Regulation of Membrane Metalloproteolytic Cleavage of L-selectin (CD62L) by the Epidermal Growth Factor Domain* 

The adhesion molecule L-selectin is cleaved rapidly from the surface of activated leukocytes by tumor necrosis factor-α converting enzyme, a cell surface metalloprotease, and also undergoes slower constitutive shedding in unactivated cells. The structural features that render it susceptible to shedding are poorly understood. We therefore analyzed the shedding of a series of mutant and chimeric L-selectin molecules. Although murine L-selectin is cleaved at a specific location in the juxtamembrane region 11 amino acids distal to the cell membrane, this cleavage has little sequence specificity. However, proline substitution at the P2 or P3 position or deletion of the epidermal growth factor (EGF) domain completely blocks the rapid phorbol ester-induced cleavage, but does not affect the slower basal proteolytic shedding. Insertion of the 15-residue membrane-proximal region (MPR) of L-selectin into the heterologous protein B7.2 results in a molecule that undergoes constitutive proteolytic turnover. In contrast, insertion of both the EGF domain and the MPR confers susceptibility to both slow constitutive shedding and the rapid proteolytic cleavage induced by phorbol 12-myristate 13-acetate. These results demonstrate that constitutive and induced L-selectin cleavage are separable processes and that the rapid phorbol ester-induced shedding requires the presence of the EGF domain, a sequence that is remote from the cleavage site.

The extracellular domains of many integral membrane glycoproteins undergo proteolytic cleavage and release from the cell surface into the surrounding fluid phase. The biologically significant proteins released in this manner include a number of lymphokines, growth factors, transcription factors, and adhesion molecules (reviewed in Refs. 1–4). Proteolytic cleavage is responsible for the regulated secretion of several cytokines derived from transmembrane precursors (5–8), and the shedding of surface receptors results in the desensitization of responsiveness to various cytokines (9–12). Membrane protein shedding is also implicated in several disease processes. Inherited mutations in the p55 TNF-α receptor lessen its sensitivity to proteolysis, cause its decreased proteolytic clearance from the cell surface, and result in a family of inherited autoinflammatory syndromes (13). Cleavage of the amyloid precursor protein (APP) at the β and γ sites, releasing the amyloidogenic Aβ fragment, is implicated in the pathogenesis of Alzheimer’s disease, and mutations that increase secretion of this peptide are associated with familial Alzheimer’s disease (14–16). The enzymes responsible for such cleavage are collectively referred to as membrane secretases or sheddases. A common mechanism for the secretion of many of these proteins has been inferred from the ability to inhibit their release with new hydroxamic acid-derived metalloprotease inhibitors (17–21) and by the identification of a mutant cell line that is defective in cleaving multiple proteins (22). More recently, a plasma membrane enzyme responsible for the shedding of TNF-α, the TNF-α converting enzyme (TACE) (23, 24), a member of the ADAM (a disintegrin and metalloprotease) family (25), was also shown to cleave TGF-α, TNF-α receptor types I and II (26, 27), Notch1 (28), the receptors for IL-6 and CSF-1 (29, 30), and APP (31). Genetic deficiency of TACE is embryonically lethal, demonstrating the importance of TACE-mediated shedding in vivo (26).

L-selectin (CD62L) is a leukocyte transmembrane glycoprotein that mediates the initial adhesive interaction of lymphocytes to specialized vascular endothelial cells in lymph node high endothelial venules and of leukocytes to endothelium at sites of inflammation. In addition to being required for the homing of lymphocytes to lymph nodes (33), L-selectin may transduce co-stimulatory signals for cell activation and for the up-regulation of integrin adhesiveness necessary for the transition from rolling to firm adhesion (34–40). The expression of L-selectin is differentially regulated by resting and activated lymphocytes at the transcriptional, mRNA, and post-translational levels (41, 42). In resting cells, L-selectin undergoes relatively slow, constitutive shedding. Stimulation by PMA or, for T cells, through the T cell receptor, induces the rapid up-regulation of shedding, releasing the soluble, ligand-binding ectodomain fragment into the supernatant and removing most of the protein from the cell surface within a few minutes (42, 43). Human neutrophils similarly shed L-selectin after treatment with chemotactic agents, phorbol esters, lipopolysaccharide, or calmodulin antagonists (44, 45). The presence of the soluble L-selectin fragment in normal serum indicates that this proteolytic shedding process also takes place in vivo (46). L-selectin shedding regulates the rolling velocity of loosely adherent leukocytes along the endothelium, an early step in leukocyte-endothelial interaction (47, 48). We have suggested that...
L-selectin cleavage may be required for de-adhesion during the transendothelial migration of lymphocytes through the high endothelial venule vessel wall and into the lymph node parenchyma (49), a proposal supported by the observation that lymphocytes down-regulate L-selectin during migration through high endothelial monolayers in vitro (50).

