Structural Requirements Regulate Endoproteolytic Release of the L-Selectin (CD62L) Adhesion Receptor from the Cell Surface of Leukocytes

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Summary

L-selectin mediates leukocyte rolling on vascular endothelium at sites of inflammation and lymphocyte migration to peripheral lymph nodes. L-selectin is rapidly shed from the cell surface after leukocyte activation by a proteolytic mechanism that cleaves the receptor in a membrane proximal extracellular region. This process may allow rapid leukocyte detachment from the endothelial surface before entry into tissues. In this study, the structural requirements for regulation of human L-selectin endoproteolytic release were examined through analysis of chimeric selectin molecules and mutant L-selectin receptors. The use of chimeric selectins and a cytoplasmic tail truncation mutant demonstrated that the extracellular membrane-proximal 15–amino acid region of L-selectin is required for endoproteolytic release. The introduction of alanine-scanning mutations within this membrane-proximal region did not prevent endoproteolytic release, indicating that a specific amino acid motif was not an absolute requirement for cleavage. Furthermore, alterations within the putative primary cleavage site (K^{283}–S^{284}) resulted in either constitutive endoproteolytic release of the receptor or inhibition of cell activation–induced shedding to variable extents. The length of the membrane-proximal region was also critical since truncations of this region completely abolished endoproteolytic release. Thus, release of L-selectin is likely to be regulated by the generation of an appropriate tertiary conformation within the membrane-proximal region of the receptor which allows recognition by a membrane-bound endoprotease with relaxed sequence specificity that cleaves the receptor at a specific distance from the plasma membrane. These observations suggest a generalized protein-processing pathway involved in the endoproteolytic release of specific transmembrane proteins which harbor widely differing primary sequences at or neighboring their cleavage sites.

Cell function can be directly controlled by increasing or decreasing cell-surface receptor expression. While surface receptor expression can be regulated by a variety of mechanisms, three mechanisms predominantly account for the rapid down-regulation of cell surface receptors: internalization, endoproteolytic release of transmembrane proteins, and glycolipid cleavage of glycosylphosphatidylinositol-anchored proteins. In addition to down-regulation of receptor expression, endoproteolytic release often generates a soluble protein that retains receptor function (1, 2). Examples of shed receptors include growth factor receptors such as the receptors for IL-1, IL-2, insulin, growth hormone, TNF (3), CSF-1 (4), epidermal growth factor, and nerve growth factor (5). Also included in this list are several immunologically important receptors, including L-selectin (CD62L) (6–12), CD14, CD43, CD44, FcεRII (CD23), FcγRII (CD32), and FcγRIII (CD16). In addition, the β amyloid precursor protein which has been implicated in the genesis of Alzheimer's disease is proteolytically shed from the cell surface giving rise to amyloid β peptide (13). These proteins are quite diverse in structure and amino acid sequence and have few unifying functional characteristics (1, 2). However, only a small fraction of all cell-surface receptors are released from the cell surface. The precise substrate specificities and the mechanisms that regulate the membrane-bound endoprotease(s) that mediate in situ cleavage of these receptors have not been completely elucidated.

L-selectin is normally expressed on the surface of most leukocytes, including lymphocytes, neutrophils, monocytes, eosinophils, hematopoietic progenitor cells, and immature thymocytes (7, 11, 14, 15). L-selectin mediates the binding of lymphocytes to high endothelial venules (HEV)[1] of peripheral lymph nodes (7, 9, 12, 14), and is also involved in leukocyte attachment to endothelium at sites of inflamma-

[1]. *Abbreviations used in this paper:* CHO, Chinese hamster ovary; EGF, epidermal growth factor; HEV, high endothelial venules; SCR, short consensus repeat; sL-selectin, shed L-selectin.

tion (16–22). Leukocytes from mice lacking L-selectin are deficient in both activities (23–25). Human L-selectin is a highly glycosylated protein of 95–105,000 M_r on neutrophils and 74,000 on lymphocytes (11, 12, 26). L-selectin is rapidly shed from the cell surface after cellular activation in vitro or after exposure to the nonphysiologic stimulator phorbol esters (PMA) (6–9, 11, 12). In addition, shedding also occurs during overnight incubation of lymphocytes at 4°C and during cell culture (27–29). It has been proposed that endoproteolytic release of L-selectin might provide a rapid means for the regulation of leukocyte deadhesion necessary to enable leukocytes to detach from bound endothelial cells before transmigration between endothelial cells and into sites of inflammation (6, 17). However, the protease(s) that mediate endoproteolytic release of L-selectin appear ubiquitous, as opposed to cell type–specific, since a broad array of L-selectin–negative cell types tranfected with L-selectin cDNA are able to shed this receptor (9, 28–31). Shed L-selectin (sL-selectin) is found in normal plasma at high levels (~1–3 μg/ml) and retains functional activity (28, 29, 32, 33). Thus, the endoproteolytic release of L-selectin may not only be a means of regulating cell-surface receptor expression, but the shed receptor may also modulate leukocyte binding to endothelium in vivo.

The selectins have a unique and characteristic domain structure that includes an extracellular C-type lectin domain, a single epidermal growth factor (EGF)-like domain, and between two and nine short consensus repeat (SCR) units homologous to domains found in complement-binding proteins (30, 34–37). The three selectins (L-, E-, and P-selectin) are closely related to each other in amino acid sequence, ranging from ~40% identity in the SCR domains up to ~65% in the lectin and EGF-like domains (15). Although the amino acid sequences of the membrane-proximal region, transmembrane region, and cytoplasmic tail of L-selectin from various mammalian species are very well conserved, the three selectins are quite divergent in these regions (Fig. 1). It is likely that endoproteolytic release of L-selectin results from cleavage of L-selectin in the membrane-proximal region of the transmembrane domain, since sL-selectin is 5–6,000 less in M_r than cell-surface L-selectin (6, 8, 9, 30). In fact, one study using radiochemical sequencing has suggested that the protease cleavage site in L-selectin is the peptide bond between K^{283} and S^{284} in the membrane-proximal region (31). However, the proposed recognition motif K^{283}SFS is not well conserved in bovine L-selectin, while this receptor is also shed (38, 39). While soluble E- and P-selectin are also found in normal plasma, they are at levels significantly lower than L-selectin (32, 40, 41). However, soluble P-selectin is proposed to be generated by alternative splicing of the mRNA transcript with deletion of the transmembrane domain (36). Nonetheless, differences between the membrane-proximal regions of the selectins may explain why L-selectin is proteolytically cleaved from the cell surface at such a rapid rate after cellular activation in contrast to E- and P-selectin.

