The Cytoplasmic Domain of P-Selectin Contains a Sorting Determinant That Mediates Rapid Degradation in Lysosomes

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Abstract. P-selectin is a cell adhesion molecule required transiently on the surface of activated platelets and endothelial cells as a receptor for leukocytes. It is stored in secretory granules in platelets, endothelial cells, and transfected neuroendocrine cells and is rapidly delivered to the plasma membrane upon exocytosis of the secretory granules. It is then rapidly internalized in endothelial cells and transfected cells. We find that in transfected neuroendocrine PC12 cells, the fraction of P-selectin that is not targeted to secretory granules is rapidly degraded. In transfected CHO fibroblasts, which lack secretory granules, P-selectin was degraded with a half time of 2.3 h in plated cells, while low density lipoprotein receptor (LDL-R) had a half life of 9 h. In cells cultured in ammonium chloride to inhibit lysosomal proteinases, P-selectin was protected from degradation and rapidly accumulated in vesicles enriched in lgp-B, a resident lysosomal membrane protein. The cytoplasmic domain of P-selectin was sufficient to confer rapid turnover on LDL-R. Deletion of 10 amino acids from the cytoplasmic domain of P-selectin extended the half life to 9.5 h and abrogated rapid lysosomal targeting in the presence of ammonium chloride, implicating this sequence as a necessary element of a novel lysosomal targeting signal. The rate limiting step in degradation occurred after internalization from the cell surface, indicating that sorting of P-selectin away from efficiently recycled proteins occurs in endosomes. We propose that this sorting event represents a constitutive equivalent of receptor down regulation, and may function to regulate the expression of P-selectin at the surface of activated endothelial cells.

Endocytosis and recycling of plasma membrane proteins are vital processes in mammalian cells. Membrane proteins that are concentrated in clathrin-coated pits, exemplified by the low density lipoprotein receptor (LDL-R),[1] are cleared from the surface very rapidly and delivered to early endosomes (also called sorting endosomes (10, 51) or CURL (13). Many of the ligands for these receptors are released from the receptors in the acidic environment of the early endosome, and the soluble contents are transported to late endosomes and then to lysosomes (14, 42). While most of the receptors recycle with high efficiency to the plasma membrane via recycling endosomes (36), occasionally some receptors escape from the early endosome recycling pathway and are transported to late endosomes. Most of these receptors are then transported to the TGN rather than to lysosomes (17, 55). This pathway presumably provides an alternative way of recycling receptors to the plasma membrane. Delivery of recycling receptors such as LDL-R to lysosomes occurs with half-times typically on the order of 0.5–1.5 d (41, 50, 55), indicating that these receptors can be internalized on the order of 100 times, and can recycle through the TGN several times, before being delivered to lysosomes.

The low probability with which recycling receptors are delivered to lysosomes is consistent with the idea that lysosomal delivery of these proteins may represent a low level of random missorting. However, certain receptors involved in signaling pathways can be modified in the presence of external signals so that they are rapidly internalized and delivered to lysosomes. Membrane proteins such as EGF receptor and CD4 are rapidly transported from the cell surface to lysosomes when they are phosphorylated upon activation of the appropriate signaling pathway, a process referred to as receptor downregulation (2, 53, 56). In these cases lysosomal delivery appears to be due not to mis-sorting, but to activation of a lysosomal targeting signal.

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1. *Abbreviations used in this paper*: CI-MPR, cation-independent mannose 6-phosphate receptor/insulin-like growth factor II receptor; LDL-R, low density lipoprotein receptor; LLP, chimeric protein consisting of the lumenal and transmembrane domains of LDL-R and the cytoplasmic domain of P-selectin.

