted to the planar cell body (14), and L/CD31 has a random topography with equivalent expression density on microvilli and the cell body (Ref. 15 and this report). L-selectin ectodomain topography on each nonactivated cell line was confirmed for this study. The same cell populations were also prepared for LVSEM analysis after exposure to PMA (10^{-6} M at 37°C) for 1 and 3 min. Gold particle distribution was determined by analysis of digital images taken with a Hitachi S-900 LVSEM (Hitachi, Tokyo, Japan). Secondary electrons were collected by an Everhart-Thornley detector, and an Autrata modified YAG detector was used for backscatter electron detection. Gold particle distribution was assessed by blinded observers on individual cells photographed at 60,000-fold magnification based on the following criteria: microvilli were classified as any identifiable projection emanating from the cell surface; to maintain uniform criteria for comparison of differently treated cell samples, other surface protrusions, such as ridges, or ruffles, which were frequently seen on cells after activation with PMA, were also classified as microvilli; microvilli were further divided into upper and lower halves based on visual estimation of the midpoint between microvillous tip and base. Regions that were not part of microvilli were designated as any identifiable projection emanating from the cell surface; to maintain uniform criteria for comparison of differently treated cell samples, other surface protrusions, such as ridges, or ruffles, which were frequently seen on cells after activation with PMA, were also classified as microvilli; microvilli were further divided into upper and lower halves based on visual estimation of the midpoint between microvillous tip and base. Regions that were not part of microvilli were designated as cell body associated.

**PMA-induced activation of L1-2 transfectants**

For dose-response studies, cell samples were pretreated at 4°C with 20 µg/ml CFSE DREG-200 Fab. A stock of 1 mM PMA was serially diluted in RPMI 1640 with 10% FBS, and 10 µl of dilution was pipetted into 96-well round-bottom tissue culture plates. Ninety microtiter plates of pre-warmed (37°C) transfectant suspension at 5 x 10^5 cell/ml was then added to each well. Samples were incubated for 15 min at 37°C, washed, fixed with 1% paraformaldehyde, and analyzed on a FACSscan flow cytometer as described above.

For kinetic studies, transfectants were incubated at 37°C in the presence of 20 µg/ml CFSE DREG-200 Fab. PMA was added to a final concentration of 10^{-6} M. Aliquots of cells were taken three times before and at nine consecutive time points after activation. Further activation of sampled cells was stopped by immediate transfer into an equal volume of ice-cold RPMI 1640 containing 10% FBS and 2% paraformaldehyde. Fixed samples were analyzed by flow cytometry as described above.

**L-selectin ELISA**

Ninety-six-well, flat-bottom microtitration plates (Linbro/Titer-Tek, ICN Pharmaceuticals, Costa Mesa, CA) were coated with 10 µg/ml mAb DREG-55 and blocked with 2% BSA in PBS. Samples of each cell line were washed and resuspended to 1 x 10^5 cells/ml in RPMI 1640 and 10% FBS. Immediately thereafter, 100 µl medium containing 10^{-5} M PMA and 10% DMSO was added to 900 µl cell suspension. Medium with DMSO alone was added to control samples. Cells were stimulated for 15 min at 37°C and then pelleted. Supernatants were added to triplicate wells of MAb DREG-55 coated plates (30 min at 37°C). After washing three times, plates were incubated with biotinylated DREG-200 (1 µg/ml). Streptavidin-coupled HRP and ABTS were added for development. Plates were incubated at room temperature for 15 min and read at 414 nm in a Titers-Tek Multiscan ELISA plate reader (Flow Laboratories, McLean, VA). Each assay included a standard dilution of a human standard human serum sample containing 1.6 µg/ml L-selectin (gift from Dr. T. K. Kishimoto, Boehringer Ingelheim) to generate a standard curve. OD_{414} measurements from supernatants of non-activated transfectants were subtracted from those of activated cells, and the resulting value was converted into activation-induced ectodomain protein concentration. To correlate the amount of L-selectin in supernatant to the loss of surface-expressed protein, aliquots of the same samples of activated and nonactivated cells were simultaneously analyzed by flow cytometry to assess the mean number of shed ectodomains per cell as described above.

