Mutational Analysis of the Membrane-proximal Cleavage Site of L-Selectin: Relaxed Sequence Specificity Surrounding the Cleavage Site

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Summary

L-selectin expression is regulated in part by membrane-proximal cleavage from the cell surface of leukocytes and L-selectin-transfected cells. The downregulation of L-selectin from the surface of neutrophils is speculated to be a process involved in the adhesion cascade leading to neutrophil recruitment to sites of inflammation. We previously reported that L-selectin is cleaved between Lys321 and Ser322 in a region that links the second short consensus repeat (SCR) and the transmembrane domain. We demonstrate that replacing this cleavage domain of L-selectin with the corresponding region of E-selectin prevents L-selectin shedding, as judged by inhibiting the generation of the 68-kD soluble and 6-kD transmembrane cleavage products of L-selectin. Unexpectedly, we found that point mutations of the cleavage site, as well as mutations of multiple conserved amino acids within the cleavage domain, do not significantly affect L-selectin shedding. However, short deletions of four or five amino acids in the L-selectin cleavage domain inhibit L-selectin downregulation. Mutations that appeared to inhibit L-selectin shedding resulted in higher levels of cell surface expression, consistent with a lack of apparent proteolysis from the cell membrane. One deletion mutant, I327N332, retains the native cleavage site yet inhibits L-selectin proteolysis as well. Restoring the amino acids deleted between I327 and N332 with five alanine residues restores L-selectin proteolysis. Thus, the proteolytic processing of L-selectin appears to have a relaxed sequence specificity at the cleavage site, and it may depend on the physical length or other secondary structural characteristics of the cleavage domain.

The recruitment of leukocytes to sites of inflammation involves a highly regulated cascade of events involving multiple adhesion proteins and chemokines (1–3). The nature of leukocyte migration requires that these adhesive interactions be transient in nature. Thus, adhesion molecules themselves must be precisely regulated. Adhesion may be rapidly induced by a variety of mechanisms, from qualitative changes in receptor activity (4, 5) to quantitative upregulation of rapidly mobilized pools of receptors (6, 7). However, mechanisms that downregulate adhesion molecules or that cause de-adhesion are less well characterized. L-selectin is a major leukocyte adhesion protein involved in lymphocyte homing to peripheral lymph nodes and in the initial "rolling" type interactions of leukocytes with inflamed endothelium (8–10). Endothelial ligands for L-selectin include sulfated mucins, such as GlyCAM-1 and CD34, and the MECA-79 antigen (11–13). Expression of L-selectin is downmodulated in part by an unusual protease activity that causes rapid and inducible cleavage of L-selectin from the cell membrane (14–18).

The selectin family of adhesion molecules is composed of three members, P-selectin, E-selectin, and L-selectin (8). The distribution and regulation of each molecule are distinct; P-selectin expression is rapidly induced by mobilization of intracellular pools to the surface of activated platelets and endothelial cells, E-selectin is synthesized de novo by cytokine-activated endothelium, and L-selectin is constitutively expressed on the surface of most leukocytes. The structure of each molecule is characterized by an NH2-terminal C-type lectin domain, an epidermal growth factor (EGF)-homologous domain, a variable number of short consensus repeats (SCRs), a transmembrane domain, and a COOH-terminal cytoplasmic domain. The extracellular domains of the selectins are highly conserved with the exception of a short sequence that links the last SCR with the transmembrane domain (19–26). Although soluble forms of E- and P-selectin have been identified, the primary mechanism for E- and P-selectin downregulation appears to be due to internalization (27, 28). L-selectin is the only member that appears to be actively downregulated from the cell surface through a rapid and inducible

1 Abbreviations used in this paper: ACE, angiotensin-converting enzyme; β-APP, β-amyloid precursor protein; EGF, epidermal growth factor; L-CDX, L-selectin cleavage domain exchange; SCR, short consensus repeat.
cleavage event. Upon activation by a variety of chemotactic factors, L-selectin is rapidly downregulated from the cell surface of neutrophils (14). A soluble form of L-selectin is present (1–2 μg/ml) in normal human serum (29). L-selectin proteolysis is unusual in that it is resistant to a variety of protease inhibitors (reference 18 and unpublished observations). Although low levels of exogenous chymotrypsin selectively cleave L-selectin from the surface of neutrophils (30), chymotrypsin inhibitors such as N-tosyl-l-phenylalanine chloromethyl ketone, PMSE, and aprotinin fail to inhibit L-selectin downregulation.