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We have examined the trafficking of P-selectin, a membrane protein that is stored in the secretory granules of platelets and endothelial cells (30, 37, 38). Previous work has shown that P-selectin is exposed rapidly after stimulation of these cells and then facilitates adhesion of leukocytes. When expressed in neuroendocrine cell lines, it is targeted to regulated secretory granules (9, 27). P-selectin is rapidly internalized from the surface of activated endothelial cells (20) and transfected CHO cells.[2] The cytoplasmic domain of P-selectin contains signals both for sorting into secretory granules and for rapid internalization (9).[2] In this paper we show that P-selectin is degraded in lysosomes rapidly after internalization from the cell surface. Mutagenesis experiments implicate a 10-amino acid cytoplasmic sequence of P-selectin in targeting P-selectin to lysosomes. Rapid transport of P-selectin from the cell surface to lysosomes may be a constitutive equivalent of receptor down regulation, a process that normally requires activation of signaling pathways.

Materials and Methods

Antibodies

Three monoclonal antibodies recognizing distinct epitopes in the lumenal domain of P-selectin, designated S12, W40, and G1 (12, 25), were pooled and used at 10 μg/ml each for western blotting an immunofluorescence microscopy. IgG isolated from polyclonal goat serum against purified P-selectin (34) was used for immunoprecipitation. Rabbit polyclonal antiserum was generated against a peptide corresponding to the 25 COOH-terminal amino acids of P-selectin (24) coupled to Keyhole Limpet hemocyanin with glutaraldehyde. Antigen-specific immunoglobulins were purified from the serum by affinity chromatography on a column of peptide coupled at 2 mg/ml to Epoxy-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) according to the manufacturer's protocol. Unbound proteins were washed from the column with PBS, and bound antibodies were eluted with 3 M KSCN; 50 mM Tris pH 7.4. The eluate was dialyzed against PBS and concentrated by ultrafiltration. Ascites containing mAb C7 (4) recognizing human LDL receptor was generated in pristane-primed Balb/c mice and was used unfractionated. Cell culture medium conditioned by hybridoma cells secreting mAb 4A4 (American Type Culture Collection, Rockville, MD), recognizing the cytoplasmic domain of the LDL receptor (59), was frozen in aliquots and used within 3 wk of thawing. IgG purified from polyclonal rabbit antiserum raised against rat cation-independent mannose 6-phosphate receptor (CI-MPR) was the generous gift of Dr. William Brown (Cornell University, Ithaca, NY). Hybridoma cells producing monoclonal antibodies 3E9 and 1B3 recognizing hamster lgp-B were kindly provided by Dr. Sandra Schmid (Scripps Research Institute, La Jolla, CA). Conditioned culture supernatants from these hybridoma lines were filtered and stored at 4°C. Polyclonal rabbit antiserum recognizing bovine chromogranin A was provided by Dr. Daniel O'Connor (University of California, San Diego, CA). Polyclonal antiserum recognizing TGN38 (35) was a gift from Dr. Paul Luzio (Cambridge University, England). Polyclonal rabbit antiserum recognizing bovine dopamine β-hydroxylase was a gift from Dr. Erik Schweitzer (University of California, Los Angeles, CA). mAb H68.4 recognizing transferrin receptor (61) was kindly provided by Ian Trowbridge (Salk Institute, La Jolla, CA). mAb SY38 recognizing synaptophysin was from Boehringer Mannheim (Indianapolis, IN). Goat anti-rabbit IgG and goat anti-mouse IgG conjugated to HRP, TRITC-, and FITC-conjugated goat anti-rabbit IgG, and FITC-conjugated goat anti-mouse IgG were from Cappel (Organon Teknika, Durham, NC). TRITC-conjugated goat anti-mouse IgG was from Fisher Biotech (Pittsburgh, PA).

