Membrane Proximal Cleavage of L-Selectin: Identification of the Cleavage Site and a 6-kD Transmembrane Peptide Fragment of L-Selectin

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Abstract. Rapid downregulation of L-selectin expression occurs in response to leukocyte activation, and it has been speculated to be an integral process in the adhesion cascade leading to neutrophil recruitment to sites of inflammation. It has previously been proposed that L-selectin is proteolytically cleaved from the cell surface; however, the nature of the cleavage site has been unknown. We have produced polyclonal antisera against the extracellular domain and against the cytoplasmic domain of L-selectin. Both antisera immunoprecipitate the intact form of L-selectin from metabolically labeled phytohemagglutinin-stimulated lymphoblasts and peripheral blood neutrophils. In addition, the anti-cytoplasmic domain serum, but not the antiectodomain serum, immunoprecipitate a 6-kD species from PMA activated lymphoblasts and formylmethionylleucylphenylalanine-activated neutrophils. Conversely, the antiectodomain serum but not the anti-cytoplasmic domain serum immunoprecipitate a 68-kD soluble form of L-selectin from the supernatant of PMA-activated lymphoblasts. The appearance of the 6-kD species on activated cells correlated with the disappearance of the intact form of L-selectin and the appearance of the soluble form of L-selectin. A third polyclonal serum generated against the membrane proximal region of the ectodomain also reacted with the 6-kD species, indicating that this is a transmembrane peptide of L-selectin. That the 6-kD species is derived from L-selectin was confirmed by immunoprecipitation of the 6-kD species from L-selectin transfectants but not from mock transfectants. Radiochemical sequence analysis defined a cleavage site between Lys^{321} and Ser^{322}, which would predict a transmembrane fragment consistent in size with the observed 6-kD fragment. A Ser-Phe-Ser motif adjacent to the cleavage site is conserved between human, mouse, and rat L-selectin, and a related motif is found proximal to transmembrane domains of other downregulated proteins, such as ACE, CD16-II, and TNF-RII, suggesting the possibility of a common recognition motif.

LEUKOCYTE localization to sites of inflammation is a precisely coordinated multistep process involving distinct families of adhesion receptors and chemokines (9, 56). Regulation of the neutrophil adhesive state is critical for leukocyte function. One major neutrophil adhesion molecule, the Mac-1 β_2 integrin (CD11b/CD18), is both quantitatively upregulated (59) and assumes an active conformation upon neutrophil exposure to chemotactic factors (10). In contrast, another major neutrophil adhesion molecule, L-selectin, is rapidly downregulated upon neutrophil activation (5, 17, 26, 28, 29). However, antibodies against either Mac-1 or L-selectin are effective in blocking neutrophil recruitment to sites of inflammation in vivo (28, 36, 62) and neutrophil adhesion to stimulated endothelial cells in vitro (18, 20, 31, 51, 52, 54). The observation that L-selectin and Mac-1 are inversely regulated upon activation provided the theoretical basis for a model of neutrophil-endothelial cell interaction (29), where L-selectin is involved in the early adhesion events, while Mac-1 is required for adhesion strengthening and transendothelial migration (1, 37, 51, 60). The precise regulation and the rapid kinetics of L-selectin downregulation is suggestive of a role in the progression of neutrophil emigration.

Normal lymphocyte traffic to peripheral lymph nodes is also mediated by L-selectin. L-selectin is downregulated on activated lymphocytes, although with a slower time course than that of neutrophils (19, 24, 25, 30). Previous studies have shown that a large fragment of L-selectin corresponding to the expected size of the extracellular portion of L-selectin can be recovered from the supernatant of activated neutrophils (5, 29) and lymphoid cells (24). A soluble form of L-selectin is found at high concentrations (1.5–2.0 μg/ml) in normal human serum (47), in culture supernatants of lym-

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phoblasts stimulated with phytohemagglutinin (PHA)[1] (55) and L-selectin-transfected embryonic kidney cells (34). Based on the rapid kinetics of L-selectin downregulation, it has been postulated that L-selectin is cleaved by an activated membrane protease (5, 24, 29). However, the mechanism of L-selectin downregulation has remained unclear, since protease inhibitors have no effect on L-selectin shedding in response to neutrophil activation (references 12, 27, 48 and unpublished observations).

Recently, the concept of membrane proximal cleavage of transmembrane proteins has emerged as a specific and inducible mechanism to rapidly modulate cell surface expression of diverse proteins (15). Many growth factors and cytokines, such as TGF-α (42), colony-stimulating factor-1 (CSF-1) (57), and Kit ligand (22), have a transmembrane bound form that can be specifically released from the cell surface by a proteolytic mechanism upon cell activation. Similarly, cell surface receptors and ectoenzymes, such as the TNF receptor (32, 44), folate receptor (16), β-amyloid protein (41, 63), and angiotensin-converting enzyme (ACE) (14) can be cleaved to release a soluble form. It is unknown whether the mechanism for rapid downmodulation of L-selectin is related to modulation of these other critical cell surface proteins.

If the ectodomain of L-selectin is released from the cell surface by specific proteolytic cleavage, then a transmembrane peptide fragment of L-selectin should be recoverable from activated leukocytes. In this report, we have raised a polyclonal antiserum against a synthetic peptide corresponding to the cytoplasmic domain of L-selectin. We demonstrate that this antiserum can recognize the intact form of L-selectin, as well as a 6-kD transmembrane fragment of L-selectin associated with activated leukocytes and L-selectin transfectants. We further identified the cleavage site by radiochemical sequence analysis of the 6-kD species. These results confirm membrane-proximal cleavage as the mechanism for rapid downregulation of L-selectin.

Materials and Methods

Polyclonal Antisera

A polyclonal antiserum against the extracellular domain of L-selectin (JK923) was generated by hyperimmunization of a rabbit with a purified preparation of a soluble, truncated form of L-selectin. An L-selectin cDNA lacking the coding sequence for the transmembrane and cytoplasmic domains was generated by PCR and subcloned into the CDM8 vector. COS cells were transfected with this construct by DEAE-dextran as previously described (11). Supernatants from transfected cells were harvested on days 3–14 after transfection, and soluble L-selectin was purified on a DREG-200 anti-L-selectin mAb column (30).