We have recently determined that L-selectin is cleaved between Lys321 and Ser322 in a short region that links the second SCR with the transmembrane domain (15). Cleavage at this membrane proximal site of the 74-kD membrane bound form of L-selectin results in the release of a soluble 68-kD L-selectin ectodomain and the formation of a 6-kD L-selectin transmembrane peptide (L-STMP) cleavage product (15). To better characterize the proteolytic event, we have constructed point mutants of the cleavage site and of conserved residues within the cleavage region. In addition, we have created a chimeric molecule that consists of the lectin, EGF, SCR1, SCR2, transmembrane and cytoplasmic domains of L-selectin, and the extracellular membrane proximal region of E-selectin. We demonstrate that replacing the L-selectin cleavage domain with the corresponding domain from E-selectin effectively inhibits downregulation of L-selectin. In addition, a series of short deletions (4–5 residues) within the cleavage domain also inhibits L-selectin shedding. In one deletion, the five deleted amino acids were restored with five alanine residues, which in turn restored L-selectin proteolysis. However, point mutations of the cleavage site and mutations of multiple conserved residues within the cleavage domain do not appear to significantly inhibit L-selectin downregulation. These results indicate that the proteolytic processing of L-selectin may depend on the physical length or other secondary structural characteristics of the cleavage domain and may not require strict sequence specificity.

Materials and Methods

Construction of L-Selectin Mutants. L-selectin mutants were generated using the Altered Sites oligonucleotide-directed mutagenesis system from Promega Biotech (Madison, WI) or by recombinant PCR. Mutations in human L-selectin cDNAs were confirmed by restriction digest in the plasmid pAlter (Promega Biotech) and subcloned into the pCDM8 vector (25). The cDNAs were screened for orientation and for the presence of the mutation by direct PCR of bacterial lysates. Briefly, 1 μl of a 500-μl overnight bacterial culture was lysed in the presence of 100 μg/ml lysozyme; 0.5% NP-40, 10 mM Tris, 1 mM EDTA at 94°C for 3 min. Samples were then subjected to 25 cycles of PCR using a forward primer specific for pCDM8 and a reverse primer specific for the mutation. PCR products were analyzed for the presence of predicted bands on 1% agarose gels. Clones that yielded the predicted band were sequenced and checked for the presence of the engineered mutations and for the absence of spontaneous mutations.

Transfection of COS Cells. COS cells (2.9 × 10⁶ cells per cuvette) were mock transfected or transiently transfected with 2 μg of mutant or wild-type L-selectin by electroporation in the presence of DEAE-dextran (31). Each cuvette was diluted into 35 ml of complete RPMI, and from this, three 100-mm tissue culture dishes and one 60-mm tissue culture dish was plated. Transfectants were allowed to recover in nonselective media supplemented with 10% FCS.

Metabolic Labeling. One 100-mm plate of COS transfectants was metabolically labeled with [35S]methionine on day 3 or 4 (day 1 = day of transfection) as previously described (15). Briefly, transfected monolayers (~75–85% confluent) were preincubated with methionine-free media to deplete intracellular methionine pools. After this preincubation, cells were pulsed with 500 μCi (5 ml at 100 μCi/ml) of [35S]methionine for 30–60 min and chased with complete RPMI medium for 30–120 min as indicated. Cell-free supernatants were harvested, and cellular debris was removed by centrifugation. COS monolayers were then lysed in 1% Triton X-100, 20 mM Tris, 150 mM NaCl, 1 mM PMSE, 1 mM aprotinin for 30 min at 4°C, and cell nuclei were removed by centrifugation at 13,000 g for 30 min.

Immunoprecipitation. Cell lysates and cell-free supernatants were immunoprecipitated as previously described (15). Cell lysates were precleared with normal rabbit serum (1:200) and protein A-Agarose. Cell supernatants were incubated with a 1:200 dilution of a rabbit polyclonal antiserum directed against the ectodomain of L-selectin (JK923) or preimmune sera, while precleared cell lysates were incubated with a 1:200 dilution of an antiserum directed against the cytoplasmic domain of L-selectin (JK564) or preimmune sera. Samples were then incubated with 20 μl of protein A-Agarose for 30 min at 4°C while being rotated end-over-end, and they were then washed extensively. After the final wash, material specifically bound to the Sepharose was eluted in tricine SDS-PAGE sample buffer (Novex, San Diego, CA) and incubated at 90°C for 5 min.