Construction of Expression Vectors

All procedures involving oligonucleotide and cDNA manipulations were performed essentially as described in Sambrook et al. (52). Except where indicated, plasmids were grown in *E. coli* strain DH5α or DH12S (GIBCO BRL, Gaithersburg, MD). P-selectin cDNA was excised from pIBI20-E4 (9) by digestion first with AflII and filling in with Klenow polymerase followed by digestion with SalI. This complete 2.5-kb cDNA was subcloned into the pCB6 expression vector (7) in which the HindIII cloning site had been replaced with an XhoI site after digestion of this vector with BamHI and filling in, then digestion with XhoI. The blunt end ligation at the 3′ end of the cDNA reconstituted the Afl II restriction site and the cDNA translation stop codon that is included in the restriction site. This vector was designated pCB6-E4. Human LDL receptor cDNA was subcloned from pLDLR2 (63) into the XbaI and SmaI sites in Bluescript SK+ phagemid (Stratagene, La Jolla, CA), and designated pBS-LDL-R. Four overlapping oligonucleotides encoding the P-selectin cytoplasmic domain (nucleotides 2424 to 2531) (24) plus a 5′ XmaI site and a 3′ HindIII site were synthesized and phosphorylated. An equimolar mixture of the oligonucleotides was annealed and ligated to pBS-LDL-R that had been linearized with XmaI. The ligated DNA was digested with HindIII, gel purified and religated to generate pBS-LDL-R/PSel. B0265 *dut-/ung-* cells were transformed with pBS-LDL-R/PSel, and single stranded DNA was recovered using VCSM13 phage (Stratagene) to infect cultures grown in 25 μM uridine. Loop-out mutagenesis was performed using the mutagenic oligonucleotide 5′CATCTTTTTGTCTGAAACGCTTTCTCCATAGAAGGAAGACCCCCAGGCAA3′ to join the sequence encoding the transmembrane domain of the LDL receptor precisely with the sequence encoding the cytoplasmic domain of P-selectin, generating the plasmid pBS-LLP. pBS-LLP was recovered from DH12S cells and restriction mapped, then sequenced from the unique internal Bgl-II site in the LDL receptor cDNA through the 3′ end at the HindIII site to confirm that the mutagenesis had produced the correct cDNA. Full length LDL receptor cDNA was subcloned using NotI and HindIII from pBS-LDL-R into pCB6 vector in which the Bgl II cloning site had been replaced with a NotI site. This plasmid was designated pCB6-LDL-R. A BglII/HindIII fragment of 0.8-kb from pBS-LLP encoding the 3′ end of the mutagenized cDNA was subcloned into pCB6-LDL-R to generate pCB6-LLP.

Cell Culture

PC12 clone A1 cells (16) were grown in DME H21 medium (Cell Culture Facility, University of California, San Francisco, CA) containing 10% horse serum and 5% FBS (Hy-Clone, Logan, UT). Transfected cells were grown in the same medium containing 400 μg/ml G418 (GIBCO BRL). CHO cells were grown in α-modified MEM containing 5% heat-inactivated FBS. Transfection and isolation of CHO cells expressing P-selectin (9) or deletion mutants C1 or C2 of P-selectin has been described.[2] These cells were grown in α-modified MEM containing 5% heat-inactivated FBS and 400 μg/ml G418. LDL-depleted serum containing HDL was prepared by adding anhydrous KBr to ice-cold heat-inactivated FBS to a concentration of 9.2% (wt/vol), centrifuging at 200,000 *g* for 18 h, removing the upper fraction to the density interface, and dialyzing the remainder extensively against 150 mM NaCl (47). Medium for CHO cells expressing LDL-R or the chimeric protein LLP (see below) was prepared by dialyzing 0.03 volumes of heat-inactivated FBS into α-MEM, then adding 5% (vol/vol) LDL-depleted serum and GMS-G medium supplement (GIBCO BRL). This was necessary for long-term growth of these cells, which could not be grown in normal serum due to the toxic effects of excessive LDL uptake in cells overexpressing the receptor. Cells were passaged by washing with PBS lacking divalent cations and containing 1 mM EDTA followed by trituration in the same buffer. Hybridoma cells were grown in alpha-modified MEM containing 10% heat-inactivated FBS.