A polyclonal antiserum against the cytoplasmic domain of L-selectin (JK564) was generated by hyperimmunization of a rabbit with a BSA-conjugated 18mer synthetic peptide corresponding to the entire cytoplasmic domain of L-selectin with an additional NH_2-terminal cysteine residue (NH_2-CRRLKKGKKSKRSMNDPY-COOH). The peptide was reduced and coupled via the sulfhydryl group of the NH_2-terminal cysteine residue to maleimide-activated BSA (Pierce Chemical Co., Rockford, IL), according to the manufacturer's instructions. The NH_2-terminal linkage provides an orientation similar to that expected of the cytoplasmic domain extending from the membrane bilayer.

A polyclonal antiserum against the membrane proximal region of the extracellular domain (JK924) was generated by hyperimmunization of a rabbit with a keyhole limpet hemacyanin–conjugated 18mer synthetic peptide corresponding to the membrane-proximal region with an additional COOH-terminal cysteine residue (NH_2-QKLDKSFSMIKEGDYNPC-COOH). The peptide was reduced and coupled to maleimide-activated keyhole limpet hemacyanin as described above. The COOH-terminal linkage provides an orientation similar to that expected of the ectodomain extending from the membrane bilayer.

Monoclonal Antibodies

The monoclonal antibody DREG-56 against the extracellular domain of L-selectin was prepared as previously described (30). CA21 hybridoma cell lines producing mAb directed against the cytoplasmic domain of L-selectin were prepared by hyperimmunization of BALB/c mice with a synthetic peptide corresponding to the entire cytoplasmic domain of L-selectin, as described above. Spleen cells were fused with the SP2/0 myeloma fusion partner, as described previously (30). Hybridoma supernatants were screened for the ability to specifically recognize immobilized cytoplasmic domain peptide. Positive clones were further screened for the ability to immunoprecipitate L-selectin. CA21 (IgG1) mAb was purified by protein G affinity chromatography.

Metabolic Labeling

COS cells were transiently transfected with cDNA encoding L-selectin (or mock transfected) by the DEAE-dextran method, as described previously (11). 3 d later, cells were metabolically labeled with [^{35}S]methionine (New England Nuclear, Boston, MA). The COS monolayers were first preincubated in methionine-free RPMI 1640 containing 10% dialyzed FCS for 20 min at 37°C to deplete intracellular pools of methionine. Cells were pulse labeled with [^{35}S]methionine (0.5–1.0 mCi/100-mm dish) for various times, as indicated. The COS cells were then chased with complete RPMI 1640. Culture supernatants were harvested and centrifuged to remove any nonadherent cells or debris. Monolayers were lysed for 30 min at 4°C in 1 ml of 1% Triton X-100 in 10 mM Tris (pH 8)–saline–0.025% azide containing 1% ovalbumin, 1 mM aprotinin, and 1 mM freshly added PMSF (lysis buffer). Cell nuclei were pelleted by centrifugation at 12,000 *g*.

Human mononuclear and PMN leukocytes were isolated from peripheral blood by Ficoll-Hypaque centrifugation and dextran sedimentation, as described previously (51). PHA-stimulated lymphoblasts were generated by incubating the mononuclear leukocytes with 2.5 μg/ml PHA (Sigma Immunochemicals, St. Louis, MO) in complete RPMI 1640 medium for 5 d at 37°C. The PHA lymphoblasts were harvested on day 5 and metabolically labeled with [^{35}S]methionine as described above. Freshly isolated human PMN were metabolically labeled as described (38). Briefly, PMN were labeled at 2×10^7 cells/ml for 5 h at 37°C. PMA (100 ng/ml) or formylmethionylleucylphenylalanine (10^{-6} M) was added in the final 30 min as indicated.

Immunoprecipitation and SDS-PAGE

Cell lysates were precleared with normal rabbit serum followed by protein A–agarose. Preimmune serum or specific serum was added to aliquots of the precleared lysates and cell-free supernatants. The samples were incubated at 4°C for 1–2 h and then 15 μl of a protein A–agarose slurry was added to each sample. Samples were rotated end-over-end at 4°C for 30 min and then washed extensively. The specifically bound material was eluted by addition of SDS-PAGE sample buffer followed by incubation at 90°C for 10 min. Eluates were resolved on tricine-SDS polyacrylamide gradient (10–20%) gels (NOVEX, Encinitas, CA). Gels were subjected to fluorography (Enhance; New England Nuclear) and autoradiography.

Western Blot Analysis

An activated human granulocyte lysate was passed over a CA21 anti-cytoplasmic domain mAb column. The column was washed extensively and eluted with 50 mM glycine (pH 2) solution containing 1% octyl thioglucopyranoside. Fractions were neutralized with Tris buffer and analyzed by dot blot analysis. Aliquots of partially purified material were subjected to SDS-PAGE on 10–20% polyacrylamide-SDS-tricine gels. Samples were transferred to polyvinylidenefluoride (PVDF) membrane (Immobilon P^{SQ}; Millipore Corp., Bedford, MA) in 10 mM CAPS (3-cyclohexylamino-1-propanesulfonic acid) buffer (pH 11) containing 10% methanol. The membrane was blocked with 2% BSA, and the transferred material was

1. *Abbreviations used in this paper*: ACE, angiotensin-converting enzyme; CSF-1; colony-stimulating factor-1; fMLP, formylmethionylleucylphenylalanine; L-STMP, L-selectin transmembrane peptide; PHA, phytohemagglutinin; PVDF, polyvinylidenefluoride; SCR, short consensus repeat.

probed with CA21 anti-cytoplasmic tail mAb and detected with an alkaline phosphatase-conjugated secondary antibody (Promega Corp., Madison, WI) followed by 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride, as described previously (30).