**Inhibition of zinc-based metalloproteases**

The hydroxamate-based metalloprotease inhibitor KD-IX-73-4 was diluted as any identifiable projection emanating from the cell surface; to maintain uniform criteria for comparison of differently treated cell samples, other surface protrusions, such as ridges, or ruffles, which were frequently seen on cells after activation with PMA, were also classified as microvilli; microvilli were further divided into upper and lower halves based on visual estimation of the midpoint between microvillous tip and base. Regions that were not part of microvilli were designated as cell body associated.

**Calmodulin inhibition**

Cells were washed, resuspended to 10^6/ml, and prestained with CFSE DREG-200 F(ab). TFP was diluted in DMSO as a 20-mM stock solution. For experiments, TFP was serially diluted and combined with cell samples to final concentrations of 25, 50, 75, and 100 µM. Aliquots of each cell line were incubated with TFP or with an equivalent amount of DMSO for 15 min at 37°C and then fixed. A second set of cells was treated with TFP in the presence of KD-IX-73-4 (50 µg/ml). Cells were analyzed by flow cytometry as described above.

**Results**

We have previously described a panel of stably transfected murine L1-2 pre-B cell lines that express the ectodomain of human L-selectin linked to the predicted TM/IC segment of WT L-selectin or CD44 or CD31 (14, 15). The different subsurface segments target ectodomains either to microvilli (WT) or the planar cell body (L/CD44) or cause a random distribution (L/CD31). These different topographies markedly affect the cells’ ability to engage with L-selectin ligands under conditions of flow, but they have no measurable impact on L-selectin-mediated rolling velocity after the cells have become tethered (14, 15). Because the velocity of L-selectin-mediated rolling is partly determined by proteolytic cleavage of the ectodomain (41, 42), the possibility remained that topographic effects on rolling velocity may have been masked by differential shedding. Moreover, it has not been determined whether L-selectin shedding requires any elements within the native TM/IC domain of L-selectin. To address these questions, shedding was induced by exposure of L1-2 cells to PMA, a potent activator of PKC. Previous studies have shown that PKC activation by PMA is an effective stimulus for L-selectin shedding for both primary leukocytes (28) and transfected cell lines, including the L1-2 cells used here (33) (U. H. von Andrian, unpublished observations).

**PMA induces dose-dependent shedding of L-selectin ectodomains from L1-2 cells independent of TM/IC domains**

Flow cytometric analysis of anti-human L-selectin mAb DREG-200 binding confirmed that L1-2 vectors cells exhibit very low fluorescence that was not different from cells stained with a nonbinding control mAb and remained unchanged after exposure to PMA (data not shown). Therefore, L1-2 vectors cells were used as a negative control to assess background fluorescence. Subclones of WT L-selectin, L/CD44, and L/CD31 transfectants were selected that expressed reproducibly high levels of L-selectin ectodomains. In all cases, ectodomains disappeared from the cell surface upon exposure to 10^{-6} M PMA for 15 min (Table I and Fig. 1A). Similar results were obtained with multiple subclones of each transfected cell line as well as with the parental polyclonal populations (not shown). Thus, it seems unlikely that the results described below were due to unique properties of individual subclones. At the relatively high PMA concentration chosen initially, all transfectants consistently lost >70% of their L-selectin ectodomains, indicating that TM/IC domains, and hence topography, are not essential to the shedding process. However, this finding alone did not exclude a more subtle role for the TM/IC domains that might affect the kinetics or sensitivity to submaximal stimulation. Therefore, we performed additional experiments to address this question.

To determine whether TM/IC domains affect the sensitivity of ectodomain shedding to mild stimulation, WT L-selectin, L/CD44, and L/CD31 transfectants were activated with serial dilutions of PMA. Ectodomain shedding was apparent in all three cell lines after exposure to PMA at concentrations as low as 2 x 10^{-9} M.
L-selectin down-regulation reached a maximum in each cell line at 4 × 10⁻⁶ M.

**Similar kinetics of L-selectin down-regulation after PMA activation**

To investigate the kinetics of PMA-induced down-regulation of L-selectin ectodomains, we exposed each of the three transfectants to PMA (10⁻⁶ M) and stopped further activation at various time points by rapid fixation in ice-cold buffer. Fig. 2 shows the time course of L-selectin down-regulation as evidenced by the loss of mAb DREG-200 binding sites. All cell lines lost >50% of their L-selectin ectodomains within 5 min. At 10 min after addition of PMA nearly 80% of surface-expressed mAb DREG-200 binding sites had been lost, on the average. Somewhat slower kinetics of shedding were observed in WT transfectants than in the two chimera transfectants. This was probably due to the fact that WT transfectants expressed fewer ectodomains before activation, which may have resulted in more limited availability of substrate for the enzyme(s) mediating proteolytic cleavage (Table I). Consistent with this idea, the time required for half-maximum ectodomain shedding was similar for each cell line (2.8 min for L/CD31, 3 min for L/CD44, and 3.8 min for WT L-selectin transfectants).