Transfection and Screening

Cells were transfected using DOTAP lipofectin reagent (Boehringer Mannheim) or Lipofectin reagent (GIBCO BRL) according to the method of Muller et al. (44). Transfected cells were passaged into selection medium two days after transfection. Clones were picked directly from the plates using 200-μl pipettes after washing once with PBS lacking divalent cations and containing 1 mM EDTA. PC12 cell clones expressing P-selectin were screened first by western blotting using the three monoclonal antibodies recognizing P-selectin, and then by immunofluorescence microscopy using the same antibodies (see below). CHO transfectants expressing LDL-R or LLP were screened by immunofluorescence microscopy using the C7 anti-LDL receptor mAb. CHO cells expressing LDL-R or the LLP chimeric protein were subcloned to obtain clones with uniform expression levels. Control experiments showed that 4A4 mAb only labeled cells expressing

2. Setiadi, H., M. Disdier, D. F. Bainton, W. M. Canfield, and R. P. McEver, manuscript in preparation.

LDL-R, and the anti-peptide antibody recognizing the cytoplasmic domain of P-selectin only labeled cells expressing LLP or P-selectin (not shown).

Immunofluorescence Labeling and Microscopy

Cells were processed for immunofluorescence microscopy at room temperature. Cells grown on 12-mm glass coverslips coated with poly-D-lysine were washed twice with PBS and fixed in 3% formaldehyde; 100 mM sodium phosphate pH 7.4 for 15 min. After washing twice in PBS containing 20 mM glycine, cells were permeabilized in 2% BSA; 0.02% saponin; 150 mM NaCl; 10 mM Hepes pH 7.4 (Blocking Buffer) for 30 min. Antibodies were applied for 1 h in Blocking Buffer containing 10% normal goat serum. Ascites fluid and antisera were diluted 1:100, culture supernatants were diluted 1:8, and purified antibodies were used at 20 μg/ml. Secondary antibodies were diluted 1:100. For epifluorescence microscopy, mouse monoclonal antibodies were detected with FITC-conjugated secondary antibodies and rabbit polyclonal antibodies were detected with TRITC-conjugated secondary antibodies. For scanning confocal microscopy, mouse monoclonal antibodies were detected with TRITC-conjugated secondary antibodies, and rabbit polyclonal antibodies were detected with FITC-conjugated secondary antibodies. These combinations minimized signal bleedthrough from one fluorescence channel to the other for each of the microscopes. Each antibody incubation was followed by three 10-min washes in Blocking Buffer. The cells were then washed twice in PBS, once in distilled water, and mounted in 90% glycerol; 10 mM Tris pH 8.2 containing 10 mg/ml *p*-phenylene diamine (Sigma Chemical Co., St. Louis, MO). Samples were viewed, and photographed on Kodak T-Max 400 film (Eastman Kodak Co., Rochester, NY), using a Nikon Diaphot epifluorescence microscope with a 100 × oil immersion planapochromatic objective lens with a numerical aperture of 1.35. Laser scanning confocal microscopy was performed on a BioRad MRC 600 scanning confocal microscope using a 60 × planapochromatic oil immersion objective lens with a numerical aperture of 1.4. The confocal optical section thickness was ∼0.6 μm, estimated using the tilted mirror method. No significant bleedthrough was observed, and no bleedthrough values were subtracted from the data. All immunofluorescence localization experiments were performed at least twice. The micrographs depict representative results.