Understanding the mechanism for regulation of L-selectin endoproteolytic release will be important for understanding how this event may regulate leukocyte migration. Blocking endoproteolytic release may also provide a way to inhibit the accumulation of leukocytes at sites of inflammation. In addition, L-selectin also serves as an excellent model system for understanding the more general process of receptor endoproteolytic cleavage. In this report, the amino acid sequence requirements for L-selectin endoproteolytic release were examined using chimeric receptors, truncation mutants, and site-specific mutants. The results revealed a primary proteolytic activity with a relaxed sequence specificity dependent on an appropriate conformation within the membrane-proximal region of L-selectin. This functional activity is remarkably similar to that of the structurally undefined endoprotease, α-secretase, which mediates the conventional secretory processing of β-amyloid precursor protein, a cell-surface molecule involved in Alzheimer's disease (13). This suggests that these two proteins and others may be cleaved by a similar, if not identical, widely distributed membrane-bound endoprotease.

Materials and Methods

Construction of Chimeric and Mutant Selectin cDNA. Chimeric L- and P-selectin cDNA (P4L and L4P, Fig. 2 *A*) were constructed as described (42). The LΔcyto (Figs. 1 and 2 *A*) mutant receptor was generated as described (43). Site-specific mutations and deletions in the membrane-proximal region were introduced by standard PCR mutagenesis protocols or two-step recombinant PCR procedures. The K283A and KE mutant receptors (Fig. 2 *B*) were generated using two "inner" complementary PCR primers synthesized based on the membrane-proximal region sequence. These primers introduced new amino acid residues and unique restriction sites. Individual "outer" primers that were complementary to either sequences at the upstream KpnI or downstream NdeI sites were used with individual inner primers to generate DNA fragments using L-selectin cDNA in pSP65 as template for the first PCR reaction. The two resulting PCR products were hybridized together and used as templates for the second PCR reaction which used only the two outer primers. These products were digested with KpnI/NdeI and cloned into the KpnI and NdeI sites of L-selectin cDNA in pSP65. The KE mutant cDNA had a SacII site at the mutation site so it was used as a PCR template to make other alanine-scanning mutants within the membrane-proximal region (Fig. 2 *A*). One PCR primer containing the necessary mutation for each new receptor was synthesized based on the membrane-proximal region sequence of the KE mutant cDNA. This primer was paired with one of the two outer primers described earlier for PCR amplification. The resulting PCR products were digested with KpnI/SacII or SacII/NdeI and subcloned into the corresponding sites within the KE mutant cDNA in pSP65. The resulting mutant receptors therefore had alanine substitutions at KE^{290} in addition to the newly introduced mutations. Both the KLD.KE and KEGDYN mutants had an introduced NotI site at the mutation sites. Thereby, joining the appropriate EcoRI/NotI fragment of the KLD.KE and KEGDYN mutants generated a mutant in which the membrane-proximal region was replaced by AAAG. This AAAG mutant cDNA served as a template for generation of the ΔK-S and ΔM-N deletion mutants in a similar manner as described above using two complementary inner primers containing sequences encoding either MIKEGDYN or KLDKSFS. All of the chimeric and mutant cDNA were subcloned into pSP65 and were verified by sequence determination.

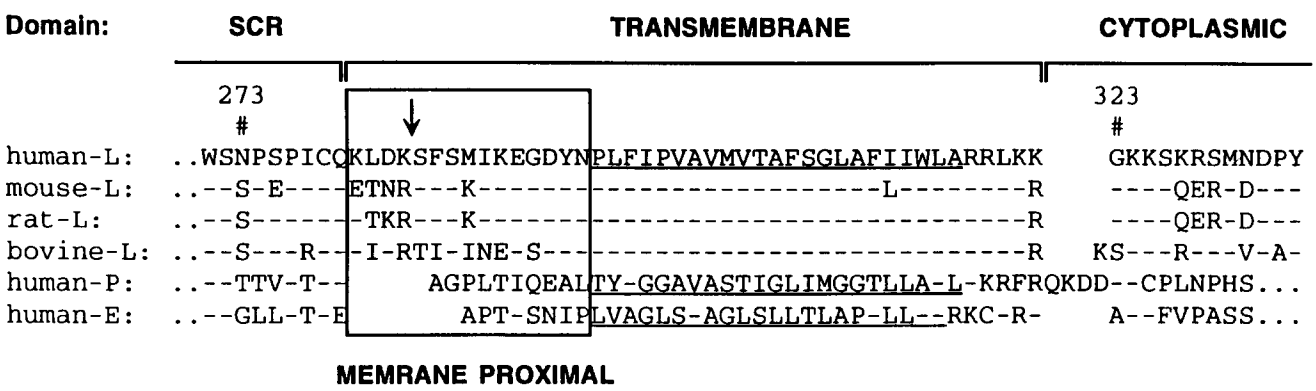
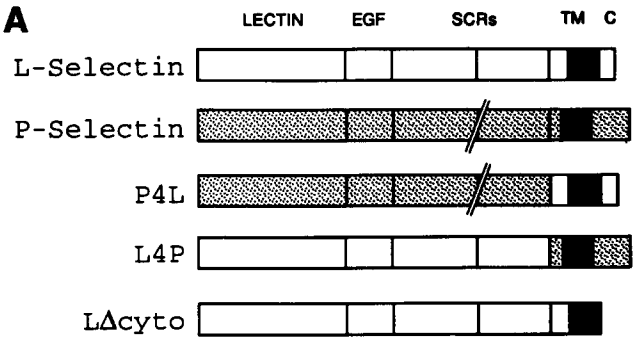


Figure 1. Amino acid sequence alignments of the membrane proximal and transmembrane regions of human (34), mouse (30), rat (37a), and bovine (38) L-selectin, human P-selectin (36), and human E-selectin (37). Amino acid positions for the mature human L-selectin protein are indicated above the sequence. The amino acid sequence for the bovine L-selectin cytoplasmic domain is not as published since an amino acid sequence that is optimal for alignment with L-selectin from other species is generated when it is assumed that an extra nucleotide was inserted between nucleotides 1214 and 1220 as published (38). The membrane-proximal region of the transmembrane domain of the selectins is boxed and the putative membrane-spanning regions are underlined. Dashes indicate amino acids identical to those found in human L-selectin with spaces introduced to generate optimal alignment. The Asn^{273} indicated by a # is the crossover point for the L4P chimeric selectin and the Gly^{323} indicated by a # is the truncation point for LΔcyto mutant receptor. Only a portion of the cytoplasmic domains of E- and P-selectin are shown as indicated by (...). The putative endopeptidase cleavage site is indicated by an arrow.