**WT and chimeric L-selectin ectodomains are released into the culture supernatant**

Having determined that all three transfectants lost surface-expressed L-selectin epitopes with equivalent sensitivity and similar kinetics upon activation, we asked whether this effect involved the same mechanism(s). To test whether ectodomains were shed by proteolytic cleavage, we measured sL-selectin in supernatants of activated and nonactivated transfectants using an ELISA (32). At the same time, the average number of shed L-selectin ectodomains was determined by FACS analysis of cell aliquots before and after activation.

**Table 1. L-selectin ectodomain shedding before and after PMA treatment (10⁻⁶ M, 15 min at 37°C) in WT L-selectin, L/CD44, and L/CD31 transfectant**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>MFI DREG-200 Binding Sites (no./cell)</th>
<th>L-Selectin Shedding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control + PMA</td>
<td>Control + PMA</td>
</tr>
<tr>
<td>WT L-sel.</td>
<td>46.3 ± 13.5</td>
<td>13.8 ± 5.4</td>
</tr>
<tr>
<td>L/CD31</td>
<td>76.1 ± 16.8</td>
<td>16.5 ± 7.4</td>
</tr>
<tr>
<td>L/CD44</td>
<td>78.6 ± 13.4</td>
<td>18.3 ± 6.5</td>
</tr>
<tr>
<td></td>
<td>112,500 ± 18,890</td>
<td>21,590 ± 8,709</td>
</tr>
<tr>
<td></td>
<td>222,000 ± 38,110</td>
<td>40,040 ± 6,055</td>
</tr>
<tr>
<td></td>
<td>171,100 ± 26,330</td>
<td>30,840 ± 6,234</td>
</tr>
</tbody>
</table>

*L1-2vector cells were used as a negative control to assess background fluorescence in all experiments. Flow cytometer settings were adjusted to yield the MFI in FL-1 for FITC-DREG-200-stained L1-2vector cells as ~4.0 using a logarithmic acquisition scale. DREG-200 binding sites were determined using a standardized microbead system (Quantum Simply Cellular). Data are shown as mean ± SEM of three independent experiments.

**FIGURE 1.** L-selectin ectodomain expression on L1-2 cells transfected with WT L-selectin, L/CD44, or L/CD31 before and after activation. A, Overlays of representative flow cytometry histograms of L1-2 transfectants stained with CFSE-conjugated anti-L-selectin mAb DREG-200 Fab before (thick solid line) and after (thin solid line) activation with 10⁻⁶ M PMA (15 min at 37°C). Histograms for L1-2vector cells (dotted line) that were stained in parallel are shown for comparison. B, Dose-response relationship of PMA-induced L-selectin shedding in WT L-selectin, L/CD44, and L/CD31 transfectants. Cells were incubated for 15 min at 37°C with different concentrations of PMA, and L-selectin ectodomain expression was assessed by FACS analysis. Data depict the change in MFI as a percentage of MFI of mock-treated control aliquots of each cell line. Data are shown as the mean ± SD (n = 3).

**FIGURE 2.** Kinetics of PMA-induced L-selectin ectodomain down-regulation of WT L-selectin, L/CD44, and L/CD31 transfectants after PMA activation. PMA (10⁻⁶ M) was added to prewarmed (37°C) cell suspensions in the presence of 20 μg/ml CFSE-DREG 200 Fab. Aliquots were removed before (0 min) and at various time points after activation and immediately transferred to ice-cold buffer containing 2% paraformaldehyde. Data are shown as the mean ± SD of the change in MFI expressed as the percentage of MFI in unactivated control aliquots (n = 7 experiments/group).
various concentrations of TFP. Shedding among cells containing tin. To address this question, we treated L1-2 transfectants with cleavage. Alternatively, calmodulin inhibition might induce shedding of L-selectin without causing cell activation (39). One pos-(39). Calmodulin inhibitors, such as TFP, induce proteolytic shedd-previously shown to bind directly to the IC domain of L-selectin
Calmodulin, an intracellular calcium regulatory protein, has been inhibition of calmodulin induces L-selectin ectodomain shedding that is partially independent of the TM/IC domain
Calmodulin, an intracellular calcium selectin ectodomain down-regulation assessed by flow cytometry, indicating that the activation-induced loss of L-selectin epitopes from the cell surface was in each case due to release of ectodomains and not to other mechanisms, such as internalization or masking of surface epitopes.