Subcellular Fractionation

Up to 7 × 10^7 PC12 A1-E4 cells (one 80% confluent 15-cm dish) expressing P-selectin were washed twice with ice cold PBS, then harvested in PBS lacking divalent cations. Cells were resuspended in 0.8 ml 0.32 M sucrose; 4 mM Hepes pH 7.4; 0.5 mM EDTA (Homogenization Buffer) and homogenized in a glass dounce homogenizer until 80–90% of the nuclei were trypan blue positive (12–15 strokes). The homogenate was centrifuged at 735 *g* for 8 min, and the supernatant was loaded onto a 12.2-ml linear gradient of 10–23% metrizamide. Stocks of 10 and 23% metrizamide were made by diluting 35% metrizamide; 4 mM Hepes, pH 7.4; 0.5 mM EDTA with Homogenization Buffer. The gradients were poured in 16 × 76 mm Quick Seal tubes (Beckman Instruments, Inc., Palo Alto, CA) containing a 0.7-ml cushion of 35% metrizamide. Gradients were centrifuged in a Beckman 70.1 Ti rotor for 2.25 h at 52,000 rpm (185,000 *g* av.). Fractions of 0.7 ml were collected from the top of the gradient. Fractions were analyzed immediately for refractive index, β-hexosaminidase activity (48) and INT-succinate reductase activity (45). For analysis of proteins labeled with $Na_2{}^{35}SO_4$, fractions were precipitated by addition of TCA to 10% final concentration. Acid precipitates were washed with acetone, dissolved in 30 μl 8 M urea, then mixed with 30 μl 2× concentrated electrophoresis sample buffer and heated in boiling water for 3 min. For Western blotting, fractions were adjusted to 1.5% SDS; 125 mM Tris pH 6.95; 1 mM EDTA using concentrated stocks, then heated in boiling water for 3 min. For immunoprecipitation, fractions were combined beginning at the top of the gradient with the first four fractions, then in sets of three through fraction 19. The pooled fractions (∼2 ml) were diluted with Homogenization Buffer to 13.5 ml and centrifuged at 60,000 rpm (330,000 *g* max.) for 5.5 h to pellet the membranes. The membranes were dissolved in 400 μl IP buffer (see below) for immunoprecipitation of P-selectin.

Metabolic Labeling

For pulse metabolic labeling with ^{35}S-amino acids, cells were washed with PBS, then incubated for 20–30 min in DME lacking methionine and cysteine containing 10% dialyzed FBS or LDL-depleted serum. Cells were labeled in the same medium containing 300–800 μCi/ml ^{35}S-amino acid mixture (TransLabel, ICN, Irvine, CA). Chase incubations were performed by washing the cells once in complete growth medium, then culturing in growth medium supplemented with 3 mM methionine and 3 mM cysteine. PC12 cells were labeled overnight with $Na_2{}^{35}SO_4$ (Amersham Corp., Arlington Heights, IL) in DME lacking sulfate and containing 10% dialyzed FBS mixed 9:1 with normal growth medium. Cells were then incubated in normal growth medium for 3 h to allow for transport of labeled proteins and proteoglycans through the constitutive secretory pathway.

Cell Surface Biotinylation

For irreversible cell surface biotinylation to measure turnover of cell surface proteins, cells were grown to 60–80% confluence on 6-cm dishes. Cells were washed three times with ice-cold PBS containing 1 mg/ml glucose (PBS-G), then labeled for 20 min on ice with 0.5 mg/ml sulfo-NHS-biotin (Pierce, Rockford, IL) dissolved just before use in PBS-G. Labeling was stopped by washing twice with ice-cold PBS containing 1 mg/ml lysine and once with ice-cold growth medium. Cells were then recultured at 37°C in complete growth medium. Reversible biotinylation with NHS-SS-biotin (Pierce) to measure the rate of internalization was performed as described previously (31).