Radiochemical Sequence Analysis

COS cells transfected with human L-selectin cDNA were metabolically labeled with [^{35}S]methionine (specific activity >1,000 Ci/mmol; 0.5 mCi/100-mm dish), as described above. COS cell transfectants were also metabolically labeled with [^{3}H]phenylalanine (specific activity = 100–130 Ci/mmol), as described above with the following modifications: the cells were pulse labeled for 5 h in phenylalanine-free RPMI 1640 and then simultaneously chased and activated by the addition of excess nonradioactive phenylalanine and PMA (100 ng/ml) for 30 min. The cells were lysed and treated as described above. PHA-stimulated lymphoblasts (5×10^6–1×10^7/ml) were metabolically labeled with either [^{35}S]methionine or [^{3}H]phenylalanine for 4–5 h, as described above. The cells were then simultaneously chased with the appropriate nonradioactive amino acid and activated with PMA (100 ng/ml) for 30 min. The 6-kD species was immunoprecipitated as described above. The material was resolved on a tricine SDS-polyacrylamide (10–20%) gradient gel (NOVEX) and then electrotransferred in Tris-glycine buffer to a PVDF membrane (Immobilon P^{SQ}; Millipore Corp.). The membrane was air dried and exposed to Kodak X-OMAT AR film. The film was subsequently developed, aligned with the Immobilon membrane, and the area of the membrane corresponding to the point of migration of the 6-kD species was excised and subjected to sequencing on a pulse-liquid protein sequencer (model 477A; Applied Biosystems, Inc., Foster City, CA). Fractions were collected from each Edman degradation cycle and analyzed in a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

Results

Identification of a 6-kD Species on PHA Lymphoblasts

We initially raised two antisera against L-selectin, one directed against purified soluble L-selectin, which lacked the transmembrane and cytoplasmic domains (antiectodomain serum JK923), and another directed against a synthetic peptide corresponding to the entire cytoplasmic domain of L-selectin (anti-cytoplasmic domain serum JK564). The antiectodomain serum specifically stained intact, unactivated leukocytes, while the anti-cytoplasmic domain serum stained only permeabilized cells (data not shown). Both antisera immunoprecipitated the intact membrane-spanning form of L-selectin (Fig. 1 *A* and data not shown).

The ability of these antisera to detect processed fragments of L-selectin on activated leukocytes was examined. The primary sequence of L-selectin predicts six methionine residues, two of which are located in the cytoplasmic and transmembrane domains. A third methionine residue is located in the ectodomain, 10 amino acids proximal to the beginning of the transmembrane domain. Therefore, we reasoned that both a transmembrane fragment of L-selectin, as well as a soluble form of L-selectin, should be detectable from activated cells metabolically labeled with [^{35}S]methionine. Lymphoblasts stimulated with PHA for 5 d actively synthesized L-selectin. The anti-ectodomain serum immunoprecipitated the intact 74-kD L-selectin molecule from biosynthetically labeled cells (Fig. 1 *A*). Although PHA blasts are actively proliferating and constitutively produce a soluble form of L-selectin (55), addition of phorbol esters (PMA) provided a further activating signal that induced a significant increase in the downregulation of newly synthesized cell surface L-selectin. This is evidenced by reduced levels of intact L-selectin in immunoprecipitates from cell lysates (Fig. 1 *B*), increased levels of soluble L-selectin in immunoprecipitates from cell-free supernatants (Fig. 1 *C*), and decreased cell surface expression of L-selectin (data not shown). The anti-cytoplasmic domain serum also recognized the intact form of L-selectin (Fig. 1, *A* and *B*), but not the soluble form (Fig. 1 *D*). These results indicated that the classical transmembrane form of L-selectin is the major species found on these cells, and that the soluble form of L-selectin lacks the cytoplasmic tail. In addition, a faint band of 6 kD could be reproducibly detected with the anti-cytoplasmic domain serum in cell lysates of day 5 PHA blasts (Fig. 1, *A* and *B*). Activation of lymphoblasts with PMA caused a marked decrease in the amount of detectable 74-kD intact L-selectin and a concomitantly marked increase in the amount of the 6-kD species (Fig. 1 *B*). Addition of the microfilament disrupting agent cytochalasin B did not inhibit or enhance the downregulation of intact L-selectin or the appearance of the 6-kD species (Fig. 1 *B*). These data suggested that the 74-kD intact L-selectin species is cleaved upon activation with PMA to yield a 6-kD cell bound fragment and a 68-kD soluble fragment, and that this cleavage does not require cytoskeletal involvement.

A 6-kD Species is Associated with PMA- and fMLP-activated Neutrophils

Neutrophils also rapidly downregulate L-selectin in response to activation by chemotactic factors or phorbol esters. Although neutrophils are a terminally differentiated cell type with few ribosomes and limited endoplasmic reticulum, others have shown that neutrophil proteins can be metabolically labeled (38). Since L-selectin mRNA has been isolated from peripheral blood neutrophils (39), we next examined if L-selectin biosynthesis could be detected. An intact form of L-selectin was immunoprecipitated with both the anti-ectodomain serum and the anti-cytoplasmic domain serum from neutrophils metabolically labeled with [^{35}S]methionine (Fig. 2). As reported previously (17, 36), the neutrophil form of L-selectin migrated more slowly than the lymphocyte form by SDS-PAGE, presumably because of additional posttranslational modifications (39). Activation of the neutrophils with either fMLP chemoattractant or phorbol esters resulted in greatly reduced amounts of intact L-selectin, with a corresponding increase in a 6-kD species immunoprecipitated with the anti-cytoplasmic domain serum (Fig. 2). This result was somewhat unexpected, since prolonged culture of neutrophils necessary for metabolic labeling can induce neutrophil shape change and degranulation, and might be expected to result in immediate L-selectin downregulation. However, these results suggested that downregulation of the newly synthesized L-selectin is greatly enhanced by a specific activating agent. Since these cultures were not chased with cold methionine, a precursor form of L-selectin (*L'*, Fig. 2) was evident in these immunoprecipitations. The L-selectin precursor was not downregulated in response to PMA or fMLP (Fig. 2), suggesting that only the mature cell surface form of L-selectin is susceptible to cleavage. The estimated size of the 6-kD species is consistent with our previous observation that ^{125}I surface labeled L-selectin released into the supernatant is ~4 kD smaller than the membrane-bound form (29).