The phorbol ester-induced proteolytic shedding of L-selectin is mediated by the same enzyme, TACE, that processes pro-TNF-α and is blocked by inhibitors of zinc-dependent metalloproteases (26, 51–53). TACE cleaves both human and mouse L-selectin at homologous sites just external to the cell membrane (Fig. 1), releasing the majority of the protein into the supernatant and leaving a membrane-retained fragment (MRF) consisting of the cytoplasmic and transmembrane domains and the first 11 amino acids of the extracellular juxtamembrane region (54, 55). Metalloproteolytic shedding of most proteins shows little specificity for the sequence around the cleavage site, and the structural features that determine transmembrane protein susceptibility to shedding are therefore poorly understood. One general feature of protease recognition is that the cleavage is positioned primarily with respect to the proximal extracellular globular domain, requiring only a susceptible membrane-proximal stalk of sufficient length to permit access of the protease to the substrate (1, 5). Indeed, it has even been proposed that membrane sequestrates will cleave any surface protein that lacks a bulky globular domain close to the cell surface (4). The shedding of human L-selectin is relatively sequence-nonspecific, and other determinants of protease recognition of membrane L-selectin have not been defined (54, 56, 57).

In the present studies, we analyzed the regulation of proteolytic shedding in transfectants expressing a series of point and domain swap mutants of murine L-selectin. Although shedding is not blocked by most point mutations in the membrane-proximal region, some mutations and deletions completely block PMA-induced proteolysis. Surprisingly, however, these mutant molecules still undergo the constitutive metalloproteolytic shedding that is characteristic of unactivated cells, demonstrating that these two processes are inherently separable and may be mediated by different enzymes. Furthermore, while the short juxtamembrane region of L-selectin is sufficient to confer susceptibility to this basal cleavage, the EGF domain is required for rapid phorbol ester-induced shedding. The EGF domain therefore likely serves as a recognition motif for the phorbol ester-stimulated protease, enhancing its interaction with substrate and increasing the rate of proteolytic cleavage.
itive for MEL-14 staining were confirmed to be Ly22-negative by staining the cells with T28. At least three clones of each transfectant with positive staining for MEL-14 or GL-1, but negative for T28, were used in these studies. Since the EGF domain deletion mutant (ΔEGF) cannot be recognized by either MEL-14 or T28, its expression was detected by Western blot analysis using the polyclonal antibody specific for the cytoplasmic domain of L-selectin. Plasma membrane expression of this mutant L-selectin was confirmed by its ability to be surface-labeled with $^{125}$I, and its smaller size distinguished it from endogenous wild-type L-selectin, as shown under “Results.”

Western Blot Analysis—Untreated or PMA (100 ng/ml)-stimulated normal lymphocytes or transfectants were lysed, precleared with non-immune rabbit IgG-Sepharose beads, and immunoprecipitated with Sepharose beads coupled to the rabbit anti-L-selectin cytoplasmic domain antibody. Eluates were separated on 10–20% Tricine-SDS gradients (Novex, San Diego, CA), transferred to Protran nitrocellulose membranes (Schleicher and Schuell), and then probed with the polyclonal anti-cyttoplasmic domain peptide antibody followed by chemiluminescence detection with Lumiglo Substrate (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland) and Kodak BioMax Light-1 film (Eastman Kodak Co.).