B

	280 294
L-Selectin	KLDKSFSMIKEGDYN
KLD.KE	AAAKSFSMIAAGDYN
K283A	KLDASFSMIKEGDYN
SFS.KE	KLDKAAAMIAAGDYN
MIKE	KLDKSFSAAAAGDYN
KE	KLDKSFSMIAAGDYN
KEGDYN	KLDKSFSMIAAAAAG
ΔK283	KLD-SFSMIKEGDYN
K283E	KLDESFSMIKEGDYN
K283P	KLDPSFSMIKEGDYN
ΔK-S	-------MIKEGDYN
ΔM-N	KLDKSFS--------
AAAG	----------AAAG

Figure 2. Structure and amino acid sequences of chimeric selectins and mutant L-selectin molecules. (*A*) Schematic structure of L-selectin, P-selectin, and chimeric receptors. Only two of the nine SCR present in P-selectin are represented. The membrane spanning region of the transmembrane domain (*TM*) is shown in black which proceeds the cytoplasmic (*C*) domain. (*B*) Mutations in the membrane-proximal region of the transmembrane domain are shown with altered amino acids underlined. Deleted amino acids are indicated by a dash.

Generation of Cell Lines Expressing Chimeric and Mutant Receptors. cDNA encoding native L-selectin, L4P, and the membrane-proximal region mutants were subcloned into the pMT2 expression vector and, along with the pSV2neo vector containing the neomycin resistance marker, were used to cotransfect the mouse pre-B cell line 300.19 (44). LΔcyto cDNA was subcloned into the pZIPneoSV vector and used to transfect 300.19 cells as reported (43). Stable transfectants of 300.19 cells were selected in medium containing 1.0 mg/ml G418 (geneticin; Sigma Chemical Co., St. Louis, MO). 20 clones from each transfection were examined by indirect immunofluorescence screening for L-selectin expression. The clones with the highest levels of expression were used in these studies. A clone of Chinese hamster ovary cells transfected with the P4L cDNA (termed PPP) was generated as described (45) and was generously provided by Dr. Bruce Furie (New England Medical Center, Boston, MA).

Antibodies. L-selectin mAbs were anti-LAM1-3 directed against an epitope within the lectin domain, anti-LAM1-1 identifying the EGF-like domain, and anti-LAM1-14 which reacts with SCR, all of the IgG_1 isotype (46). Anti-L-selectin mAbs were purified by salt fractionation followed by anion exchange chromatography, with the mAb concentration determined by light absorption. Purified mAbs were used for ELISA and ascites fluid diluted to the optimal concentration was used for indirect immunofluorescence staining. The AC1.2 mAb reactive with P-selectin was generously provided by Dr. Bruce Furie.

Cell Cultures and Induction of Endoproteolytic Release. 300.19 cells were cultured in RPMI 1640 medium containing 10% FCS, 2% L-glutamine, penicillin, streptomycin, and 2-mercaptoethanol. CHO-P4L cells were cultured in α MEM medium containing 10% FCS, 2% L-glutamine, penicillin, and streptomycin. All cells were incubated at 37°C in 5% CO_2 with 100% humidity. To induce endoproteolytic release for cell-surface analysis, ~0.5 × 10^6 cells were cultured in 0.4 ml of medium containing 50 ng/ml PMA in a test tube at 37°C for 30 min before analysis. To induce endoproteolytic release for ELISA analysis, ~2 × 10^6 cells were cultured in 0.4 ml of medium containing 50 ng/ml PMA in a test

tube at 37°C for 30 min. After centrifugation, the supernatant was saved and the pellet was lysed at 4°C for 30 min in 0.4 ml of lysis buffer (50 mM Tris-HCl, 0.5% NP-40, 150 mM NaCl, 0.1% BSA, 5 mM EDTA, 1 mM PMSF, pH 7.5). For studying spontaneous endoproteolytic release, ~2 × 10^6 cells were washed and cultured for 24 h in 10 ml of culture medium. Aliquots (1.5 ml) of the cell suspension were centrifuged and the supernatant and the cell pellet lysed as described above were used for ELISA analysis.

Indirect Immunofluorescence Analysis. Indirect immunofluorescence analysis was carried out after washing the cells three times. Cells (0.5 × 10^6) were resuspended in 100 μl of media containing optimal concentrations of mAb, and incubated for 30 min at 4°C. After washing, the cells were treated with FITC-conjugated goat anti-mouse Ig antibodies (Southern Biotechnology Associates, Birmingham, AL) for 20 min at 4°C. The cells were washed, and fixed (1.5% formalin in PBS), and single-color fluorescence analysis was carried out by flow cytometry (ELITE™; Coulter Immunology, Hialeah, FL). 10,000 cells were analyzed in each sample and the relative mean fluorescence intensity of cells was determined on a linear scale.

sL-Selectin ELISA. The sandwich ELISA used to quantitate sL-selectin levels in culture supernatant fluid and cell lysates was as described (29). Briefly, wells of 96-well microtiter plates were coated with the anti-LAM1-5 mAb, blocked, and the test samples were added to triplicate wells. After washing, the plates were incubated with biotinylated anti-LAM1-3 mAb, followed by avidin-horseradish peroxidase and finally developed. The OD of the reaction mixture was quantitated using an ELISA reader. Each assay included the titration of a previously quantified plasma sample that was used to generate a standard curve. The relative concentration of sL-selectin in individual samples was calculated by comparing the mean OD obtained from triplicate wells to a semilog standard curve of titrated plasma using linear regression analysis.