**Figure 3.** sL-selectin in supernatant correlates with PMA-induced L-selectin ectodomain down-regulation on WT L-selectin, L/CD44, and L/CD31 transfectants. Multiple transfected subclones expressing different baseline levels of WT and chimeric L-selectin (each identified by a differ-ent symbol) were maximally stimulated with PMA and sL-selectin was quantified in the supernatant using an ELISA as described in Materials and Methods. An sL-selectin standard curve (based on a human serum standard) was used to determine the concentration of sL-selectin in supernatants after subtraction of background levels from nonactivated samples. The average number of down-regulated L-selectin ectodomains per cell was determined by concurrent FACS analysis.

**Figure 4.** Inhibition of L-selectin cleavage in WT L-selectin, L/CD44, and L/CD31 transfectants by the metalloprotease inhibitor KD-IX-73-4. All samples were activated with PMA (10^-6 M) for 15 min. Subsequently, KD-IX-73-4 was removed from one aliquot, and these cells were again activated with PMA. As a control, some cells were treated with the inhibitor without addition of PMA. Bars represent the mean ± SD (n = 3) of MFI values expressed as percentage of the MFI of control cells treated with DMSO.

**Figure 5.** Effect of calmodulin inhibition by TFP on the expression of L-selectin ectodomains on L1-2 transfectants. Transfectants expressing WT L-selectin (triangles), L/CD44 (circles), or L/CD31 (squares) were treated with increasing concentrations of TFP in the presence (open symbols) or the absence (filled symbols) of the metalloprotease inhibitor KD-IX-73-4. Cells were fixed after 10 min, and L-selectin ectodomain expression was assessed by flow cytometry. At 50 μM TFP, all cells showed a significant loss of ectodomains in each cell line compared with DMSO-treated controls (p < 0.05 vs sham treated) upon exposure to 50 μM TFP (Fig. 5). In contrast, at higher TFP concentrations (75 and 100 μM), WT L-selectin shedding was significantly more complete differing TM/IC domains was readily detectable and occurred to a similar degree (p < 0.05 vs sham treated) upon exposure to 50 μM TFP (Fig. 5). In contrast, at higher TFP concentrations (75 and 100 μM), WT L-selectin shedding was significantly more complete

To determine whether the mechanisms of ectodomain shedding are similar for WT and chimeric molecules, each transfectant was activated in the presence of the metalloprotease inhibitor KD-IX-73-4 (50 μM). Consistent with previous reports (35, 39, 41), KD-IX-73-4 was highly efficient in blocking shedding of WT L-selectin (Fig. 4). Importantly, KD-IX-73-4 was equally potent in preventing PMA-induced shedding from L/CD44 and L/CD31 transfectants. Without PMA, inhibitor-treated cells showed slightly elevated ectodomain expression compared with untreated cells, perhaps due to inhibition by KD-IX-73-4 of a low constitutive sheddase activity in L1-2 cells. Down-regulation of L-selectin ectodomains following cell activation with PMA was restored after KD-IX-73-4 was thoroughly washed away, indicating that KD-IX-73-4 did not irreversibly alter cell function.

**Inhibition of calmodulin induces L-selectin ectodomain shedding that is partially independent of the TM/IC domain**

calmodulin inhibition might induce shedding independent of its interaction with the IC domain of L-selectin. To address this question, we treated L1-2 transfectants with various concentrations of TFP. Shedding among cells containing
than that of the chimeras. In the presence of KD-IX-73-4, calmodulin inhibition by TFP did not induce significant shedding in any cell line, suggesting that the proteolytic mechanism(s) of L-selectin down-regulation by TFP was closely related to or identical with that induced by PMA.