$^{125}$I Labeling of Cell Surface Proteins and Analysis of Surface Protein Shedding—Cell surface proteins were labeled with $^{125}$I (Amersham Pharmacia Biotech) using the glucose oxidase-lactoperoxidase method (63). Briefly, cells were washed thoroughly with phosphate-buffered saline. Three to $6\times 10^6$ cells were labeled with 1 mCi of $^{125}$I in phosphate-buffered saline containing 1 mM KI, 5 mM glucose, 0.4 unit of glucose oxidase, and 20 μg of lactoperoxidase. After labeling, cells were washed extensively and resuspended in Hanks’ balanced salt solution containing 0.2% bovine serum albumin. In mock labeling experiments, treating cells with unlabeled I$^-$ under oxidizing conditions did not cause any down-regulation of L-selectin (data not shown). For analysis of PMA-induced shedding, about $3\times 10^7$ $^{125}$I-labeled cells were left untreated or were treated with 100 ng/ml PMA, with or without pretreatment with Ro 31-9790 for 20 min. Cells incubated in the same concentration of Me$_2$SO (0.1%) were used as a negative control. After treatment with Ro 31-9790 for 20 min, cells incubated in the same conditions. To remove the various treatments, the cells were lysed. Lysates and supernatants were precleared with Sepharose conjugated to irrelevant rat IgG2a antibody and then immunoprecipitated with protein G beads precoated with MEL-14 or, for B7.2 chimeras, with GL-1 antibody. Eluates from Western blot analysis using the polyclonal antibody specific for the cytoplasmic domain of L-selectin shows the same size in-munoprecipitated proteins, the antigens were eluted by boiling the beads in incubation buffer (20 mM sodium phosphate, pH 7.5, 50 mM EDTA, 0.02% sodium azide) containing 0.5% SDS and 5% β-mercaptoethanol and digested with N-glycanase (Oxford GlycoSciences, Wakefield, MA). Labeled proteins were detected by exposing dried gels to Kodak X-Omat AR-5 film at ~70°C.

RESULTS

Rapid PMA-induced Shedding of L-selectin from Normal Murine Lymphocytes—Treatment with phorbol esters that activate protein kinase C causes rapid L-selectin shedding from the surface of normal lymphocytes (43). As illustrated in Fig. 2A, L-selectin expression on normal lymphocytes, detected by flow cytometric analysis using the mAb MEL-14, decreases rapidly after PMA treatment, with about 90% loss in 30 min. This down-regulation is not affected by treatment with inhibitors of serine and several other proteases (43). However, pretreatment of the cells with Ro 31-9790, a zinc-dependent metalloprotease inhibitor, blocks this down-regulation, suggesting that it is mediated by a metalloproteolytic secretase. To confirm that the down-regulation of L-selectin was due to a proteolytic mechanism, PMA-treated and untreated cells were immunoprecipitated with polyclonal antibody specific for the cytoplasmic domain of L-selectin and then examined by Western blot analysis, probing the membrane with the same polyclonal antibody (Fig. 2B). In untreated normal lymphocytes, there is a broad 95-kDa band corresponding to intact L-selectin. In addition, there is a 6-kDa band that represents the protein fragment remaining in the membrane after L-selectin shedding. The presence of this MRF in the lysates of untreated cells shows that L-selectin undergoes base-line constitutive shedding in the absence of PMA, as we have demonstrated previously (43). After PMA treatment, the 95-kDa band decreases in intensity, as expected, while that of the MRF increases at 5, 10, and 15 min after PMA treatment, reflecting its generation during proteolytic cleavage. Then, the intensity of the MRF band decreases as the fragments are gradually cleared from the cell surface. These data show that, upon PMA stimulation, L-selectin in normal murine lymphocytes is proteolytically cleaved and shed by a membrane metalloprotease in a process similar to that shown in human cell lines (54). Based upon the mobility of the MRF bands, the proteolytic cleavage site appears to be the same for both the slow basal cleavage and the rapid PMA-stimulated shedding.

Expression of Mouse L-selectin Mutants in the TK-1 Cell Line—To determine the structural properties of L-selectin required for its shedding, we analyzed its expression in the murine lymphoma cell line TK-1 transfected with cDNAs encoding wild-type and mutant L-selectin. As was observed with normal lymphocytes, the expression of wild-type L-selectin transfectants decreased markedly after 30-min treatment with PMA (Fig. 3A). This down-regulation was completely inhibited by the metalloprotease inhibitor Ro 31-9790. Western blot analysis of the wild-type L-selectin transfectant shows the same size in-
same, or similar, metalloproteolytic mechanisms. The more rapid PMA-induced shedding are mediated by the L-selectin shedding and that both the slow basal shedding and phorbol ester stimulation induces the rapid up-regulation of activated and resting cells, respectively. These data show that starting membrane antigen appeared in the supernatants of L-selectin was markedly inhibited; only 5% and /H11021 were pretreated with the metalloprotease inhibitor Ro 31-9790, lane 10 into the supernatant fraction (\[50x125\]). When transfectants without PMA, only 12% of the surface L-selectin has been shed (ding of L-selectin is relatively slow; after 60-min incubation basal shedding and supernatant with anti-L-selectin, cell surface proteins on the wild-type L-selectin transfectant. Solid line — to Fig. 2B.