Frozen Section HEV-binding Assays. Rat lymph nodes were obtained from freshly killed Lewis rats, snap-frozen in isopentane/liquid nitrogen, and stored at −70°C in isopentane until use. The HEV assay was as described (47). Each cell type (5 × 10^6 cells) was incubated on three 12-μm sections/slide at 64 rpm for 25 min at 4°C. The excess cells were gently removed, and the slides were placed vertically in ice-cold fixative (2.4% glutaraldehyde in PBS) overnight. The slides were then counterstained with Gill's hematoxylin, overlaid with glycerol gelatin, and coverslips were applied. Each slide was scored for the number of test cells bound/HEV and 100–200 HEV were counted for each experiment.

Results

Construction and Expression of Selectin Chimeras and L-Selectin Mutants. The chimeric P4L receptor was generated with the lectin, EGF, and SCR domains of P-selectin attached to the transmembrane (including membrane-proximal region) and cytoplasmic domains of L-selectin (Fig. 2 *A*). L4P is a similar domain swap mutant except the extracellular domain of L-selectin is attached to the transmembrane and cytoplasmic domains of P-selectin. A cytoplasmic tail deletion mutant of L-selectin (LΔcyto) was also generated by introducing a stop codon at amino acid position G^{324} in the cytoplasmic domain (Figs. 1 and 2 *A*) as described (43). Site-specific mutations and deletions within the membrane-proximal region of the transmembrane domain of L-selectin were also generated (Fig. 2 *B*) using PCR-based mutagenesis protocols.

Table 1. *Cell-surface Expression of Chimeric and Mutant L-Selectin Receptors*

	mAb Reactivity (Mean linear fluorescence channel number)				
Receptor	Control	LAM1-1	LAM1-3	LAM1-14	AC1.2
L-selectin	0.2	3.5	11.0	3.5	–
LΔcyto	0.4	2.1	10.7	1.9	–
P4L	0.4	–	–	–	1.0
L4P	0.4	1.6	2.5	1.1	–
KLD.KE	0.3	1.5	3.9	1.0	–
K283A	0.2	0.2	0.2	0.2	–
SFS.KE	0.3	4.5	10.7	5.7	–
MIKE	0.3	0.8	2.2	0.6	–
KE	0.3	2.0	5.6	1.8	–
KEGDYN	0.3	1.2	2.4	1.1	–
ΔK283	0.2	11.3	19.3	10.7	–
K283E	0.3	4.6	9.0	5.6	–
K283P	0.2	3.9	8.1	4.3	–
ΔK-S	0.2	6.7	17.9	7.6	–
ΔM-N	0.4	12.2	30.5	12.3	–
AAAG	0.4	13.0	18.0	4.1	–

All results shown are for cDNA-transfected 300.19 cells, except P4L which is expressed in CHO cells. Background staining levels for untransfected 300.19 cells and CHO cells were ~0.3 fluorescence channel units. Similar results were obtained in at least three experiments.

The chimeric and mutant cDNA were used for stable transfection of an L-selectin deficient mouse pre-B cell line, 300.19, and for transient transfection of COS cells. Cell-surface expression of the chimeric selectins and L-selectin mutants by 300.19 cells or COS cells was assessed by either indirect immunofluorescence staining using L- or P-selectin-specific mAb with flow cytometry or by immunoperoxidase staining, respectively. COS cells transiently expressed each of the receptors on the cell surface at high levels, while receptor expression by different 300.19 clones varied from low to high as is common for stable cDNA-transfected cells (Tables 1 and 2, Fig. 3). P4L was not expressed at high levels by 300.19 cells, so stably transfected CHO cells and transiently transfected COS cells expressing this receptor were studied. CHO cells transfected with L- or P-selectin behaved similarly to 300.19 and COS cells transfected with these receptors (data not shown) so there is no reason to suspect that results for P4L from CHO cells differ from results obtained in other cell types. The K283A mutant receptor was not expressed at detectable levels on the cell surface of either 300.19 cells or COS cells, but was expressed at high levels intracellularly (Tables 3 and 4, data not shown). Other mutations, such as ΔM-N, resulted

Table 2. *Changes in Cell-surface Receptor Expression after PMA Exposure*

Receptor	Mean linear fluorescence channel number			Percent shed*
	Control	– PMA	+ PMA	
L-selectin	0.46	12.75	1.84	89
LΔcyto	0.43	11.30	1.15	93
P4L	0.41	0.94	0.48	87
L4P	0.44	2.64	4.86	—‡
KLD.KE	0.42	3.84	1.13	79
K283A	0.21	0.21	0.21	—‡
SFS.KE	0.44	5.49	2.06	68
MIKE	0.44	2.30	0.56	93
KE	0.45	5.99	0.68	96
KEGDYN	0.43	2.27	0.57	92
ΔK283	0.26	32.95	25.20	24
K283E	0.36	23.40	16.45	30
K283P	0.26	8.75	7.30	17
ΔK-S	0.41	35.40	34.55	2
ΔM-N	0.34	64.05	63.20	1
AAAG	0.38	18.05	16.60	8

* Percent shed = 100[1 – MF(+PMA)/MF(–PMA)] with control levels subtracted from mean fluorescence channel numbers before the calculations. All values represent the means observed from duplicative cell samples. Similar results were obtained in at least three experiments.
‡ Percent shed for L4P could not be calculated since surface expression increased with PMA stimulation and K283A was not detectable on the cell surface.