The 6-kD Species is a Specific Product of L-Selectin

To confirm that the 6-kD species is directly related to

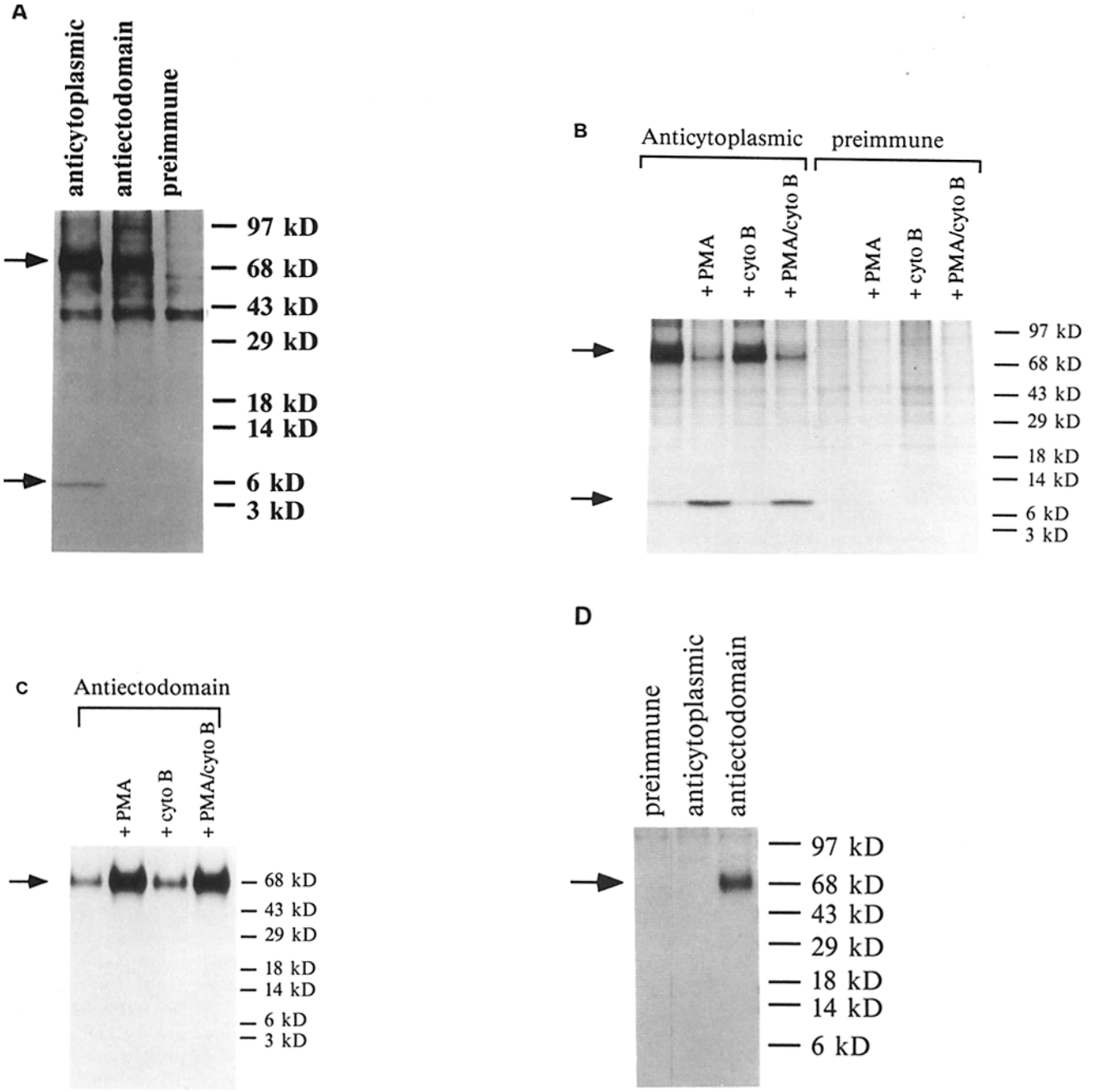

Figure 1. Identification of a 6-kD species in metabolically labeled PHA lymphoblasts. Peripheral blood lymphocytes were stimulated with PHA for 5 d. Lymphoblasts were pulse labeled with [^{35}S]methionine for 15 min and chased with cold methionine for 30 min. Cells were treated with PMA (100 ng/ml) or cytochalasin B for 30 min, as indicated. (*A* and *B*) Labeled cells were lysed in 1% Triton X-100 solution, and cell lysates were immunoprecipitated with antiserum directed against the ectodomain of L-selectin (JK923), the cytoplasmic domain of L-selectin (JK564), or with preimmune serum, as indicated. *Upper arrow*, the position of the intact form of L-selectin; *lower arrow*, the position of the putative 6-kD transmembrane cleavage product. The 42-kD band observed in all lanes, including the preimmune control, of *A* is likely to be actin, and it is more prominent in this figure because the gel was intentionally overexposed to bring out the 6-kD band. (*C* and *D*) Cell-free supernatants were immunoprecipitated with antisera against the ectodomain or the cytoplasmic domain of L-selectin or with preimmune serum, as indicated. *Arrow*, the position of the soluble form of L-selectin. Immunoprecipitates were subjected to SDS-PAGE on 10–20% gradient gels and visualized by autoradiography with fluorography.

L-selectin, we compared COS cells that were transfected with L-selectin cDNA vs those that were mock transfected. Lasky et al. (34) have previously shown that L-selectin transfectants produce a soluble form of L-selectin. We confirmed this observation and extended it to demonstrate that the anti-cytoplasmic domain serum immunoprecipitated both the intact L-selectin and the 6-kD species from L-selectin transfectants, but not mock transfectants (Fig. 3 *A*). Moreover, the anti-cytoplasmic domain serum did not react with the soluble L-selectin from COS cell supernatants (Fig. 3

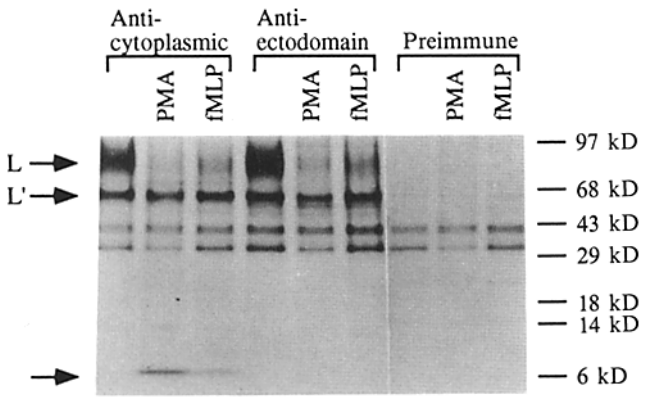

Figure 2. The 6-kD species is associated with activated neutrophils. Peripheral blood neutrophils were metabolically labeled with [^{35}S]methionine for 5 h without chase, as described previously (38). Cells were treated with fMLP or PMA in the last 30 min of the labeling period, as indicated. Cell lysates were immunoprecipitated with antisera against the cytoplasmic domain, the ectodomain, or with preimmune serum, as indicated. Immunoprecipitates were analyzed as described in Fig. 1. L → denotes the position of the mature cell surface form of the neutrophil L-selectin. L' → denotes the position of the biosynthetic precursor form of L-selectin. *Lower arrow*, the position of the putative 6-kD cleavage product. Note that the neutrophil form of L-selectin migrates slower than the lymphocyte form of L-selectin because of differences in post-translational modifications, as previously described (20, 40, 45). Immunoprecipitates were subjected to SDS-PAGE on 10–20% gradient gels and visualized by autoradiography with fluorography.