Immunoprecipitation

PC12 cells expressing P-selectin were washed twice with PBS, harvested in PBS containing 1 mM EDTA, and lysed in IP buffer (1% NP-40; 0.5% deoxycholate; 150 mM NaCl; 10 mM Tris HCl pH 7.8; 1 mM EDTA) on ice. Typically, cells from an 80–90% confluent 6-cm dish (∼5 × 10^5 cells) were lysed in 300–400 μl buffer. Nuclei and insoluble material were pelleted at 30,000 *g* for 10 min, and the supernatant was adjusted to 0.25% SDS. Polyclonal goat anti–P-selectin antibodies were added in the ratio of 6 μg IgG for each 5 × 10^5 cells. After 1–2 h on ice, 25 μl of a 25% slurry of protein G–Sepharose (Pharmacia) in IP Wash buffer (1% NP-40; 0.2% SDS; 150 mM NaCl; 10 mM Tris HCl pH 7.8) was added, and the mixture incubated with constant gentle mixing for 60 min. The Sepharose beads were washed 4 times with IP Wash buffer and once with 50 mM NaCl; 5 mM Tris HCl pH 7.4. Bound proteins were eluted into electrophoresis sample buffer (29) containing 4% SDS and 20 mM DTT. CHO cells were lysed on the plates in 400 μl IP buffer for immunoprecipitation of P-selectin or IP buffer containing 1 mg/ml BSA for immunoprecipitation of LDL-R or the chimeric protein LLP. SDS was added to 0.25% final concentration to the cleared lysates for precipitation of P-selectin. Precipitations were performed with 6 μl C7 ascites plus 2 μg anti–P-selectin peptide antibody (for LLP) or 40 μl 4A4 culture supernatant (for LDL-R) for each 5 × 10^5 cells (one 6-cm dish). P-selectin was immunoprecipitated from lysates of membranes recovered from metrizamide density gradient fractions using 30 μg polyclonal goat anti–P-selectin per sample. Samples were incubated overnight at 4°C, followed by the addition of 15 μl protein G–Sepharose for 1 h with continuous mixing.

Electrophoresis, Western Blotting, and Phosphorimager Analysis

Immunoprecipitates were separated on SDS-polyacrylamide gels containing 9% acrylamide (29). Prestained molecular weight markers (Sigma Chemical Co.) were used, and biotinylated markers (Sigma Chemical Co.) were included where appropriate. Gels containing ^{35}S-labeled proteins were fixed in 30% methanol; 10% acetic acid in water and dried. Radioactivity in the gel bands was quantitated using a Molecular Dynamics phosphorimager and ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA). Gels were subsequently exposed to Kodak XAR-5 x-ray film at −80°C. For Western blotting, proteins were transferred to nitrocellulose filter paper (Schleicher and Schuell, Keene, NH), blocked in PBS; 0.05% Tween 20; 5% non-fat milk solids, and probed with antibodies diluted in the same buffer. After washing off unbound HRP-conjugated secondary antibodies, the filters were washed twice with PBS, impregnated with ECL (Amersham Corp.) chemiluminescence substrate, and exposed to x-ray film. Biotinylated proteins transferred to nitrocellulose were detected using ^{125}I-streptavidin as described (17, 33) and radioactive streptavidin bound to the bands was quantitated using the phosphorimager.

Analysis of half-Lives

Turnover experiments were analyzed by plotting the log of the phosphorimager volume for each gel band (with background from a blank lane on the gel subtracted) vs. chase time, and obtaining a slope *m* for the plot by

regression analysis. All measurements presented were obtained from at least four time points. Turnover was assumed to be a first order process, with $t_{1/2} = 0.693/k$ where $k = -2.3\ m$. Variation between duplicate samples analyzed in early experiments averaged 3-4%. While the half-life measurements were consistent for each clone, correlation coefficients for the regression analyses varied. The half-lives and correlation coefficients shown in Tables I and II represent data from one experiment for each clone, as described in the legends.

Results

Expression of P-Selectin and LDL-R in PC12 Cells and CHO Cells

We have found that recycling of several membrane proteins from the cell surface through the TGN occurs at equal rates, suggesting that this pathway is not selective for a subset of internalized membrane proteins (17). However, since some granule membrane proteins may recycle to the TGN (40), we were interested in determining whether this specialized class of proteins might be targeted to the TGN more efficiently than plasma membrane receptors for re-incorporation into nascent secretory granules. To address this question, we expressed P-selectin as well as LDL-R in both the PC12 neuroendocrine cell line and in CHO cells. CHO fibroblasts expressing P-selectin (9) and PC12 cells expressing human LDL-R (17) have been described. The pCB6 expression vector (7), which has yielded high expression levels in PC12 cells with a variety of cDNAs, was used for expression of P selectin in PC12 Clone A1 cells (16), and for expression of LDL-R in CHO cells (see Materials and Methods). One PC12 A1 clone exhibiting a high level of P-selectin expression and normal morphology, judged by immunofluorescence and phase contrast microscopy, was selected for further study. CHO cells expressing uniformly high and low levels of LDL-R expression were isolated (see below).