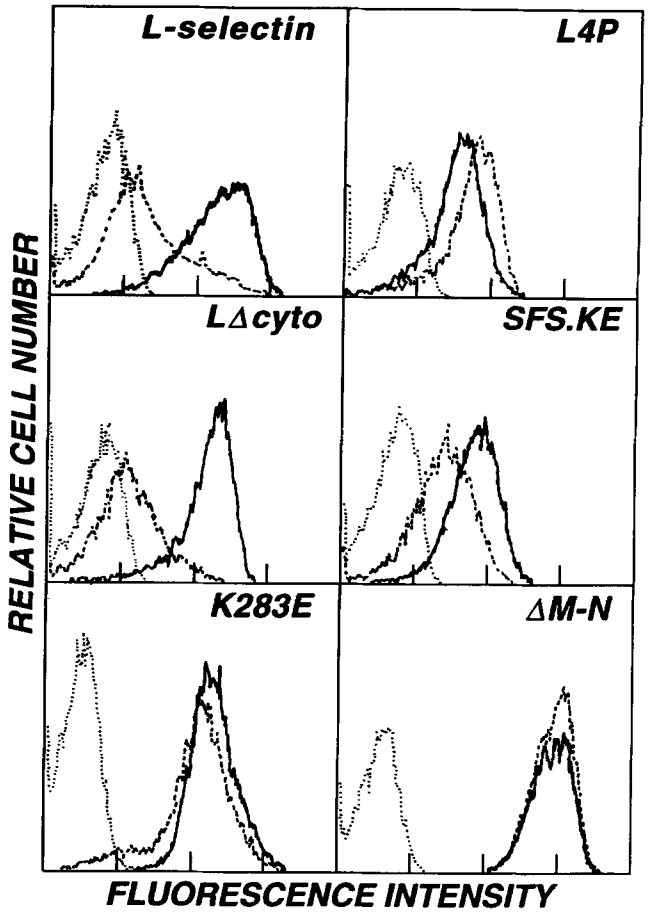


Figure 3. Cell-surface expression of selected chimeric and mutant L-selectin molecules before and after culture with PMA. Solid histograms indicate the fluorescence intensity of cells stained with saturating amounts of the anti-LAM1-3 mAb and assessed by flow cytometry; dashed lines indicate staining after PMA treatment; dotted histograms indicate background staining with an unreactive control mAb.

in significantly higher levels of L-selectin being expressed on the cell surface in both 300.19 cells and COS cells. In all cases, cultured cells were examined when grown at low density, since high density cultures have a tendency to shed L-selectin at an increased rate (7).

Structural and Functional Integrity of L-Selectin Mutants. The structural integrity of the chimeric selectin and L-selectin mutant proteins was examined by analysis with mAbs specific for epitopes located within the lectin, EGF, and SCR domains (42). The LAM1-3 (lectin), LAM1-1 (EGF), and LAM1-4 (SCR) mAb all bound to each of the transfected cells with similar relative fluorescence intensities when compared with wild-type L-selectin as determined by flow cytometry analysis (Table 1). Expression of P4L was assessed using the AC1.2 mAb specific for P-selectin SCR domains (48). Analysis with these mAbs demonstrate that the overall structure of the chimeric and mutant receptors was not dramatically altered by changes in membrane-proximal domains or residues.

The functional activity of receptors with changes in the membrane-proximal region was examined by determining whether replacement of the entire membrane-proximal region with four amino acids, AAAG, affected L-selectin function. 300.19 cells expressing the AAAG mutant receptor bound to rat peripheral lymph node HEV (6.3 ± 3.6 cells bound/HEV) as well as wild-type L-selectin–transfected cells (5.1 ± 0.7), while untransfected 300.19 cells did not bind (0.6 ± 0.1). That the membrane-proximal region of L-selectin was not required for cell adhesion suggests that other modifications within this region should not inhibit receptor function. As previously reported, the L4P and P4L chimeric receptors and LΔcyto each retain the appropriate carbohydrate/ligand-binding specificities (42, 43, 45).

The L-Selectin Transmembrane Domain Is Required for Endoproteolytic Release. The region of L-selectin necessary for endoproteolytic release was determined using cells transfected with chimeric selectins (Fig. 2 *A*). Receptor endoproteolytic release was assessed by cell-surface immunofluorescence staining with flow cytometry analysis after PMA (50 ng/ml) exposure for 30 min at 37°C (Fig. 3, Table 2), by ELISA to quantitate the amount of receptor released into the culture supernatant fluid after PMA exposure (Table 3) and spontaneously released into the supernatant fluid during culture (Table 4). Quanti-

Table 3. *Quantitation of PMA-induced Endoproteolytic Release of Receptor by ELISA*

Receptor	Quantity of L-selectin (total ng)*			Ratio‡
	Cell lysate	Supernatant	Supernatant	
	−PMA	*−PMA*	*+PMA*	
L-selectin	37.8	3.4	22.8	0.55
LΔcyto	12.6	1.3	8.5	0.61
L4P	13.2	<0.4	<0.4	<0.03
KLD.KE	6.2	3.4	4.4	0.46
K283A	9.3	3.1	5.0	0.40
SFS.KE	8.5	3.4	5.2	0.43
MIKE	3.8	1.0	3.0	0.63
KE	8.8	2.8	7.0	0.60
KEGDYN	7.9	2.7	6.6	0.63
ΔK283	35.6	1.6	1.8	0.05
K283E	17.6	1.3	1.9	0.10
K283P	14.0	1.4	1.6	0.10
ΔK-S	60.4	<0.4	<0.4	<0.01
ΔM-N	86.8	<0.4	<0.4	<0.01
AAAG	30.5	<0.4	<0.4	<0.01

* Cells (~2 × 10^6) were cultured in 0.4 ml of medium containing 50 ng/ml PMA at 37°C for 30 min. After centrifugation, the cell pellet was resuspended in 0.4 ml of lysis buffer. The total quantity of L-selectin present in the supernatant fluid and cell lysate was determined by ELISA.
‡ Ratio = sL-selectin/(total L-selectin) = Sup(+PMA)/[Lysate(−PMA) + Sup (−PMA)]. Similar results were obtained in at least three experiments.

Table 4. *Spontaneous Endoproteolytic Release of Receptors during 24-h Cultures*

Receptor	Quantity of L-selectin (ng)*		
	Supernatant fluid	Cell Lysate	Sup/Lysate
L-selectin	27.0	54.4	0.5
LΔcyto	31.5	34.0	0.9
L4P	<1.5	12.4	<0.1
KLD.KE	13.8	7.5	1.8
K283A	10.7	6.9	1.5
SFS.KE	11.0	8.1	1.4
MIKE	12.8	8.4	1.5
KE	5.0	4.6	1.1
KEGDYN	13.8	13.2	1.1
ΔK283	9.7	30.0	0.3
K283E	5.7	14.5	0.4
K283P	13.7	13.8	1.0
ΔK-S	<1.5	37.5	<0.04
ΔM-N	<1.5	68.0	<0.02
AAAG	<1.5	20.1	<0.07

* Cells (~2 × 10^6) were washed, resuspended in 10 ml of culture medium, and cultured for 24 h. Cells from 1.5-ml aliquots of the cultures were harvested, pelleted, and resuspended in 0.5 ml of lysis buffer. The total quantity of L-selectin present in the supernatant fluid and cell lysate was determined by ELISA. Similar results were obtained in at least three experiments.

tation of cell-associated L-selectin was also carried out by ELISA analysis of detergent-solubilized cells (Tables 3 and 4). This multiparameter analysis allowed determination of the relative amount of receptor shed independent of the levels of cell-surface receptor expressed by the different cDNA-transfected clones.