**Shedding occurs without redistribution of ectodomains following PMA activation**

Although the data presented above clearly show that TM/IC domains are not relevant for PMA- or TFP-induced ectodomain shedding, this does not prove unequivocally that L-selectin topography is also irrelevant for this process. Even though ectodomain distribution is determined by TM/IC domains on resting cells (14, 15), it remained theoretically possible that the activating stimuli caused a redistribution of L-selectin on the cell surface independent of TM/IC domains. To address this possibility, we investigated the distribution of colloidal-gold labeled anti-L-selectin Abs on each TM/IC domains. To address this possibility, we investigated the distribution of colloidal-gold labeled anti-L-selectin Abs on each TM/IC domains. To address this possibility, we investigated the distribution of colloidal-gold labeled anti-L-selectin Abs on each TM/IC domains. To address this possibility, we investigated the distribution of colloidal-gold labeled anti-L-selectin Abs on each TM/IC domains. To address this possibility, we investigated the distribution of colloidal-gold labeled anti-L-selectin Abs on each TM/IC domains. To address this possibility, we investigated the distribution of colloidal-gold labeled anti-L-selectin Abs on each TM/IC domains. To address this possibility, we investigated the distribution of colloidal-gold labeled anti-L-selectin Abs on each TM/IC domains. To address this possibility, we investigated the distribution of colloidal-gold labeled anti-L-selectin Abs on each TM/IC domains. To address this possibility, we investigated the distribution of colloidal-gold labeled anti-L-selectin Abs on each TM/IC domains. To address this possibility, we investigated the distribution of colloidal-gold labeled anti-L-selectin Abs on each TM/IC domains. To address this possibility, we investigated the distribution of colloidal-gold labeled anti-L-selectin Abs on each TM/IC domains. To address this possibility, we investigated the distribution of colloidal-gold labeled anti-L-selectin Abs on each TM/IC.

We noted that upon PMA activation, either with or without protease inhibition, cells underwent striking morphological changes. Microvilli appeared to retract, while ridge-like structures took form, seemingly replacing most of the finger-like microvilli that were typically seen on resting cells (Fig. 6). Gold particles were quantified and assigned to one of three different surface domains based on the following criteria. Membrane projections were separated into halves. The top half was defined as the tip and the distal 50% of the microvilli, or peak of any ridge, or other detectable projection/protrusion from the cell surface. The lower half was defined as the surface area from the base of a projection to the border of the top half. Gold particles were assigned to the cell body when they were not detectably associated with a surface projection. As previously described, WT L-selectin ectodomains were expressed preferentially on the tips of microvilli. In contrast, cells transfected with the L/CD44 chimera displayed L-selectin ectodomains preferentially on the cell body (14). The ectodomains on cells expressing L/CD31 chimera were randomly distributed; half were found on the cell body, whereas the remainder were almost evenly split between the top and bottom halves of the microvilli.

**FIGURE 6.** Scanning electron micrographs of L1-2 cells before (pre) and 1 and 3 min after PMA treatment. Cell morphologies are representative of most cells in three independent preparations of each cell line. PMA consistently induced a striking shortening of microvilli and the appearance of ridge-like structures within 3 min after treatment. Similar features were also observed after 10 min of PMA treatment (data not shown). No differences were seen between the different transfectants used in this study and untransfected L1-2 cells (the micrographs show L/CD44 cells). Scale bars are 2.7 μm.

**Discussion**

Here we have examined the role of the TM/IC domain of L-selectin during activation-induced shedding. Using stably transfected L1-2 cells expressing WT or chimeric L-selectin with different subsurface components and different surface distribution patterns (14, 15), we show that the ectodomain alone is sufficient for normal PMA-induced proteolytic cleavage by one or more hydroxamate-sensitive proteases.

Although many surface molecules can be shed by leukocytes in response to a variety of stimuli (43–45), L-selectin shedding is unusual in that it occurs extremely rapidly and with marked efficacy. Previous work has demonstrated that L-selectin shedding is triggered by many diverse stimuli. Some of the many shedding-inducing signals include numerous inflammatory cytokines and chemotaxtactants (27), most of which signal through G protein-coupled receptors and induce PKC activation (46); extracellular ATP (47); C-reactive protein (48); cross-linking of β2 integrins (49) or of the L-selectin ectodomain itself (50, 51); and a number of pharmacologic reagents that activate PKC directly (52), act as inhibitors of calmodulin (39), or possess thiol-oxidizing or -blocking activity (53). In addition, upon activation of T cells by Ag, L-selectin expression is down-modulated by reduced transcription of the L-selectin gene (52, 54).