SDS-PAGE and Autoradiography. Immunoprecipitated samples were run on tricine-SDS polyacrylamide 10–20% gradient gels (Novex). Gels were then fixed in 30% methanol, 10% acetic acid, 1% glycerol, and treated with an autoradiography enhancer (Emulsion A/B; DuPont-NEN, Boston, MA), dried in cellophane, and exposed to x-ray film (X-Omat; Eastman Kodak Co., Rochester, NY) at −70°C.

ELISA for Soluble L-Selectin. Supernatants were collected from COS transfectants on day 3 or 4 posttransfection. Cellular debris was removed by centrifugation at 2,000 rpm for 15 min, followed by syringe filtration through a 0.2-μm filter. 96-well plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with 100 μl DREG-55 (anti-L-selectin) at 10 μg/ml and blocked with 2% BSA. Filtered supernatants were serially diluted and applied to 100-μl aliquots to the wells, and the presence of a soluble cleavage product was detected using 100 μl biotinylated DREG-200 (anti-L-selectin) at 1 μg/ml, a streptavidin-horseradish peroxidase conjugate, and 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) substrate per the manufacturer's instructions (Zymed Labs, Inc., South San Francisco, CA). Soluble L-selectin levels were calculated by a log-logit analysis curve generated from a serial titration of a known standard of purified soluble L-selectin (Bender Medsystems, Vienna, Austria). Plates were read on a plate spectrophotometer (Molecular Dynamics, Sunnyvale, CA) at 405 nm.

FACS® Analysis. COS transfectants were washed with Ca²⁺/Mg²⁺-free PBS containing 2 mM EDTA, followed by addition of 2 ml tissue culture grade trypsin-EDTA solution (GIBCO BRL, Gaithersburg, MD) for 1–2 min. The trypsin was quenched by the addition of 5 ml PBS containing 5% goat serum and 2 mM EDTA. The cells were harvested in Ca²⁺/Mg²⁺-free PBS containing 5% goat serum and 2 mM EDTA by repeatedly pipetting and washed twice with 5% goat serum in PBS. Low doses of trypsin have pre-
Previously been shown not to affect L-selectin expression (reference 30 and data not shown). Cells were then incubated with 50 μg/ml DREG-56 or DREG-200 for 30 min at 4°C and washed twice with goat serum/PBS. Cells were then incubated with a 1:100 dilution of phycoerythrin-conjugated F(ab')2 fragments of goat anti–mouse IgG (Tago Inc., Burlingame, CA) at 4°C for 30 min and washed twice with goat serum/PBS. Cells were suspended at ~1 × 10^6 cells per ml in 1% paraformaldehyde/RPMI and were analyzed by flow cytometry on a FACScan® (Becton Dickinson & Co., Mountain View, CA). To confirm surface expression of the L-selectin mutants, duplicate plates of COS transfectants were stained in situ (without harvesting) and analyzed by fluorescence microscopy.

Radiochemical Sequencing. Radiochemical sequencing was performed as previously described (15). Briefly, COS cells were transfected with mutated L-selectin cDNA and metabolically labeled with [35S]methionine. The 6-kD transmembrane cleavage product was immunoprecipitated and the material was subjected to SDS-PAGE as described. The sample was then electrotransferred from the gel to a polyvinylidifluoride membrane (Immobilon P®; Millipore Corp., Bedford, MA), which was then air dried and exposed to x-ray film at −70°C (Kodak X-OMAT). Once the film was developed, it was aligned with the original Immobilon membrane, and the area of the membrane corresponding to the location of the 6-kD species was excised and subjected to radiochemical sequencing on a pulse-liquid protein sequencer (model 477A; Applied Biosystems Inc., Foster City, CA). Fractions collected from each degradation cycle were analyzed in a liquid scintillation counter.

Analysis of Downregulation of Mutated L-Selectin Clones. L-selectin mutants were transiently transfected into COS cells on day 1 and stained with a phycoerythrin-conjugated anti-L-selectin antibody on day 3 or 4. Cell-free supernatants were saved for analysis by trapping ELISA. A 100-mm plate was metabolically labeled with [35S]methionine on day 3 or 4 after transfection, and radiolabeled cellular lysates were immunoprecipitated with JK564 rabbit anti-L-selectin cytoplasmic domain serum. Cell-free supernatants were immunoprecipitated with JK923 rabbit anti-L-selectin ectodomain serum. Mock transfectants and preimmune sera were used as nega-

![Figure 1](image-url)