Inhibition of Both Constitutive and PMA-induced Shedding of L-selectin by a Metalloprotease Inhibitor—To examine the processes of PMA-induced and constitutive shedding of L-selectin, cell surface proteins on the wild-type L-selectin transfectant were labeled with \[125\]I and then incubated further in the presence or absence of PMA. L-selectin was then immunoprecipitated from the cell surface and supernatant with anti-L-selectin mAb and analyzed by gel electrophoresis. As shown in Fig. 4, broad 95-kDa bands, corresponding to the intact cell surface molecule, are detected in lysates of unactivated cells at time 0 or after 30-min incubation in the absence of PMA. Stimulation of cells for 30 min with phorbol ester results in a 94% decrease in the intensity of the lysate band (lane 5), similar to the decrease observed by flow cytometry, and the appearance of a slightly more mobile band in the supernatant, corresponding to the shed ectodomain (lane 6). The basal shedding of L-selectin is relatively slow; after 60-min incubation without PMA, only 12% of the surface L-selectin has been shed into the supernatant fraction (lane 10). When transfectants were pretreated with the metalloprotease inhibitor Ro 31-9790, both the PMA-induced and constitutive shedding of \[125\]I-labeled L-selectin was markedly inhibited; only 5% and /H11022 of the starting membrane antigen appeared in the supernatants of activated and resting cells, respectively. These data show that phorbol ester stimulation induces the rapid up-regulation of L-selectin shedding and that both the slow basal shedding and the more rapid PMA-induced shedding are mediated by the same, or similar, metalloproteolytic mechanisms.

Conservation of the Primary Sequence around the Cleavage Site Is Not Required for PMA-induced Shedding of Murine L-selectin—To determine the specificity of murine L-selectin cleavage, point mutations within the MPR of mouse L-selectin were analyzed for their effects on shedding. The expression of wild-type L-selectin and its mutants before and after PMA stimulation was determined by flow cytometric analysis using MEL-14 mAb, and shedding of L-selectin molecules was evaluated as the percentage decrease of L-selectin expression after PMA treatment, as summarized in Fig. 5. L-selectin mutants with both conserved (R321K, S322T) and nonconserved (N320A, R321I, R321A, S322I, F323A) point mutations of the P1, P2, P1’, and P2’ positions shed well (>70%) after PMA stimulation. A molecule with alanine substitutions of residues 329–332 also sheds in response to PMA. Shedding by all of the L-selectin mutants was inhibited by the metalloprotease inhibitor, and Western blots of the representative mutants R321I, F323A, and S322I showed 6-kDa MRF bands (data not shown), indicating that their shedding is mediated by the same proteolytic mechanism as for wild-type L-selectin. Thus, shedding of the murine L-selectin molecule has little specificity for the amino acid sequence at the substrate cleavage site.

Different Structural Requirements for Constitutive and PMA-induced Shedding—In contrast to the down-regulation of most L-selectin point mutants, proline substitution mutations at the P2’ and P3’ positions (F323P and S324P), which could markedly change the secondary structure of the MPR, completely block shedding induced by PMA (Figs. 5 and 6A). Western blot analysis of the F323P transfectant revealed the expected 95-kDa band, but, surprisingly, also showed a strong band identical to the 6-kDa MRF of the wild-type and other mutants (data not shown). Neither of the bands in F323P is affected by PMA treatment. Since this fragment arises only after the proteolytic cleavage of L-selectin, the following experiment was carried out to examine directly whether the F323P protein undergoes shedding from the cell surface. F323P transfectant cells were labeled with \[125\]I, washed, treated with PMA, and the cells lysates and supernatants were then immunoprecipitated with MEL-14. As shown in Fig. 6B, soluble ectodomain was released into the supernatant of unstimulated cells, and the amount shed did not increase with the addition of PMA. After
Regulation of L-selectin Shedding by the EGF Domain

A summary of flow cytometric analyses of the PMA-induced down-regulation of L-selectin mutants. The proteolytic cleavage site of mouse L-selectin is indicated (**), and the P1, P2, P3, P1′, P2′, and P3′ positions relative to this cleavage site are marked. Dashes indicate amino acids identical to those found in wild-type L-selectin, and stars indicate deletion of amino acids from wild-type L-selectin. Untreated or PMA-treated transfectants were analyzed flow cytometrically after staining with MEL-14. Mean linear fluorescence intensity (MFI) of the untreated and PMA-treated cells was determined, and the percentage of shedding was calculated as 100 × [1 − MFI PMA/MFI H11002]. All results are derived from experiments on at least three independent transfectant cell lines and are expressed as the mean ± S.D.