L-selectin shedding has also been observed in several settings in vivo. For example, neutrophils shed L-selectin before or during transendothelial migration in inflamed lung tissue (55). Painful stimuli can also induce L-selectin shedding from circulating neutrophils, which resulted in reduced neutrophil migration into inflamed joints and improved clinical symptoms in a rat arthritis model (56). In this situation, shedding was thus beneficial by limiting tissue inflammation. The anti-inflammatory effects of glucocorticosteroids and nonsteroidal anti-inflammatory drugs may also be due in part to induction of L-selectin shedding (57, 58). However, in some pathologic conditions, such as septicemia, excessive L-selectin shedding from circulating neutrophils may contribute to dysfunctional neutrophil migration (59, 60). Similarly, systemic treatment of mice with bacterial superantigens induced a rapid loss of L-selectin from reactive T cells (61).

The shed ectodomain, sL-selectin, is readily detectable in normal human serum and exerts anti-adhesive effects at concentrations above ~10 μg/ml (62). Up to 2 mg/ml of sL-selectin has been reported in serum of patients with myeloproliferative disorders and certain lymphomas (63, 64). Thus, even though the physiologic role of L-selectin shedding is still controversial, there are numerous clinical settings where this phenomenon may either serve as a protective feedback mechanism or exacerbate existing pathologies. For these reasons, it is important to develop a thorough understanding of the cellular and molecular mechanisms that regulate this process.

The L-selectin sheddase has not been identified. As mentioned above, TACE-deficient thymocytes do not lose L-selectin when stimulated with PMA (37). Interestingly, TACE has a punctate distribution on human leukocytes (38) indicating that it, like L-selectin (25), is not homogeneously expressed over the entire cell circumference. However, it is not known whether TACE mediates L-selectin shedding from mature leukocytes. Another study has suggested that a protein disulfide isomerase may be involved (53).
Nevertheless, given the conspicuous distribution of TACE and considering the rapidity at which shedding occurs on normal leukocytes (6) as well as L1-2 transfectants (this report), it seemed reasonable to speculate that the L-selectin sheddase and/or molecules that regulate its activity might be targeted to the same surface domain as WT L-selectin, i.e., to microvilli. We surmised that if such a topographic association played a role, there should be detectable differences between the kinetics and/or sensitivity at which differentially distributed ectodomains are shed from L1-2 transfectants.

Contrary to our expectations, a particular surface topography is clearly not required for L-selectin shedding to occur, at least in response to PKC activation. Regardless of ectodomain distribution, the magnitude and kinetics of PMA-induced shedding were equivalent in all transfected cell lines. Moreover, PMA-mediated activation of cells in suspension did not induce a significant redistribution of the transfected molecules. This was important to rule out, because a previous study had shown that both L-selectin and CD44 can undergo capping upon extensive cross-linking, and both are redistributed to pseudopodia on migrating lymphocytes (65). Moreover, full-length CD31 has been localized preferentially to cell-cell junctions in endothelial monolayers and a variety of transfected cell lines (66). Although these distribution patterns of CD31 and CD44 are probably controlled by interactions that require their

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**FIGURE 7.** LVSEM micrographs of immunogold-labeled L-selectin ectodomains. L1-2 transfectants expressing WT L-selectin (A), L/CD44 (B), or L/CD31 (C) were stained with mAb DREG-200, followed by 12 nm colloidal gold-conjugated second-stage Ab. Gold particles appear as bright white dots. Transfectants were treated with PMA (10⁻⁶ M) in the presence of KD-IX-73-4. No change in ectodomain topography was apparent after PMA treatment. KD-IX-73-4 had no detectable effect on the appearance of L1-2 cells or on the topography of transfected surface molecules (data not shown). Original magnification, ×60,000.