PMA-induced endoproteolytic release of L-selectin by 300.19 cells transfected with wild-type L-selectin resulted in an ~90% loss of cell-surface L-selectin expression (Fig. 3, Table 2). Concomitant with this, there was a sevenfold increase in the levels of sL-selectin found in the supernatant fluid of cells cultured in the presence of PMA (Table 3). Also, one-third of L-selectin generated by cells cultured for 24 h was found in the supernatant fluid as spontaneously shed receptor (Table 4). Endoproteolytic release of LΔcyto and the P4L receptor was similar to that of wild-type L-selectin, with an ~90% loss of cell-surface receptor after PMA stimulation (Tables 2–4). In contrast, cell-surface expression of L4P increased approximately twofold after PMA treatment (Fig. 3, Table 2), presumably because cell activation results in mobilization of P-selectin to the plasma membrane from intracellular storage granules (49). Identical results were obtained using P-selectin–transfected 300.19 cells and COS cells (data not shown). L4P was not shed from the cell surface since it was not detected in the supernatant fluid of transfected cells cultured with PMA or cells cultured for 24 h (Tables and 3 and 4). In all cases, similar results were obtained with mutant receptors transiently transfected into COS cells (data not shown). Collectively, these results indicate that the extracellular lectin, EGF, and SCR domains, and the cytoplasmic domain of L-selectin are not required for regulation of L-selectin endoproteolytic release. Therefore, the exon encoding the transmembrane domain must encode the sites of cleavage and/or regulatory elements that promote endoproteolytic cleavage.

Role of the Membrane-proximal Sequence in Endoproteolytic Release. The role of the primary structure of the transmembrane domain in L-selectin endoproteolytic release was examined by systematically substituting each amino acid in the membrane-proximal region with alanine residues (Fig. 2 *B*). Essentially, these mutations generated three sets of results. A first group of 300.19 cells expressing L-selectin mutations with alanine residues substituted for KE, KEGDYN, and MIKE were similar to wild-type L-selectin–transfected cells where >90% of the cell-surface receptor was shed after PMA exposure (Table 2). Similarly, a large fraction of the receptor was shed into the supernatant fluid (Tables 3 and 4). A second group of mutations in the KLD.KE or SFS.KE sequences re-

duced cell-surface receptor endoproteolytic release to 79 and 68%, respectively (Fig. 3, Table 2) with a small decrease in receptor release into the supernatant fluid after PMA exposure (Table 3). These subtle differences were most evident when the amount of PMA needed to induce optimal cell-surface receptor endoproteolytic release was determined. A PMA concentration of 10 ng/ml induced >80% endoproteolytic release of L-selectin and KE, KEGDYN, or MIKE mutants (Fig. 4, and data not shown), while a PMA concentration of 50 ng/ml was required to induce maximal endoproteolytic release of SFS.KE and KLD.KE mutants (Fig. 4).

The third type of result was obtained with the K283A mutant receptor which changed a basic residue within the putative cleavage site to a neutral residue. This mutant receptor was not expressed at detectable levels on the cell surface (Tables 1 and 2), but was released into the supernatant fluid at levels comparable to L-selectin (Tables 3 and 4). Since this receptor was expressed within the cytoplasm of the transfected cells (Tables 3 and 4), it is likely that the receptor is immediately cleaved after expression on the cell surface. Nonetheless, high levels of sL-selectin accumulated in the culture supernatant fluid for all alanine substitution mutants examined (Table 4). Similar results to those described above for each mutant receptor were also obtained with transiently transfected COS cells (data not shown). These results suggest that receptor endoproteolytic release is independent of the specific sequence MIKEGDYN within the membrane-proximal region of the transmembrane domain, whereas residues within the sequence KLDKSFS are more critical, although not absolutely required, for regulation of receptor endoproteolytic release.

Endoproteolytic Release Requires a Unique Structure of the Cleavage Site. Since the K283A mutation resulted in unregulated endoproteolytic release of L-selectin, the limit of tolerance for amino acid substitutions at this location was examined. Deletion of K^{283} (ΔK283) resulted in a 73% reduction in cell-surface receptor endoproteolytic release in 30-min cultures containing PMA (Table 2). Changing K^{283} to an acidic glutamic acid residue (K293E) also reduced endoproteolytic release by ~70% (Fig. 3, Table 2). Similarly, changing K^{283} to proline (K283P), a residue that is known to induce profound local conformational changes in proteins, reduced release by ~80%. Consistently, less receptor was shed into the culture medium from ΔK283-, K283P-, and K283E-transfected cells than from wild-type L-selectin–transfected cells during 30-min cultures stimulated with PMA (Table 3). These mutations also reduced the sensitivity of L-selectin to PMA-induced endoproteolytic release since even high concentrations of PMA caused only low level endoproteolytic release of these receptors (Fig. 4). However, in 24-h cell cultures not containing PMA, the relative amount of soluble ΔK283 and K283E receptor was not much less than that of wild-type L-selectin, while the K293P mutant was spontaneously shed to a high level, similar to the alanine-scanning mutants (Table 4). Collectively, these data suggest that the kinetics of receptor cleavage are delayed by these mutations, but that high concentrations of spontaneously shed receptor can accumulate in culture supernatant fluid over 24 h despite cleavage at a slower rate. Alternatively, spontaneous cleavage could result from mechanisms distinct from those that are PMA-induced. Nonetheless, these results and the results obtained with the alanine-scanning mutations, suggest that a unique local structure rather than sequence at the cleavage site needs to be preserved for L-selectin to be optimally recognized and/or cleaved by the endoprotease.

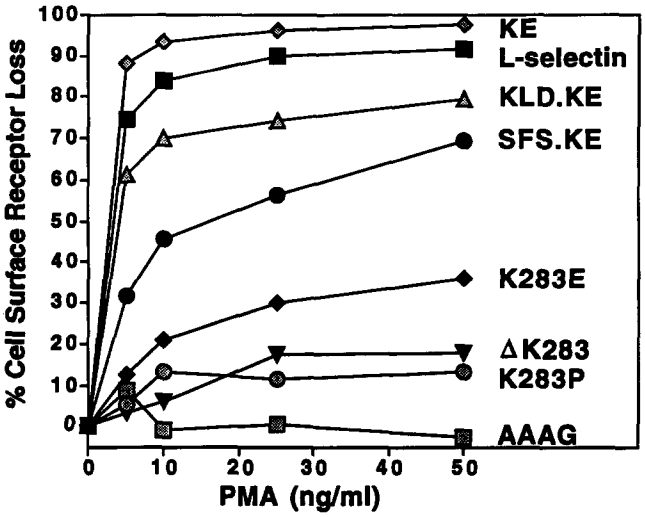


Figure 4. Dose dependence of PMA-induced endoproteolytic release of L-selectin. Cells were cultured with or without PMA for 30 min. The percentage of cell surface L-selectin that was lost was determined as in Table 2.