3 h of incubation, the unstimulated cells released 79% of the mutant L-selectin. Thus, L-selectin F323P undergoes constitutive proteolytic cleavage, but this shedding does not increase in the presence of PMA. The rate of this basal shedding is similar to that of wild-type L-selectin in unstimulated cells (see the following section).

An Eight-amino Acid Deletion in the MPR Blocks Both Constitutive and PMA-stimulated Shedding of Murine L-selectin—Deletions that shorten the length of the juxtamembrane region of several proteins, including human L-selectin, inhibit their proteolytic shedding (1, 3, 56, 57). To determine whether murine L-selectin is similarly affected by the MPR length, eight amino acids in the membrane-proximal region (Lys325 to Asn332) were deleted. This mutant protein (∆K-N) was expressed at high levels and was completely resistant to PMA-induced down-regulation (Fig. 7A). To determine whether the ∆K-N deletion mutant undergoes constitutive shedding, transfected cells were labeled with 125I and then incubated for 3 h in the absence of PMA. As shown in Fig. 7B, 69% of the wild-type L-selectin appears in the supernatant after 3 h in vitro. In contrast, less than 2% of the ∆K-N molecules were released into the supernatant, and the lysate contained virtually all of the mutant L-selectin that was present at time 0. Thus, truncation of the membrane-proximal region abolishes both constitutive and PMA-induced release of L-selectin, even though the cleavage site sequence is intact. These results suggest that a minimal MPR length of at least eight amino acids is required for the cleavage of cell surface L-selectin, both the slow constitutive shedding and the rapid shedding induced by PMA.

The Roles of the EGF, SCR, Transmembrane, and Cytoplasmic Domains in L-selectin Shedding—Domains other than the juxtamembrane region could be important for recognition of L-selectin by TACE. To identify potential sites of interaction with the protease, a series of domain deletion and domain swap mutants were generated as shown in Fig. 8A. The expression of these L-selectin mutants was determined by flow cytometric analysis, except the ∆EGF deletion mutant. This molecule was detected in Western blots using the anti-L-selectin cytoplasmic domain antibody. The shedding by these L-selectin mutants is inhibited by the metalloprotease inhibitor (data not shown).

The sequence of the transmembrane domain of L-selectin is highly conserved throughout evolution (56), suggesting that it may have some role other than membrane anchoring. Intramolecular recognition can occur within the plane of the membrane, and some membrane secretases cleave their substrate proteins within the transmembrane domains (4). However, replacing the transmembrane domain of L-selectin with that of P-selectin, which shares no sequence homology (Fig. 1), had little effect on PMA-induced shedding (Fig. 8A). Other work has suggested a role for the cytoplasmic domain in the regulation of L-selectin cleavage (57) and in lymphocyte adhesion.
Regulation of L-selectin Shedding by the EGF Domain

EGF transfectants were surface-labeled with $^{125}$I and then were precipitated with anti-L-selectin cytoplasmic domain antibody, and the precipitated antigens were separated on a 8% SDS-PAGE gel prior to autoradiography. The amount of PMA-induced shedding was calculated as 100 - [intensity (band of untreated cells) / intensity (band of PMA-treated cells)] x 100%

**FIG. 8. Role of L-selectin domains in shedding.**

**A.** Structures of wild-type L-selectin and the domain deletion and domain swap mutants are illustrated together with a summary of the immunofluorescence analyses of their PMA-induced shedding. TMp denotes the transmembrane domain of mouse P-selectin. The amount of PMA-induced shedding was determined by immunofluorescence flow cytometry, and the results are expressed as the mean ± S.D. of at least nine determinations. Shedding of the ΔEGF mutant was determined by Western blot analysis. The intensity of intact ΔEGF bands before and after PMA treatment were determined densitometrically. The percentage of shedding was calculated as 100 x (1 - [intensity (+PMA)/intensity (-PMA)]). **B.** Western blot analysis of shedding of L-selectin deletion mutants. TK-1 cells transfected with wild-type, ΔEGF, or ΔSCR cDNA were left untreated or treated with PMA for 30 min. The lysates were immunoprecipitated and analyzed as described in the legend to Fig. 2B. **C.** Shedding of the ΔEGF mutant analyzed by $^{125}$I surface labeling. ΔEGF transfectedants were surface-labeled with $^{125}$I and then were treated with PMA or Me$_2$SO (control). The cell lysates were immunoprecipitated with anti-L-selectin cytoplasmic domain antibody, and the precipitated antigens were separated on a 8% SDS-PAGE gel prior to autoradiography.