B). These results strongly indicate that the 6-kD species is a specific product of L-selectin and that COS cells can constitutively process L-selectin in a manner similar to that of activated leukocytes.

Western Blot Analysis of the 6-kD Species

During the course of these experiments, a series of mouse mAb, CA21, directed against the cytoplasmic domain of L-selectin was developed. The CA21 mAb, like the JK564 polyclonal anti-cytoplasmic domain serum, immunoprecipitated the 6-kD species from lysates of L-selectin–transfected COS cells (Fig. 4 *A*).

It was possible that the 6-kD species coprecipitated with L-selectin and was not directly related to a fragment of L-selectin. This seemed unlikely, given the correlation between the appearance of the 6-kD species and the disappearance of the intact L-selectin on activated leukocytes. However, to formally exclude this possibility, lysates of activated neutrophils were subjected to affinity chromatography followed by Western blot analysis. The CA21 mAb reacted directly with both the intact form of L-selectin and the 6-kD species by Western blot analysis (Fig. 4 *B*). Control mAb showed no reactivity under these conditions (data not shown). These results indicated that the 6-kD species is related to the cytoplasmic domain of L-selectin, and they also confirmed that activated neutrophils can produce the 6-kD species.

The 6-kD L-Selectin Product is a Transmembrane Peptide

A 6-kD COOH-terminal fragment of L-selectin would indicate a transmembrane peptide of ~50–55 amino acids. This would map the putative cleavage site to a short region of the ectodomain, between the COOH terminus of the second short consensus repeat (SCR) and the beginning of the transmembrane domain (Fig. 5 *A*). Interestingly, this region is well conserved between mouse (34, 49), rat (61), and human L-selectin (8, 11, 50, 58), but is not conserved between L-, E-, and P-selectin (6, 23) (Fig. 5 *A*). Although soluble forms of E-selectin and P-selectin have been described, neither protein is regulated in the same manner as L-selectin. To confirm that the 6-kD species is a transmembrane product of L-selectin, we produced a third polyclonal serum (JK924) against a synthetic peptide (NH_2-QKLDKSFSMIKEGDY-NPC-COOH) corresponding to the sequence of this putative membrane-proximal cleavage domain with an additional COOH-terminal cysteine residue, as described in Materials and Methods. Both the JK924 anti-cleavage domain serum and the JK564 anti-cytoplasmic domain serum immunoprecipitated the 6-kD species from cell lysates of L-selectin transfectants (Fig. 5 *B*). In some experiments using the

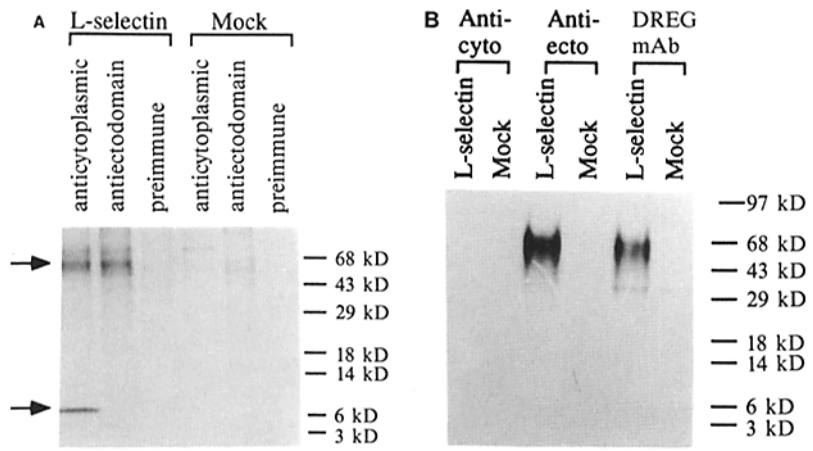

Figure 3. The 6-kD species is a fragment of L-selectin. COS cells were transiently transfected with a cDNA clone encoding the membrane form of L-selectin or were mock transfected, as indicated. On day 3 of posttransfection, cells were pulse-labeled with [^{35}S]methionine for 60 min and chased for 120 min. (*A*) Cell lysates were immunoprecipitated with antisera directed against the cytoplasmic domain of L-selectin, the ectodomain of L-selectin, or with preimmune serum, as indicated. *Upper arrow*, the position of the intact form of L-selectin; *lower arrow*, the position of the putative transmembrane cleavage product. (*B*) Cell-free supernatants of labeled COS cells were immunoprecipitated with polyclonal antiserum or the DREG-56 mAb, as indicated. *Arrow*, the position of the soluble form of L-selectin. Immunoprecipitates were subjected to SDS-PAGE on 10–20% gradient gels and visualized by autoradiography with fluorography.