Length of the Membrane-proximal Region Is Critical. Since proteolytic cleavage does not require a specific sequence within the membrane-proximal region of L-selectin, the distance of the cleavage site from the cell membrane could also affect endoproteolytic release. To test this possibility, the amino- or carboxy-terminal half of the membrane-proximal region of L-selectin was deleted (Fig. 2 *B*). The resulting mutants, ΔK-S and ΔM-N, were not shed from the cell surface of transfected 300.19 cells (Fig. 3, Table 2) and sL-selectin was not detectable in the supernatant fluid of PMA-stimulated cells (Table 3) or 24-h cultured cells (Table 4). Similarly, when the membrane-proximal region was eliminated and replaced with AAAG (Fig. 2 *B*), L-selectin was not released from the cell surface of transfected 300.19 cells (Tables 2–4) regardless of the level of PMA used to stimulate endoproteolytic cleavage (Fig. 4). Thus, even though the putative protease recognition motif was present in the ΔM-N mutant receptor, a certain length of the membrane-proximal region (at least >8 amino acids long) was absolutely essential for endoproteolytic release of the receptor. Identical results were obtained with transiently transfected COS cells in each case (data not shown).

Discussion

Mutagenesis experiments carried out in this study are consistent with release of L-selectin from the cell surface through a proteolytic mechanism. Chimeric P- and L-selectin receptors, in conjunction with an L-selectin cytoplasmic domain trun-

cation mutant (Fig. 2), localized cleavage sites between Q^{279} and G^{323} (Fig. 1). The L4P chimera contained the entire extracellular domain of L-selectin except the transmembrane and cytoplasmic domains, but was not shed at detectable levels (Fig. 3, Tables 2–4). In contrast, the reciprocal receptor P4L was shed (Tables 2–4). Although the P4L chimeric receptor contained a portion of the last SCR of L-selectin, this region is not a potential site for endoproteolytic release since the receptor would remain membrane attached through the disulfide bond of Cys^{278} at the end of the SCR. Although the cytoplasmic domain of L-selectin is critical for receptor function (43), it is unlikely to regulate receptor cleavage since deletion of the entire cytoplasmic domain (LΔcyto) failed to affect endoproteolytic release (Tables 2–4). Thus, all of the structural elements that regulate endoproteolytic release of L-selectin appear to be contained within the transmembrane domain. Although a role for the membrane-spanning portion of L-selectin in regulation of endoproteolytic release was not revealed by these studies, it is likely that it is involved in some aspect of receptor function since there has been complete conservation within this region during recent mammalian evolution (Fig. 1).

Sites of L-selectin cleavage are likely to reside within the amino-terminal portion of the transmembrane domain. Deletion of K^{283}, or changing K^{283} to glutamate or proline, dramatically reduced the kinetics of PMA-induced endoproteolytic release by 70–80%, confirming that amino acid 283 is likely to be at or near the primary receptor cleavage site (Fig. 3 and 4, Tables 2 and 3). However, changing K^{283} to glutamate or proline did not significantly inhibit the amount of sL-selectin that accumulates in the supernatant fluid of cultured cells (Table 4). Substitution of K^{283} with a neutral residue dramatically increased the level of spontaneous endoproteolytic release (Tables 2–4). Substitution of amino acids proximal to the putative primary cleavage site, KLD^{282}, and SFS^{286} reduced PMA-induced endoproteolytic release by 20–30% (Table 2, Fig. 4), with considerable endoproteolytic release of the receptor into the supernatant fluid of cultured cells (Tables 3 and 4). Mutations within the MIKEGDYN amino acid sequence, more than three residues away from the putative K^{283} cleavage site, did not have a detectable effect on endoproteolytic release (Fig. 3, Tables 2–4). Therefore, while K^{283} appears important for receptor shedding, the K^{283} SFS motif or residues near this amino acid motif were not required for endoproteolytic release. This is consistent with the lack of the KSFS motif in bovine L-selectin which is nonetheless shed from the cell surface (39). Therefore, cleavage of L-selectin appears to be mediated by an endoprotease with relaxed sequence specificity.

Although cleavage of L-selectin did not require a specific primary amino acid sequence, this study suggests that two related factors contribute to protease susceptibility. First, a particular structure that can be generated by a series of sequences within the cleavage site appears essential. Substitution of K^{283} with glutamate or proline sufficiently altered the secondary or tertiary structure of this region so that it greatly reduced the kinetics of endoproteolytic release. Similarly, but with opposite effects, changes resulting from the K283A mutation made the receptor immediately susceptible to spontaneous cleavage such that it was not detectable on the cell surface, while it was found at normal levels in the cytoplasm and supernatant fluid. A second and directly related constraint is that the cleavage site must be at a certain distance from the plasma membrane. The mutant ΔM-N receptor was not shed, possibly because its unaltered cleavage site was too proximal to the membrane. This suggests that in addition to conformational constraints, the active site of the endoprotease may be restricted to a certain distance above the membrane. Therefore, the most likely explanation for why L-selectin is endoproteolytically released from the cell surface at a high rate while P- and E-selectin are not is likely to be a combination of sequence and length constraints on tertiary structure within the membrane-proximal regions of these receptors.