The EGF mutant was examined further to determine whether it is indeed expressed on, and shed from, the cell surface, using surface labeling and precipitation with the polyclonal anti-cytoplasmic domain antibody. As shown in Fig. 8C, bands corresponding to the full-length surface molecule were obtained from lysates of both resting and PMA-activated cells. Furthermore, there was no decrease in band intensity after PMA treatment, confirming that this construct is completely insensitive to PMA-induced proteolytic cleavage, as suggested by the Western blot experiments.

The Membrane-proximal Region of L-selectin by Itself Can Fully Confer Constitutive Shedding upon an Unrelated Surface Protein—Since only the 8-amino acid deletion in the MPR of L-selectin (ΔK-N) is sufficient to block all constitutive shedding, and neither the EGF or SCR domains affect this basal cleavage, it is possible that the 15-amino acid MPR sequence is sufficient to mediate constitutive shedding from the cell surface, but not the rapid, inducible shedding. To test this hypothesis, a chimeric CDNA (B7.2/MPR) was constructed in which the MPR of L-selectin was inserted into the membrane-proximal sequence of murine B7.2, which has no sequence homology to L-selectin. As shown in Fig. 9A, treatment with PMA for 30 min does not induce any decrease of wild-type B7.2 expression, and only slight (11%, mean of six experiments) down-regulation of B7.2/MPR was observed. Thus, the MPR of L-selectin can confer at most a very low level of PMA-induced shedding upon the surface protein B7.2. Interestingly, when cells are incubated for 3 h in the presence of the metalloprotease inhibitor in the absence of PMA stimulation, the expression of the chimeric molecule, but not wild-type B7.2, increases by 1.5-fold (Fig. 9B), consistent with blocking basal proteolytic shedding.

To determine whether the B7.2/MPR chimeric molecule undergoes constitutive cleavage, transfectedants were surface-labeled with $^{125}$I, incubated in the absence of stimulation, and the lysates and supernatants immunoprecipitated using anti-B7.2 antibody. As shown in Fig. 9C (left panel), 83% of the labeled B7.2/MPR was released into the supernatant (mean of two experiments) after 3-h incubation in the absence of stimulation, similar to the 69% constitutive shedding of wild-type L-selectin (Fig. 7B). It has previously been shown that electrophoretic estimation of the relative molecular weights of intact amphipathic membrane glycoproteins and shed fragments is inaccurate unless the proteins are first deglycosylated (1, 43). Thus, to assess more precisely the size difference between the surface protein and the shed fragment, both the cell lysate and supernatant-derived eluates were N-deglycosylated before electrophoretic analysis. Bands from enzymatically deglycosylated proteins demonstrate the small 1.5-kDa difference between membrane B7.2 and B7.2/MPR (Fig. 9C, right panel). The apparent molecular mass of the shed B7.2/MPR fragment is 8.5 kDa smaller than the cell surface form, indicating that
cleavage occurs at, or very near, the same R-S site in the MPR as in L-selectin. No soluble antigen was detected in the supernatant of wild-type B7.2 transfectants.

The EGF Domain of L-selectin Is Sufficient for Rapid (Induced) Proteolysis of MPR-containing Proteins—The above data showed that an intact MPR is necessary for shedding and that the EGF domain is required for optimal PMA-induced rapid proteolytic cleavage of L-selectin. We next addressed the question of whether the EGF domain by itself is sufficient to render an MPR-containing protein susceptible to PMA-induced shedding. A series of chimeric molecules were generated based on the B7.2/MPR molecule, as illustrated in Fig. 10A. B7.2/MPR expressing TK-1 transfectants were left untreated or were incubated at 37 °C in the absence (solid line) or presence (dotted line) of the metalloprotease inhibitor Ro 31-9790 following by staining with anti-B7.2 mAb. The shaded histograms represent the negative control staining with isotype-matched antibody. C, constitutive shedding of the B7.2/MPR chimera detected by surface 125I labeling. Untreated B7.2 and B7.2/MPR transfectants labeled with 125I were incubated at 37 °C for 3 h. The cell lysates (L) and culture supernatants (S) were immunoprecipitated with anti-B7.2 antibody (left panel) or treated with N-glycanase prior to the immunoprecipitation (right panel). The resulting eluates were separated on 8% (left) or 10% (right) SDS-PAGE gels prior to autoradiography. Immunoprecipitation with an irrelevant isotype-matched antibody was used as negative control (Ig).