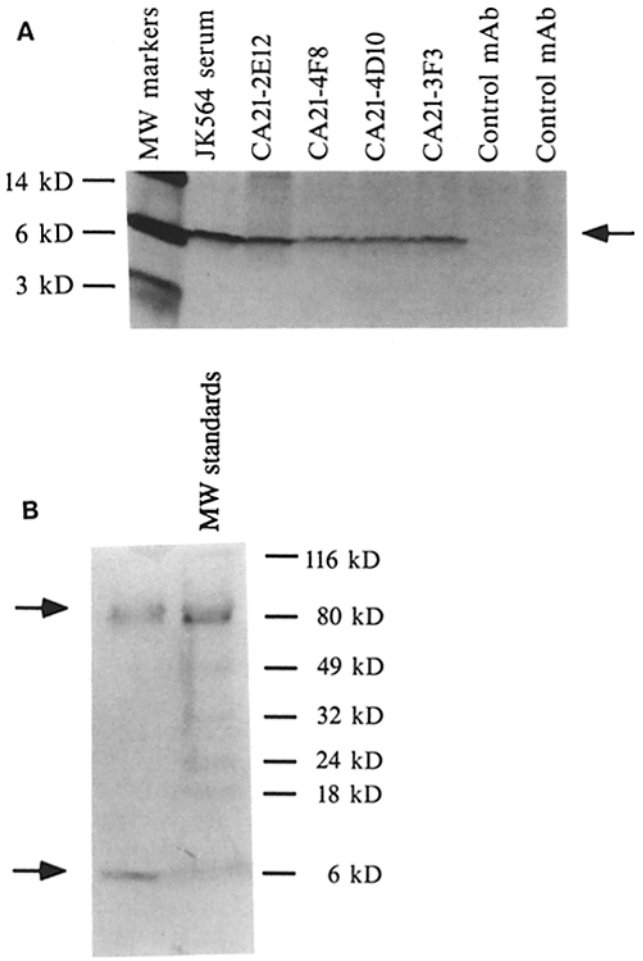

Figure 4. CA21 mAb directed against the L-selectin cytoplasmic domain detects the 6-kD species. (*A*) COS cells were transfected with full-length L-selectin cDNA and labeled with [^{35}S]methionine, as described in the Fig. 3 legend. Cell lysates were immunoprecipitated with JK564 anti-cytoplasmic domain serum, the CA21 series of mAb (CA21-2E12, CA21-4F8, CA21-4D10, CA21-3F3), or irrelevant control mAb, as indicated. Immunoprecipitates were subjected to SDS-PAGE and autoradiography. The figure was truncated to show only the relevant area of the gel. *Arrow*, the position of the 6-kD putative cleavage product of L-selectin. (*B*) Lysates of activated neutrophils were partially purified on a CA21-2E12 anti-L-selectin cytoplasmic domain mAb affinity column, subjected to SDS-PAGE and transferred to an Immobilon P^{SQ} membrane. The membrane was blocked in 1% BSA and probed with CA21-2E12 mAb. Bands were visualized by addition of a second stage goat anti-mouse IgG-alkaline phosphatase conjugate followed by development with BCIP and NBT. Prestained molecular weight standards were used to monitor transfer efficiency. *Upper arrow*, the position of the intact neutrophil L-selectin; *lower arrow*, the position of the 6-kD putative cleavage product of L-selectin.

JK924 serum, an 18-kD band was found to be variably associated with L-selectin. This 18-kD band, unlike the 6-kD species, did not react directly with any of our antibodies by Western blot analysis (unpublished observation). Thus, the 6-kD species reacted with two independent antisera, one directed against membrane-proximal extracellular determinants and the other directed against cytoplasmic domain determinants. These results indicated that the 6-kD species is a transmembrane peptide fragment of L-selectin.

Identification of the Cleavage Site

To determine the actual cleavage site, the 6-kD species was purified for NH_2-terminal sequence analysis. Since the deduced amino acid sequence of L-selectin is known (8, 11, 50, 58), we performed radiochemical sequence analysis of the 6-kD species from L-selectin-transfected COS cells biosynthetically labeled with [^{35}S]methionine. The cell lysate was immunoprecipitated with the anti-cytoplasmic domain serum, subjected to SDS-PAGE, and transferred to PVDF membrane. The 6-kD band was visualized by autoradiography, excised, and subjected to protein sequencing. Radioactive peaks were observed at cycles 4 and 20 (Fig. 6 *A*), indicating methionine residues at these positions. A spacing of 16 amino acids between the two methionine residues is found only between Met^{325} and Met^{341} of the L-selectin sequence, suggesting that L-selectin was cleaved between Lys^{321} and Ser^{322}. No radioactivity was observed with mock-transfected COS cells (data not shown). To confirm this predicted cleavage site, we performed radiochemical sequence analysis with COS transfectants biosynthetically labeled with another radiolabeled amino acid, [^{3}H]phenylalanine. Radioactive peaks were observed at cycles 2, 14, and 24 (Fig. 6 *B*), indicating phenylalanine residues at these sites. The spacing between the observed peaks was consistent only with the spacing between Phe^{323}, Phe^{335}, and Phe^{345} of L-selectin, again indicating a cleavage site between Lys^{321} and Ser^{322}. Cleavage at this site would produce a transmembrane peptide with a predicted molecular mass of 5,823 D, which is in excellent agreement with the observed relative molecular mass of 6,000 by SDS-PAGE. To confirm that this is a natural cleavage site, and not an artifact of the COS expression system, we biosynthetically labeled PHA lymphoblasts with [^{35}S]methionine and [^{3}H]phenylalanine. Again, radioactive peaks were observed at cycles 4 and 20 with [^{35}S]methionine (Fig. 6 *C*), and prominent peaks were observed at cycles 2 and 14 with [^{3}H]phenylalanine (data not shown). These results confirm that L-selectin is cleaved between Lys^{321} and Ser^{322}, and they indicate that COS cells process L-selectin in a manner indistinguishable from that of PHA lymphoblasts.

Discussion

We have identified a 6-kD L-selectin transmembrane peptide (L-STMP) on activated leukocytes and on L-selectin transfectants. The 6-kD L-STMP reacted with monoclonal antibodies and polyclonal antiserum directed against the cytoplasmic domain of L-selectin, as well as with an antiserum directed against a 17-amino acid region of the ectodomain adjacent to the transmembrane domain. Appearance of the 6-kD L-STMP on activated leukocytes correlated with the disappearance of the intact membrane-bound L-selectin and with the appearance of a soluble form of L-selectin in culture supernatants. Previous studies have estimated that the soluble form of L-selectin is 4 kD (29) to 12 kD (24) smaller than the intact membrane form. However, the precise nature of L-selectin processing has been difficult to determine because the large, heavily glycosylated ectodomain migrates as a diffuse band by SDS-PAGE. In contrast, the L-STMP migrated as a sharp band with an apparent molecular mass of 6.0–6.5 kD. The size of the 6-kD form would indicate a putative cleavage site in a short region between the COOH