Figure 1. Exchange of the membrane-proximal region of L-selectin with the corresponding region of E-selectin prevents L-selectin proteolysis. (A) Schematic representation of the membrane-proximal cleavage domain of L-selectin in relation to the whole molecule (SCR, short consensus repeat; TM, transmembrane domain; Cy, cytoplasmic domain). The estimated molecular weight of the intact molecule and the predicted sizes of the L-selectin cleavage fragments are indicated. The sequence of the cleavage domain of human, mouse, and rat L-selectin are aligned, and identical residues within the cleavage domain are boxed. The predicted cleavage site is indicated with an inverted triangle, and the P1, P2, P3, Pi, Pi', and P3' positions of the cleavage site are indicated (36). The corresponding regions of E-selectin and the L-CDX mutant are shown. (B) The L-CDX mutant is expressed on the cell surface of COS cell transfectants. COS cells were mock transfected or transfected with L-selectin or L-CDX cDNA, as indicated, and then stained for cell surface expression with DREG-200 mAb and a goat anti–mouse phycoerythrin second-stage antibody. Cells were harvested and analyzed by flow cytometry. Mean fluorescence values (MFV) of the whole population and the percentage of cells falling in the G1 gate are indicated. (C) The 6-kD transmembrane cleavage product of L-selectin is not efficiently produced by L-CDX transfectants. COS cells were transfected with wild-type L-selectin, L-CDX, or mock-transfected as indicated. On day 3 after transfection, cells were pulse labeled with [35S]methionine for 60 min and chased for 30 min. Cell lysates were immunoprecipitated with antiserum directed against the cytoplasmic domain of L-selectin, and the lower arrow denotes the position of the 6-kD transmembrane cleavage product, as previously described (15). (D) Radiolabeled soluble L-selectin is not detectable in the supernatants of L-CDX transfectants. COS cells were transfected with wild-type L-selectin, L-CDX, or mock-transfected as indicated. On day 3 after transfection, cells were pulse labeled with [35S]methionine for 60 min and chased for 30 min. Cell lysates were immunoprecipitated with an antisera directed against the cytoplasmic domain of L-selectin or with preimmune serum, as indicated. The upper arrow denotes the position of the intact membrane-bound L-selectin, and the lower arrow denotes the position of the 6-kD transmembrane cleavage product, as previously described (15). (E) ELISA analysis for soluble L-selectin. Cell-free supernatants were harvested from COS 3 d after transfection and serially diluted. Soluble L-selectin present in these supernatants was trapped with DREG-55 anti-L-selectin mAb and detected with biotinylated DREG-200 anti-L-selectin mAb, as described in Materials and Methods. Soluble L-selectin levels were quantitated by constructing a standard curve using a purified soluble L-selectin standard.
Results

An L-Selectin Chimera Containing the E-Selectin Membrane-proximal Region is Resistant to Proteolysis. We have previously shown that L-selectin is cleaved within a short membrane proximal region (cleavage domain) that links the second SCR with the transmembrane domain (15). The extracellular domains of L-, E-, and P-selectin are highly conserved (19-26); however, the short cleavage region of L-selectin has no homologous counterpart in E- or P-selectin (Fig. 1 A). L-selectin is the only member of the family that appears to be actively regulated through a rapid and inducible cleavage event from the cell surface (14, 16, 27, 28). To determine if the cleavage domain is critical for L-selectin proteolysis, a chimeric molecule termed L-selectin cleavage domain exchange (L-CDX) was constructed, consisting of the lectin, EGF, SCR1, SCR2, transmembrane, and cytoplasmic domains of L-selectin, as well as the extracellular membrane proximal domain of E-selectin (Fig. 1 A).