Numerous membrane proteins are proteolytically cleaved and shed from the surface of leukocytes and other cell types (1, 3). Some of these proteins, such as the mannose receptor, are shed constitutively and the rate of shedding is unaffected by cell activation (65). The cleavage of other proteins is induced or accelerated by cell activation, and it has been estimated that up to 4% of cell surface proteins is shed after phorbol ester stimulation (22). Many of these proteins are cleaved by metalloproteolytic enzymes that are members of the ADAM family, although other classes of proteases are responsible for the shedding of some proteins (1). TNF-α is constitutively shed at a high rate, almost completely removing the cytokine from the cell surface. This cleavage, mediated by TACE, is accelerated by guest on November 6, 2017 http://www.jbc.org/ Downloaded from
moderately by phorbol esters (66). In contrast, murine lymphocytes undergo relatively slow basal shedding from unactivated cells, and its rate of proteolytic release increases dramatically after activation by PMA or anti-CD3 mAb (42, 43). This rapid release is mediated by TACE (26) and is blocked by metalloprotease inhibitors (Fig. 2A). In the current study, we have demonstrated that the slow constitutive shedding and rapid inducible shedding of L-selectin are separable processes with distinct structural requirements and that optimal shedding of L-selectin depends upon sequences remote from the proteolytic cleavage site in the EGF domain.

Even though many integral membrane proteins are processed by plasma membrane secretases, it is clear that most proteins are not cleaved from the cell surface and therefore that components of the proteolytic process must recognize structural features of the relevant substrates. The structural features of substrate proteins required for cleavage are incompletely understood. One characteristic common to membrane metalloproteolytic secretases is that they have relatively low specificity for the amino acid sequence of the substrate (1, 3). Most mutations introduced into the MPR of murine (Fig. 5) and human (56, 57) L-selectin do not block proteolytic cleavage. Similarly, a 30-amino acid substitution in the juxtamembrane region of TGF-α (67), TNF-α (7), and APP (68) do not inhibit their proteolysis. However, introduction of a proline into this region of L-selectin, which would be expected to disrupt its secondary structure, markedly inhibits its cleavage (Fig. 6), as it does for APP and the p55 and p75 TNF-α receptors (69–71). Thus, at least some overall structure of this substrate region must be maintained for protease recognition. The inherent substrate activity of the MPR of L-selectin was demonstrated by its ability to confer constitutive shedding upon the unrelated transmembrane glycoprotein B7.2 (Fig. 9). The juxtamembrane regions of several other shed proteins, including APP, TGF-α, and the TNF-α receptor I, are also sufficient to mediate shedding when inserted into heterologous membrane proteins (70, 72). In an examination of the cleavage of synthetic substrate oligopeptides by purified TACE, it was shown that cleavage of the TNF-α peptide was 2250 times more efficient than that of L-selectin (26). This strong interaction between the processing enzyme and the juxtamembrane region of TNF-α may explain why TNF-α undergoes so much more rapid constitutive shedding than does L-selectin.

One structural feature generally required for the cleavage of membrane metalloprotease substrates is that they have a stalk region of a minimal length between the plasma membrane and the first globular ectodomain (1, 3). This appears to be necessary for the proteolytic enzyme to gain access to the substrate’s cleavage site. Juxtamembrane amino acid deletions block cleavage of several proteins, regardless of whether the sequence at the cleavage site remains intact, if the remaining stalk is shorter than a minimal length, generally about 10–12 amino acids. A 19-residue deletion in the membrane-proximal region of APP, which includes the cleavage site, results in a new cleavage site 12 amino acids external to the transmembrane domain, the same distance from the membrane as with wild-type APP (68). Only 11 of the original 35 amino acids of the APP juxtamembrane region are required for proteolytic processing to occur. Similar results found with TNF-α and the p75 TNF-α receptor have confirmed the general principle that the shedding enzyme requires a certain stalk length for access and that it positions itself for cleavage primarily with respect to the plasma membrane and the proximal extracellular domain (1). This distance from membrane requirement may pertain only to metalloproteases, as the asparyl protease responsible for the β-secretase processing of APP cleaves only with respect to a strict amino acid sequence specificity (73). The lack of PMA-induced down-regulation of the L-selectin ΔΚ-N deletion mutant is in keeping with the requirement for a minimal MPR length for proteolytic cleavage by TACE.