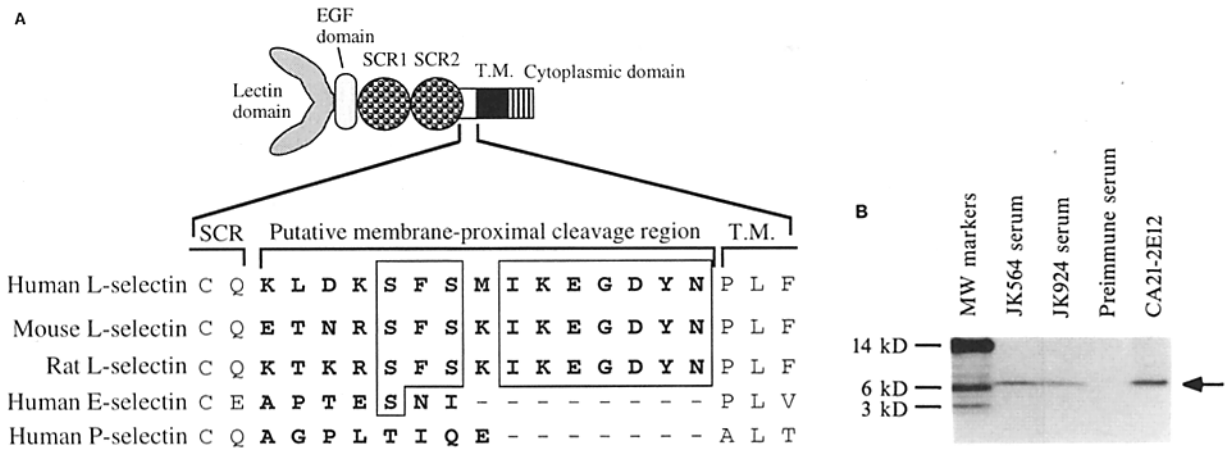

Figure 5. The 6-kD species is an L-selectin transmembrane peptide (*L-STMP*). (*A*) Sequence of the putative membrane-proximal cleavage region of human L-selectin, based on the size estimate of the 6-kD species, aligned with the corresponding regions of mouse and rat L-selectin, as well as human E-selectin and P-selectin. Conserved residues in this region are boxed, and the COOH terminus of the last SCR domain and the NH_2 terminus of the transmembrane domain are indicated. (*B*) A rabbit antiserum (JK924) was raised against a synthetic peptide corresponding to the putative cleavage region as described in Materials and Methods. Lysates of L-selectin–transfected COS cells metabolically labeled with [^{35}S]methionine were immunoprecipitated with JK924 antisera against the putative cleavage region, JK564 anti–cytoplasmic domain serum, CA21 anti–cytoplasmic domain mAb, or preimmune serum, as indicated. The figure was truncated to show only the relevant area of the gel. *Arrow*, the position of the 6-kD cleavage product of L-selectin.

terminus of the second SCR and the beginning of the transmembrane domain. This estimate is also consistent with recognition of the 6-kD species by an antiserum directed against a synthetic peptide corresponding to this membrane proximal extracellular region. Radiochemical sequence analysis of the L-STMP molecule predicts a cleavage site between Lys^{321} and Ser^{322}. Cleavage at this site would yield a transmembrane peptide fragment with a predicted molecular mass of 5,823 D, consistent with our observed size estimate. Although the extracellular domains of L-, E-, and P-selectin are well conserved (6, 8, 11, 23, 50, 58), this short membrane proximal region including the cleavage sequence is highly divergent. This is consistent with the observation that only L-selectin is rapidly downregulated in this manner. Taken together, these results strongly support the model that L-selectin is cleaved at a membrane proximal site.

It has previously not been possible to formally demonstrate a proteolytic mechanism involved in L-selectin downregulation because of its lack of sensitivity to protease inhibitors. Although the addition of exogenous chymotrypsin can selectively remove L-selectin from the cell surface, a variety of chymotrypsin inhibitors had no affect on L-selectin downregulation on activated cells (27). L-selectin downregulation has also been reported to be resistant to the serine protease inhibitors, aprotinin, or PMSF (12), and to the metalloprotease inhibitor phosphoramidon (48). Moreover, we have expanded the search and have found that L-selectin downregulation is insensitive to a battery of inhibitors of serine proteases (PMSF, 4-amidinophenyl methyl sulfonyl fluoride, aprotinin, antipain, leupeptin, tosyl-L-lysine chloromethyl ketone, and tosyl-L-phenylalanine chloromethyl ketone), metalloproteases (EDTA, phosporamidon, leupeptin, antipain, and bestatin), aspartic proteases (pepstatin), and thiol proteases (E64) (unpublished observations). However, the identification of the 6-kD transmembrane fragment and the soluble extracellular fragment of L-selectin on neutrophils and lymphocytes clearly indicate that the transmembrane form of L-selectin is the dominant form, and that downregulation occurs via a proteolytic mechanism.

Membrane proximal cleavage has recently emerged as a specific mechanism to rapidly modulate cell surface expression of a limited but diverse subset of transmembrane proteins. Other leukocyte membrane proteins, including CD14 (3), CD16 (21), CD43 (4, 12, 46), CD44 (2, 12), and TNF receptor (32, 44), have been reported to be downmodulated upon leukocyte activation with PMA. Moreover, this mechanism is not limited to leukocytes, but rather appears to be involved in regulating cell surface expression of proteins on many cell types (15). Proteins as diverse as the β-amyloid precursor protein (41, 63), the angiotensin-converting enzyme (14), the folate receptor (16), growth factor and cytokine receptors, such as TNF receptor (32, 44) and CSF-1 receptor (13), as well as growth factors and cytokines themselves, such as TNF (33), TGF-α (42), CSF-1 (57), and Kit ligand (22), have transmembrane forms that are cleaved to release soluble forms.