Immunofluorescence microscopy (data not shown) and FACS® analyses (Fig. 1 B) indicated that the L-CDX chimera was expressed on the cell surface of transfected cells, and multiple transfection experiments revealed that L-CDX was consistently expressed at higher surface levels than wild-type L-selectin (Fig. 1 B). Proteolysis of L-selectin was evaluated by the presence or absence of L-selectin cleavage products in metabolically labeled cell lysates and cell-free supernatants. We have previously shown that leukocytes and COS cells proteolytically cleave the 74-kD membrane-bound L-selectin to form a 68-kD soluble fragment and a 6-kD transmembrane fragment (15). Antiserum JK564 directed against the L-selectin cytoplasmic domain immunoprecipitated the 74-kD cell surface form of L-selectin, as well as the 6-kD transmembrane cleavage fragment from lysates of wild-type L-selectin transfectants (Fig. 1 C, lane 2). Antiserum JK923 directed against the ectodomain of L-selectin immunoprecipitated the 68-kD soluble fragment of L-selectin from cell-free supernatants of wild-type L-selectin transfectants (Fig. 1 D, lane 2). Immunoprecipitation of lysates from L-CDX transfected cells with JK564 yielded large amounts of intact L-CDX; however, the amount of the 6-kD transmembrane cleavage fragment was greatly reduced (Fig. 1 C, lane 3). In addition, cell-free supernatants of L-CDX immunoprecipitated with JK923 did not yield a detectable soluble 68-kD species (Fig. 1 D, lane 3). To confirm these results, unlabelled cell-free supernatants were analyzed by a trapping ELISA to detect the released soluble L-selectin fragment. The amount of soluble L-selectin detected in the supernatants of L-CDX transfected cells was ~32-fold less than that detected in the supernatants of wild-type L-selectin transfectants (Fig. 1 E). Both the trapping mAb (DREG-55) and the detecting mAb (DREG-200) recognize the L-CDX molecule expressed on the cell surface of transfectants (Fig. 1 B and data not shown). These data indicate that replacing the membrane-proximal cleavage region of L-selectin with the corresponding E-selectin domain effectively inhibited L-selectin downregulation. In addition, the increased surface expression of L-CDX over that of wild-type L-selectin was consistent with a lack of proteolysis from the cell surface.

Point Mutations of the Cleavage Site Do Not Inhibit L-Selectin Downregulation. L-selectin is cleaved between Lys 321 and Ser 322 in lymphoblasts and in L-selectin-transfected COS cells (15). To determine whether the Lys and Ser at the PI and PI' positions of the cleavage site direct the specificity of cleavage, we mutated these amino acids to alanine. Clones K321A and S322A (Fig. 2 A) were expressed on the cell surface in amounts comparable with that of wild-type L-selectin, as determined by flow cytometry (data not shown). Antiserum directed against the cytoplasmic domain of L-selectin immunoprecipitated both the full-length cell surface form of the mutant L-selectin, as well as a 6-kD transmembrane cleavage product (Fig. 2 B, lanes 3 and 4), indicating that the K321A and S322A mutants are cleaved as efficiently as wild-type L-selectin (Fig. 2 B, lane 2). A soluble form of L-selectin could be detected in the cell-free supernatant of K321A and S322A transfecteds by immunoprecipitation of radiolabeled material and by ELISA assay of cold supernatants (data not shown).
shown). A double-point mutation that simultaneously changed both the lysine and serine residues to alanine (K321S/AA, Fig. 2A) was also cleaved normally (Fig. 2C, lane 7). Similarly, more radical point mutations that changed the net charge at the cleavage site by mutating the lysine or serine residues to glutamic acid (K321E and S322E, Fig. 2C, lanes 4 and 6) caused little change in L-selectin proteolysis. In all point mutations, the 6-kD transmembrane and the 68-kD soluble cleavage products were indistinguishable in size from the corresponding fragments of wild-type L-selectin (Fig. 2C), suggesting that the location of the cleavage site was not grossly altered. These results indicate that proteolysis of L-selectin from the surface of COS cells is not strictly dependent on the sequence at the cleavage site.

**Mutations of Conserved Amino Acids in the Membrane-proximal Cleavage Region Do Not Inhibit L-Selectin Downregulation.** The cleavage domain of L-selectin spans a 15-amino acid region that links the transmembrane domain and the last SCR domain of L-selectin. 10 of these amino acids are conserved across the rat, mouse, and human L-selectin sequences (Fig. 1A), suggesting evolutionary pressure to maintain these sequences. To determine if these sequences contribute to a potential protease docking site, these residues were mutated singly or in combination to alanine residues. Mutants F323A, S324A, I326A, K327A, Y331A, S322FS/AAA, and K327EGDY/AAAAA are illustrated in Fig. 3A. In addition, the glutamic acid residue at position 328 was mutated to glutamine (E328Q) to mimic an E-to-Q mutation found in a homologous position of the β-amyloid precursor protein from patients with Alzheimer's disease, Dutch type (32). The E-to-Q mutation in β-APP prevents proteolysis at the normal membrane proximal site and results in aberrant processing leading to the formation of amyloid plaques. All of the L-selectin mutants were expressed on the cell surface in amounts comparable with that of wild-type L-selectin (data not shown). Lysates of the single-point mutants yielded both the full-length cell surface form of the mutant L-selectin and the 6-kD transmembrane cleavage product using JK564 anticytoplasmic domain serum (Fig. 3B). Supernatants from each mutant also yielded a corresponding soluble 68-kD cleavage product when immunoprecipitated with JK923 antiectodomain serum (Fig. 3C). The 6-kD and 68-kD species immunoprecipitated from each mutant were indistinguishable in size from those immunoprecipitated from wild-type L-selectin.