The amino acid substitutions examined in this study could affect L-selectin proteolytis in at least two indirect ways. First, primary structural changes could produce cleavage sites recognized by proteases not normally involved in L-selectin endoproteolytic release. This is not likely as the primary site for proteolytic cleavage suggested in this study using a mutational approach is in agreement with that obtained by others using radiochemical sequencing (31). Second, primary structural changes could provide better, or worse, substrates of normal or ectopic endoproteases, which would alter the rates of L-selectin cleavage. Undoubtedly this occurs as was dramatically revealed by the K283A mutant receptor. However, receptor cleavage was examined in a number of ways to control for these influences. First, receptor shedding from the cell surface was assessed after brief PMA activation of the cells which specifically augments release of this receptor (Figs. 3 and 4, and Tables 2 and 3). Second, accumulation of receptor in the supernatant fluid as well as that retained in the cell was assessed after brief PMA exposure (Fig. 3, Table 3). Third, spontaneous accumulation of shed receptor in the supernatant fluid was assessed after culture of transfected cells for 24 h to assesses receptor shedding in the event that the kinetics of shedding were inhibited (Table 4). Fourth, the dose response of cells to various concentrations of PMA was assessed to determine the relative susceptibility of mutant receptors to activation-induced shedding. Therefore, although primary structural changes in L-selectin appeared to alter the secondary and high order conformation of the receptor and also altered the kinetics of endoprotease-mediated cleavage, the results with each of these assay systems were consistent, further strengthening the validity of the conclusions for the primary L-selectin cleavage site.

Cleavage of L-selectin is most likely due to a membrane-bound protease with an extracellular catalytic domain constitutively present on the membrane (9). Further, this endoprotease activity must be widely distributed since identical results were obtained in this study with mutant receptors transfected into a mouse pre–B cell line, monkey kidney fibroblasts (COS cells), and CHO cells. Similar results have also been obtained with radiochemical sequencing of L-selectin released from COS cells and human lymphocytes (31). Since endoproteolytic release of L-selectin is increased rapidly in

response to cellular activation and PMA exposure, cellular stimulation may activate endoproteases already present within the membrane and thereby enhance proteolytic activity. The existence of widely distributed, PMA-sensitive endoproteolytic pathways has been suggested for the cleavage of transforming growth factor receptor (50), macrophage colony-stimulating factor receptor (4), LAR (51), and β amyloid protein (13). Alternatively for L-selectin, cellular activation or ligand binding may induce conformational changes in the membrane-proximal region of the receptor that expose nascent sites which are highly susceptible to enzymatic cleavage. This is supported by the immediate endoproteolytic release of the K283A mutant receptor from the cell surface without cellular stimulation. Ligand binding or cell activation may direct the membrane-proximal region of L-selectin into a protease-susceptible conformation similar to that induced by the K283A mutation. This is likely to be physiologically relevant since cellular activation up-regulates the affinity of L-selectin for ligand through conformational changes in receptor structure or organization within the membrane (52). This process would allow for a rapid increase in receptor affinity that would then be rapidly down-modulated through receptor release from the cell surface.

The failure to completely eliminate shedding by changing K^{283} to glutamate or proline could be explained solely by primary structural changes that alter the conformation of the receptor and thereby only inhibit the kinetics of endoproteolysis. However, a second explanation for these data is that the K^{283} recognition region is the primary but not the sole endoprotease cleavage site and that these changes thereby reveal additional normal and/or unusual cleavage sites. In this case, mutations of K^{283} may abolish endoproteolytic release at that site, but cleavage at a secondary and perhaps less efficient site could still occur. This may be likely since our analysis of the primary data published in the study that identified a single putative cleavage site for L-selectin after K^{283} suggests a potential secondary cleavage site for the receptor within the membrane-proximal region (31). Our analysis of their published radiochemical sequencing data finds a minor [^{35}S]methionine peak at cycle 14 and two minor [^{3}H]phenylalanine peaks at cycle positions 8 and 18. These results suggest receptor cleavage at a secondary site after K^{289} (Fig. 1). Again, the amino acid sequence surrounding this potential cleavage site is not conserved in bovine L-selectin (39) and changes in this sequence do not affect L-selectin shedding (Tables 2–4). That additional minor cleavage sites exist within the membrane proximal region of L-selectin cannot be ruled out, but all of the results obtained in this study are consistent with a primary cleavage site within the amino-terminal portion of the membrane-proximal region and a potential secondary cleavage site within the carboxy-terminal portion of the membrane-proximal region. In both cases, the enzyme or enzymes that mediate endoproteolytic release of L-selectin from the cell surface appear flexible in their sequence specificity and were up-regulated after PMA activation of receptor-expressing cells. Further, the structure of both cleavage sites and their relationship to membrane-spanning and SCR domains appears critical to shedding as the ΔK-S receptor was not shed from the cell surface even though the region containing K^{298} remained intact.

The molecular basis of receptor endoproteolytic release has only been examined in a small number of cases (1, 2). The best studied example of receptor endoproteolytic release is β amyloid precursor protein, which as been directly implicated in the genesis of Alzheimer's disease (13). Cleavage of β-amyloid precursor protein is through unidentified enzymes designated α- and β-secretase that cleave at different sites within the protein (13). Cleavage of β-amyloid precursor protein by α-secretase is remarkably similar to what has been observed with L-selectin. First, cleavage of β-amyloid precursor protein is a physiologic process observed in all cell lines that express this protein and in cDNA-transfected cells (53–55). Second, as with L-selectin, an array of protease inhibitors has failed to inhibit β-amyloid precursor protein cleavage from intact cells (11, 56–58). Third, β-amyloid precursor protein cleavage is accelerated in response to cell activation or PMA stimulation (59). Fourth, both proteins are primarily cleaved at a peptide bond after a lysine residue ~12 amino acids above the membrane. There is little sequence specificity for α, secretase cleavage since most amino acids substitutions in the cleavage site do not affect receptor proteolysis (53–55). However, substitutions with helix-destabilizing amino acids at and near the cleavage site inhibit endoproteolytic release (60). Fifth, natural mutations in critical residues of the β-secretase cleavage site actually accelerate endoproteolytic release and the accelerated development of Alzheimer's disease (61, 62). Finally, the shed forms of both receptors have been found in normal body fluids. Similar to the level of heterogeneity observed with the cleavage sites of L-selectin, β-amyloid precursor protein cleavage generates additional minor peptide species due to endoproteolytic cleavage at sites in addition to the predominant cleavage site (13). Therefore, it is possible that α-secretase is a ubiquitous membrane endoprotease that also cleaves L-selectin and other unidentified membrane proteins. These observations suggest a generalized protein processing pathway involved in the secretion of specific integral membrane proteins that may be mediated by a common mechanism or family of membrane bound endoproteases that exhibit a relaxed amino acid sequence specificity. Identification and characterization of these endoproteases could therefore have broad implications for an array of cell-surface receptor systems and therapeutic agents.

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