Examination of domain deletion mutants and chimeras revealed additional structural information about the regulation of L-selectin shedding. Deletion of the EGF domain resulted in a molecule (ΔEGF) that does not down-regulate in response to PMA (Fig. 8). Conversely, when the EGF domain was inserted into B7.2/MPR, which undergoes primarily constitutive shedding, it became susceptible to PMA-induced rapid cleavage (Fig. 10). These experiments demonstrate that the EGF domain of L-selectin, while not required for its basal proteolytic turnover, is necessary for optimal PMA-induced rapid cleavage. In contrast to the EGF domain, insertion of the SCR regions into B7.2/MPR resulted in a molecule (B7.2/MPR/SCR) in which the PMA-induced shedding was increased by only 16%. Thus, the SCR domains of L-selectin are not essential for either basal or stimulated shedding.

EGF domains frequently mediate protein-protein interactions and are present in other membrane proteins that are shed, including TGF-α (74), EGF (8), heparin-binding EGF-like growth factor (75), and Notch1 (76). This is consistent with our previous suggestion that the EGF domain of membrane-bound L-selectin contains a docking site for the protease (49). This would be analogous to the membrane-distal region of angiotensin-converting enzyme that contains a recognition motif that is required for its cleavage by a membrane secretase (77). Resolution of the crystal structure of TACE has revealed several large insertion loops that extend out from the surface of the catalytic domain, but separate from the active site, and that have been postulated to be sites of interaction with substrate (3, 78). TACE is thought to interact with a docking site in the base of the compact cone region of TNF-α, a site distant from that of its scissile bond in the juxtamembrane stalk (78). The binding of TACE to an analogous docking site on L-selectin would have an even more dramatic effect on cleavage of L-selectin than of TNF-α because of the much lower apparent affinity of interaction of the MPR of L-selectin for the protease. Thus, when the EGF domain of L-selectin binds to TACE, the overall avidity of interaction with substrate would be enhanced, and the shedding rate would increase correspondingly.

Another potential site of substrate interaction with the enzyme is within the transmembrane region of L-selectin, similar to the interactions of proteins with γ-secretases. However, the transmembrane domain substitution chimeric molecule sheds well in response to PMA, demonstrating that the transmembrane region is not involved in protease recognition. Similarly, since the B7.2/MPR/EGF chimeric protein undergoes rapid shedding, the lectin domain of L-selectin is not required for its proteolytic release.

The nature of the signals and structural changes that lead to activation of the rapid shedding process are poorly understood. Several lines of evidence have led to the general belief that the sheddase exists in the membrane in a constitutively active state and that the induction of shedding therefore involves a change in the substrate to a conformation more susceptible to proteolysis. Thus, TACE continuously cleaves pro-TNF-α at a high rate in unstimulated cells. Purified TACE also cleaves some substrate peptides and proteins in the soluble phase (23, 24, 26). In addition, a point mutation in the MPR of human L-selectin (K283A) causes this molecule to be shed continuously from untreated cells (56). We propose that the activation of L-selectin to a protease-sensitive conformation exposes a cryptic contact site within the EGF domain and thereby facilitates its interaction with TACE, as described above. The in-
ability of proline substitution mutants to undergo PMA-induced shedding would then result from their inability to adopt the conformation necessary for the docking of the EGF domain to TACE, while still permitting the slower PMA-mediated constitutive cleavage. It is possible that a conformational change may also occur in the MPR under activating conditions and that the proline substitution mutations prevent this. This would be consistent with the small PMA inducibility of shedding of the B7.2/MPR chimera. Since the cytoplasmic tail of L-selectin is not required for the activation (or inhibition) of proteolytic processing, the transition of L-selectin to the more proteolytically susceptible conformation could arise from the PMA-induced interaction with the extracellular domain of a separate regulatory membrane protein. The recent demonstration that perturbation of surface thiol groups induces shedding suggests that a membrane protein sulfide isomerase could be such a regulatory protein (80).

The distinct structural requirements for basal and stimulated shedding suggest that these activities may be mediated by different secretases, with only the enzyme mediating activation-dependent shedding (TACE) being blocked by the proline substitution in the MPR. Both basal and PMA-induced cleavage sites are identical, or at least very close (within 1–2 amino acids), and both processes are blocked by the metalloprotease inhibitor Ro 31-9790 and Drs. Ann Ager (London) and Stanley Perlman (Iowa City) for numerous helpful discussions.
Regulation of L-selectin Shedding by the EGF Domain

J. Immunol. 150, 952–963
Regulation of Membrane Metalloproteolytic Cleavage of L-selectin (CD62L) by the Epidermal Growth Factor Domain

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doi: 10.1074/jbc.M103748200 originally published online May 24, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103748200

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