A Ser-Phe-Ser motif adjacent to the cleavage site is conserved across species, and a related motif is found proximal to the transmembrane domains of three proteins that are also cleaved from cell surfaces: ACE (33), CD16-II (34), and TNF receptor II (TNF-RII) (35). However, mutating all three residues to alanine (S322FS/AAA) had no appreciable effect on L-selectin proteolysis, as judged by the formation of the 6-kD transmembrane fragment (Fig. 2C, lane 8) and the 68-kD soluble fragment (data not shown). Similarly, simul-
taneously mutating five conserved residues K327EGDY to alanine residues (K327EGDY/AAAAA) had no effect on L-selectin proteolysis (see Fig. 5 A and results below). The appearance of the 6-kD transmembrane species and the soluble 68-kD species indicate that mutating conserved residues in the cleavage domain does not significantly inhibit the down-regulation of L-selectin.

Short Deletions Within the Cleavage Domain Inhibit L-Selectin Proteolysis. The E-selectin extracellular membrane-proximal region is eight amino acids shorter than the 15-amino acid L-selectin region (Fig. 1 A). In the L-CDX chimeric molecule, this difference in size may allow for steric or structural interference with L-selectin proteolysis. We constructed a number of clones containing deletions of four to five consecutive amino acids at various locations throughout the membrane-proximal cleavage domain. Deletions spanning the cleavage site (K318ΔF323, L319ΔM325, and D320AΔI326) significantly prevented L-selectin proteolysis, as demonstrated by the lack of 6-kD transmembrane (Fig. 4 B) or 68-kD soluble (Fig. 4 C) cleavage products. The four-amino acid deletion (K318ΔF323) showed a small but detectable amount of the 6-kD cleavage product (Fig. 4 B, lane 3). In contrast, the other five amino acid deletions showed no detectable 6-kD cleavage product. All deletion mutants showed no detectable amounts of the 68-kD soluble cleavage product in supernatants of radiolabeled cells (Fig. 4 C). A low but measurable amount of soluble L-selectin could be detected by trapping ELISA in supernatants accumulated over a course of 3 d (data not shown). The deletion mutants were expressed on the cell surface, as judged by immunoprecipitation of the intact cell surface form of the L-selectin deletion mutants radiolabeled cell lysates (Fig. 4 B, upper arrow) and by immunofluorescence microscopy. FACS® analysis of these cells indicated that the mean fluorescence of cells stained was higher for the deletion mutants than for wild-type L-selectin (Table 1). To determine whether deletion of the actual cleavage site was necessary for inhibition of proteolysis, a five amino acid deletion (I326ΔN332) of the COOH-terminal side of the cleavage domain was generated. This deletion mutant retains the native cleavage site, yet L-selectin proteolysis was significantly inhibited (Fig. 4, B and C, lane 6). These data indicate that short deletions of the cleavage domain of L-selectin significantly inhibit proteolysis. This suggests that a minimal physical length of the cleavage domain may be important for accessibility to the site by the putative protease.

Restoring the Deleted Amino Acids in I326ΔN332 with Five Alanine Residues Restores L-Selectin Proteolysis. The truncation mutant I326ΔN332 retains the native cleavage site of L-selectin, yet it is not cleaved from the surface of transfected cells. To assess whether the native length and/or sequence of the cleavage domain were required for L-selectin proteolysis, we restored the five deleted amino acids in the I326ΔN332 mutant with five alanine residues (clone K327EGDY/AAAAA, depicted in Fig. 3 A). FACS® analysis indicated that native L-selectin and K327EGDY/AAAAA transfectants have comparable cell surface expression (data not shown). Restoration of the deletion with five alanine residues restores the ability of the mutant L-selectin to be cleaved. Immunoprecipitation of cell lysates of K327EGDY/AAAAA yielded a transmembrane cleavage product that migrated slightly faster than the wild-type cleavage product (Fig. 5 A, compare lanes 2 and 3). Cell-free supernatants immunoprecipitated with antiserum directed against the extracellular domain of L-selectin showed the expected 68-kD soluble cleavage product (Fig. 5 B). In contrast, the original deletion mutant I326ΔN332 showed no detectable 6-kD transmembrane or 68-kD soluble cleavage products (Fig. 5, A and B, lane 1).