**FIGURE 8.** Effect of PMA on the distribution of L-selectin ectodomains on L1-2 transfectants expressing WT L-selectin, L/CD44, or L/CD31. Cells were incubated at 37°C in the presence of the metalloprotease inhibitor KD-IX-73-4, and aliquots were prepared for LVSEM analysis before and 1 or 3 min after the addition of PMA (10⁻⁶ M). The frequency of gold particles in different surface domains was determined on individual cells. The numbers of cells/gold particles analyzed are shown for each experimental group.
similarly dramatic loss of L-selectin expression only in WT transfectants, whereas a substantial fraction of chimeric molecules were not cleaved at TFP doses up to 100 μM (higher doses exerted toxic effects on L-2 cells and, therefore, could not be tested). This suggests that calmodulin may regulate L-selectin sheddase activity through two distinct modes. One pathway may be more sensitive to TFP inhibition and is independent of calmodulin interactions with the TM/IC domain of L-selectin. This idea is consistent with recent studies on calmodulin inhibitor-mediated cleavage of other membrane proteins, such as the receptor tyrosine kinase TrkA, which was shed even after the intracellular calmodulin binding site of TrkA was mutated or deleted (72). In addition, a second pathway that is only triggered by relatively high doses of TFP appears to control shedding of a subpopulation of L-selectin molecules in a fashion that requires the WT TM/IC domain. Because this region is absent in L/CD44 and L/CD31, ectodomains are only partially shed when the chimera transfectants are exposed to TFP. However, this high dose TFP-sensitive calmodulin pathway does not appear to play a role during PKC-induced shedding, because PMA had nearly complete and equivalent effects on all transfectants in every treatment modality tested. Thus, it seems likely that PKC activates the L-selectin sheddase(s) through signaling pathways that are at least in part independent of calmodulin inhibition.

Taken together, the results of this study demonstrate that the ectodomain of L-selectin alone is sufficient for normal PMA-induced proteolytic cleavage by one or more hydroxamate-sensitive proteases. Because microvillous WT L-selectin, cell body-expressed L/CD44, and randomly distributed L/CD31 were cleaved with equivalent sensitivity and kinetics and were not redistributed on the surface of activated cells in suspension, we conclude that the sheddase(s) appears to have equal access and activity on all aspects of the leukocyte surface. Subtle differences in transfectant responses to calmodulin inhibition raise the possibility that calmodulin influences L-selectin shedding through two distinct pathways, one requiring interactions with the cytoplasmic tail of L-selectin, and the other independent of such intracellular aspects of the leukocyte surface. Subtle differences in adhesive behavior as discussed above, some studies have found that inhibition of L-selectin shedding reduces rolling velocity (41, 42). Thus, the differential tethering ability of our transfectants cannot be explained by differences in shedding, nor is it likely that differential shedding during rolling could have masked subtle topography-dependent differences in rolling velocity or jerkiness.

Our data also reveal a complex relationship between PKC, calmodulin, L-selectin, and the L-selectin sheddase(s). Previous work has shown that calmodulin can associate with the juxtamembrane region in the cytoplasmic tail of L-selectin and that calmodulin inhibits such as TFP induce shedding (39, 72). Because calmodulin and PKC are thought to obstruct each other’s function (73), it seems plausible that PKC agonists may exert their effects by deactivating a poorly understood process by which calmodulin protects L-selectin from proteolytic cleavage. However, it has been unclear whether calmodulin is essential in PKC-induced shedding and to what extent calmodulin interactions with L-selectin are linked to its role in this event.

The present experiments show that both chimeric and WT L-selectin transfectants shed L-selectin similarly upon exposure to 50 μM TFP, but this effect was much less complete than the near total loss of surface-expressed ectodomains that was observed after maximal PKC activation. Using high doses of TFP, we observed a similarly dramatic loss of L-selectin expression only in WT transfectants, whereas a substantial fraction of chimeric molecules were not cleaved at TFP doses up to 100 μM (higher doses exerted toxic effects on L-2 cells and, therefore, could not be tested). This suggests that calmodulin may regulate L-selectin sheddase activity through two distinct modes. One pathway may be more sensitive to TFP inhibition and is independent of calmodulin interactions with the TM/IC domain of L-selectin. This idea is consistent with recent studies on calmodulin inhibitor-mediated cleavage of other membrane proteins, such as the receptor tyrosine kinase TrkA, which was shed even after the intracellular calmodulin binding site of TrkA was mutated or deleted (72). In addition, a second pathway that is only triggered by relatively high doses of TFP appears to control shedding of a subpopulation of L-selectin molecules in a fashion that requires the WT TM/IC domain. Because this region is absent in L/CD44 and L/CD31, ectodomains are only partially shed when the chimera transfectants are exposed to TFP. However, this high dose TFP-sensitive calmodulin pathway does not appear to play a role during PKC-induced shedding, because PMA had nearly complete and equivalent effects on all transfectants in every treatment modality tested. Thus, it seems likely that PKC activates the L-selectin sheddase(s) through signaling pathways that are at least in part independent of calmodulin inhibition.

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