A protease involved in membrane proximal cleavage of cell surface proteins has not been identified in any system. Moreover, it is not clear whether these diverse transmembrane proteins are cleaved by one common membrane proximal protease activity or multiple related or unrelated activities. It is unusual that COS cells, which do not normally express L-selectin, can process L-selectin into fragments that are indistinguishable from those found with activated leukocytes. Transfection of COS or CHO cells with genes encoding transmembrane forms of TGF-α (43), Kit ligand (22), TNF receptor (32), and ACE (14) also results in release of soluble proteins, suggesting the possibility of a common proteolytic pathway. However, the rapid kinetics of L-selectin shedding in response to a physiogical stimulus are unusual. On neutrophil surfaces, CD43 and CD44 are downregulated during a 15–60-min period in response to PMA

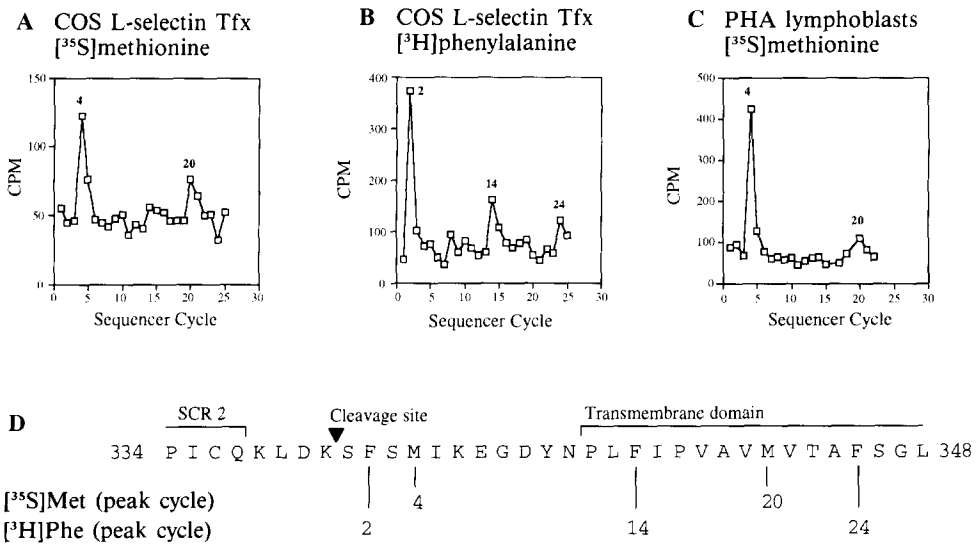

Figure 6. Identification of the cleavage site by radiochemical sequencing. COS cells transfected with L-selectin were metabolically labeled with [^{35}S]methionine (*A*) or [^{3}H]-phenylalanine (*B*), and PHA lymphoblasts were labeled with [^{35}S]methionine and activated with PMA (*C*). The 6-kD species was immunoprecipitated with the JK564 anti-cytoplasmic domain serum, subjected to SDS-PAGE, and transferred to PVDF membrane. The 6-kD band was visualized by autoradiography, excised, and subjected to a pulse-liquid protein sequencer. Fractions from each cycle were collected and analyzed. Assignment of methionine and phenylalanine residues were made based on radioactive peaks from [^{35}S]methionine and [^{3}H]phenylalanine labeled cells, respectively, and aligned with the L-selectin sequence (*D*).

(46), but this is in contrast to cleavage of L-selectin within 5 min. Moreover, PMA is an extremely potent and nonphysiological activator of protein kinase C, that may artificially accelerate the kinetics of downregulation of some proteins or may cause downregulation of proteins that are not normally shed. Physiological stimuli, such as TNF and fMLP, are very poor modulators of CD43 and CD44 cleavage, but cause efficient downregulation of L-selectin (4). Thus, it is unclear under what conditions CD43 and CD44 would normally be rapidly downregulated.

The mechanism involved to downmodulate L-selectin expression is distinct from that of many other downregulated proteins in its sensitivity to protease inhibitors. As discussed above, L-selectin cleavage appears to be insensitive to a battery of common protease inhibitors. In marked contrast common serine protease inhibitors have been shown to inhibit release of pro-TGFα, KL-1, CD14 and CD44 (3, 12, 42), while metalloprotease inhibitors have been shown to inhibit release of folate receptor and CD16-II (16, 21). Interestingly, downregulation of CD43 appears to be sensitive to both serine and metalloprotease inhibitors (4, 12, 46). These results suggest that the mechanism to downregulate L-selectin is distinct from the mechanism involved in downregulation of these other cell surface proteins, and they suggest at least three distinct protease activities: one susceptible to serine protease inhibitors (3, 12, 42), another susceptible to metalloprotease inhibitors (16, 21), and a third that is resistant to most common protease inhibitors (35, 44).

Moreover, the cleavage sites for some of these proteins have been determined, but have no significant regions of homology to each other or to the putative cleavage site of L-selectin. The cleavage of TGF-α and Kit ligand has been mapped to a region rich in small apolar residues, such as alanine and valine (42). In contrast, the cleavage region of L-selectin is strikingly devoid of these residues, and the actual cleavage site (K↑SFS) occurs after a charged lysine residue. It is possible that sequence specificity at the cleavage site is not necessary, but that positional effects or a protease docking site may be more critical (7). The Ser-Phe-Ser (SFS) motif after the cleavage site of L-selectin is conserved between human, mouse, and rat L-selectin (Fig. 5 *B*). Interestingly, an SFS motif is found eight residues proximal to the transmembrane domain of CD16-II (45), and a related Ser-Phe-Leu motif is found 18 residues and 16 residues proximal to the transmembrane domains of TNF receptor type II (32) and ACE (53), respectively. It is unknown whether these motifs are involved in the downregulation of these proteins, but it suggests the possibility of a common recognition sequence.

Finally, we cannot formally exclude the possibility that the protease activity is intrinsic to L-selectin. There is precedent for autoproteolytic activity in proteins, although these proteins typically have conserved consensus sequences of one of the major protease families. L-selectin lacks any obvious homology to known proteases, although we cannot exclude the possibility of a novel or unusual protease. Letellier et al. (35) have provided evidence that the IgE Fc receptor, which like L-selectin has a C-type lectin domain, may have autoproteolytic activity.

The physiological requirement for rapid L-selectin downregulation remains to be proven. It was originally postulated that L-selectin downregulation may be necessary for neutrophils to progress from initial contact to transendothelial migration (29). Alternatively, L-selectin downregulation may serve as a protective mechanism to prevent neutrophils activated in circulation from entering an inappropriate tissue site. More recently, Palecanda et al. (40) showed that crosslinking of L-selectin causes its downregulation, and proposed that shedding of L-selectin may be a continuous process during neutrophil rolling and normal leukocyte trafficking. The continuous release of L-selectin during leukocyte trafficking and neutrophil rolling in response to subclinical stimuli may account in part for the high levels of circulating L-selectin in normal serum (47). The finding that cultured neutrophils actively synthesized L-selectin suggests the possibility that at least some of the neutrophil L-selectin can be replenished by de novo synthesis. The identity of the natural cleavage site may be useful in designing sequence-specific protease inhib-

itors to determine the biological consequences of interfering with this precisely regulated processing event and as a tool to identify the natural protease.

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