Since the 6-kD cleavage product of the K327EGDY/AAAAA migrated faster than the native cleavage product, we used radiochemical sequence analysis to determine if the cleavage site was shifted. We have previously shown that the 6-kD cleavage product of wild-type L-selectin transfected cells metabolically labeled with [35S]methionine yields radioactive methionine peaks at cycles 4 and 20, indicating cleavage between Lys321 and Ser322 (15). The cleavage site was verified by radiochemical sequencing of cells labeled with [3H]phenylalanine and other tritiated amino acids (reference 15 and data not shown). Radiochemical sequence analysis of the 6-kD product from [35S]methionine-labeled K327EGDY/AAAAA mutant also yielded peaks at cycles 4 and 20 (Fig. 5 C), indicating that cleavage occurs at the native site between Lys321 and Ser322. The apparent size difference of the K327EGDY/AAAAA mutant 6-kD product may be a result of anomalous migration of the small alanine-rich peptide in gel electrophoresis.

These results indicate that restoring the native length, but not the native sequence, of the L-selectin cleavage region restores L-selectin proteolysis. In addition, L-selectin proteolysis occurs at the native cleavage site, between Lys321 and Ser322, despite the five-residue mutation. These data may suggest that L-selectin proteolysis occurs at a defined length from the membrane and further support the model that L-selectin proteolysis may depend on the physical length, but not necessarily the sequence, of the cleavage domain.

Table 1. Cell Surface Expression of the L-Selectin Deletion Mutants

| Mutant       | MFV* |
|--------------|------|
| Mock         | 18.5 |
| L-selectin   | 147.0|
| K318ΔF323    | 475.4|
| L319ΔM325    | 451.4|
| D320AΔI326   | 436.3|
| I326ΔN332    | 401.7|

* Mean fluorescence value of the total population.
requirement for L-selectin proteolysis. In contrast, short deletions within the cleavage region have a profound affect on L-selectin proteolysis. It is possible that the cysteine residue at position 316 of L-selectin, which contributes to an intrachain disulfide bridge in the second SCR, may allow for sterical or structural interference with the protease and prevent cleavage at upstream sequences.

Discussion

L-selectin downregulation involves an unusual and yet-unknown proteolytic mechanism. Most proteases can be classified into one of four basic protease families—serine protease, metalloprotease, aspartic protease, or cysteine protease—based on their inhibition by specific mechanism-based protease inhibitors (36). However, previous studies have shown that broad panels of common protease inhibitors have no effect on L-selectin proteolysis (reference 18 and unpublished observations). Moreover, most proteases also show some degree of sequence specificity in the P1 or P1′ positions of the cleavage site (36). We have previously shown that the cleavage event occurs between Lys321 and Ser322 in a short region that links the last SCR with the transmembrane domain (15). In this report we demonstrate further that L-selectin proteolysis is remarkably resilient to point mutations of the cleavage site. The lysine at the P1 position is suggestive of a possible trypsinlike specificity, yet a mutation that radically changes the net charge of the P1 residue from a basic lysine residue to an acidic glutamic acid is still efficiently cleaved. 10 of 11 amino acid residues downstream of the cleavage site are conserved across mouse, rat, and human L-selectin (21-26, 37), suggesting a possible conserved protease docking site. The S322FS motif was of particular interest, since a related motif is found proximal to the transmembrane domains of other cleaved membrane proteins, such as ACE (33), CD16-II (34), and TNF receptor type II (35). However, mutation of the SFS motif to three alanine residues had no dramatic effect on L-selectin proteolysis. Similarly, alanine scanning mutagenesis of other conserved residues showed no strict sequence requirement for L-selectin proteolysis. In contrast, short deletions within the cleavage domain have a dramatic impact on L-selectin proteolysis. The I326ΔN332 deletion mutant retains the native cleavage site but is not effectively cleaved from the cell surface. Restoration of the deleted amino acids with five alanine residues restores L-selectin proteolysis. These results suggest that the proteolytic cleavage of L-selectin appears to have a relaxed sequence specificity at the cleavage site, but that a minimal physical length of the cleavage domain may be important for the accessibility to the site by the putative protease.

The apparent relaxed sequence specificity of the cleavage site is reminiscent of that described for several other cell surface molecules that are known to undergo membrane-proximal proteolysis. Membrane-anchored precursors of growth factors, such as TGF-α, EGF, CSF-1, TNF-α, and the c-kit ligand, are cleaved to release a soluble form of growth factor (38, 39). The cleavage region of TGF-α and TNF-α must be extensively mutated to inhibit proteolysis (39-41). However, the protease activity involved in cleaving these membrane-anchored growth factors shows specificity for small aliphatic residues, such as alanine and valine, and can be inhibited by serine protease inhibitors (39). Other proteins such as the β-amyloid precursor protein (β-APP) (42, 43), the folate receptor (44), TNF receptor (35, 45), IL-6 receptor (46), and ACE (47) are also released from cell membranes by a regulated proteolytic mechanism. The proteolysis of ACE (47), IL-6 receptor (46), and the TNF receptor (45), like that of L-selectin, appears resistant to a broad panel of protease inhibitors. Interestingly, rabbit ACE is cleaved between an arginine and a serine residue (47), and β-APP (42, 43), is cleaved between a lysine and a leucine residue. These cleavage sites are homologous to the lysine and serine residues at the P1 and P1′ positions, respectively, of the L-selectin cleavage site. Also, like L-selectin, these other receptors are cleaved when expressed in COS or Chinese hamster ovary cells, and the cleavage specificity is retained (42, 43, 46, 47). Despite the apparent similarity of these cleavage sites, the cleavage regions of β-APP, ACE, and L-selectin can be mutated without dramatic effect on the efficiency of proteolysis. Moreover, large deletions in the membrane proximal cleavage regions of β-APP and ACE have no affect on their proteolysis (42). Cryptic cleavage sites in upstream sequences of β-APP are activated; however, cleavage occurs at a similar distance from the transmembrane domain as found in the native cleavage site, suggesting that the physical distance from the membrane is critical. In contrast, small deletions in the L-selectin cleavage region have a profound affect on L-selectin proteolysis. It is possible that the cysteine residue at position 316 of L-selectin, which contributes to an intrachain disulfide bridge in the second SCR, may allow for sterical or structural interference with the protease and prevent cleavage at upstream sequences.

Figure 5. Restoration of the I326ΔN332 deletion with five alanine residues also restores susceptibility to proteolysis. The five amino acid residues deleted in the I326ΔN332 mutation were replaced with five alanine residues. COS cells were mock transfected, transfected with wild-type L-selectin, the I326ΔN332 deletion mutant, or the restored K327-EGDY/AAAAA mutant, as indicated. (A) Cell lysates were analyzed for the presence of the 6-kD transmembrane cleavage product, and (B) cell-free supernatants were analyzed for the presence of the soluble L-selectin cleavage fragment. (C) The K327-EGDY/AAAAA mutant is cleaved between Lys 321 and Ser 322. The 6-kD transmembrane cleavage product was isolated from [35S]methionine-labeled transfected subjected to SDS-PAGE and transferred to polyvinyldifluoride membrane. The 6-kD band was visualized by autoradiography, excised, and subjected to a pulse-liquid protein sequencer. Assignment of methionine residues was made based on radioactive peak fractions and aligned to the cleavage region of L-selectin, as described previously (15).
If the primary sequence of the L-selectin cleavage site does not direct proteolysis, then the protease must use some other docking site or other means of recognizing its substrate. In the case of TGF-α, Bosenberg et al. (48) elegantly demonstrated that the COOH-terminal valine of the cytoplasmic domain was required for proteolysis at the extracellular juxtamembranous site. However, this is not likely to be a universal domain was required for proteolysis at the extracellular juxtamembranous site. However, this is not likely to be a universal theme for all cleaved proteins, since cytoplasmic tail truncation of the TNF receptor does not affect its proteolytic release from the cell membrane. Similarly, we have found that truncation of the last five residues of the cytoplasmic tail of L-selectin does not prevent proteolysis, however, the cytoplasmic tail may influence the efficiency of proteolysis (Kahn, J., and T. K. Kishimoto, unpublished observations). Understanding how a protease recognizes L-selectin as a substrate may provide a handle for identifying a putative protease. It is worth noting that a physiologically relevant protease has not been identified in the case of any protein that undergoes regulated membrane-proximal proteolysis. If a common proteolytic mechanism exists, then identification of such a protease could provide important insight into the regulation of inflammation (L-selectin, TNF receptor), hypertension (ACE), or the pathogenesis of Alzheimer’s disease (β-APP).

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