Accumulation of P-Selectin in Secretory Granules in PC12 Cells

The steady-state distribution of P-selectin in PC12 A1 cells expressing P-selectin was examined by laser scanning confocal immunofluorescence microscopy. As described previously for transfected AtT-20 cells (9, 27), P-selectin (Fig. 1 *B*) was concentrated in many of the same structures containing chromogranin A (Fig. 1 *A*), a soluble content protein of regulated secretory granules in PC12 cells. A fraction of the P-selectin expressed in these cells co-fractionated on density gradients with secretogranin II and dopamine β-hydroxylase, markers of chromaffin granules (see below). Thus, targeting of P-selectin to regulated secretory granules occurred normally in transfected PC12 A1 cells.

Rapid Turnover of P-Selectin in PC12 Cells and CHO Cells

The rate of turnover of P-selectin expressed in transfected PC12 A1 cells and CHO cells was compared to the rate of LDL-R turnover in these cells measured by pulse-chase metabolic labeling and immunoprecipitation. Cells were labeled in suspension with ^{35}S-amino acids for 30 min, then aliquoted into culture dishes in chase medium as described in Materials and Methods. After a 60-min incubation to allow export from the endoplasmic reticulum and full processing of oligosaccharide side chains in the Golgi apparatus, dishes were harvested at intervals extending to 23 h. Proteins were quantitatively immunoprecipitated from cell lysates, the precipitates resolved on SDS-polyacrylamide gels, and the radioactivity in the P-selectin or LDL-R bands quantitated by phosphorimager analysis. An autoradiograph of a typical experiment measuring P-selectin turnover is shown in Fig. 2. The turnover of P-selectin in CHO cells labeled in suspension exhibited simple first order kinetics with a half time of 1.3 h (Table I). All three of the P-selectin bands immunoprecipitated from CHO cells were degraded at the same rate, suggesting that the minor bands do not represent degradation products of the predominant band. Rather, they most likely represent molecules that were terminally glycosylated to a lesser degree than the predominant form. LDL-R was degraded more slowly with a half time of 6.6 h in these cells. The turnover of P-selectin in PC12 cells was biphasic. During the first 6 h of chase, approximately two thirds of the labeled protein was degraded with a half time of 3.1 h. Analyzing the turnover from 7 to 23 h of chase showed that approximately one third of the labeled protein was degraded with a longer half-time of 9.7 h (Table I). These

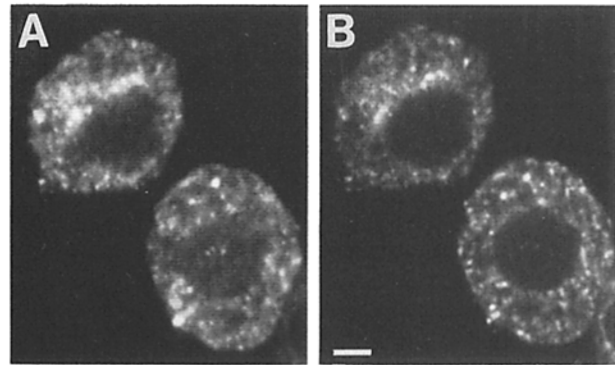

Figure 1. P-selectin is concentrated in regulated secretory granules in transfected PC12 A1 cells. PC12 A1 cells expressing P-selectin were processed for indirect immunofluorescence localization of chromogranin A, a soluble content protein of regulated secretory granules, using a polyclonal rabbit antiserum (*A*), and P-selectin, using mAb S12 and mAb W40 (*B*). Laser scanning confocal microscopy showed that P-selectin was concentrated in many of the structures containing chromogranin A, and was also present in some structures lacking chromogranin A. Bar, 2 μm.

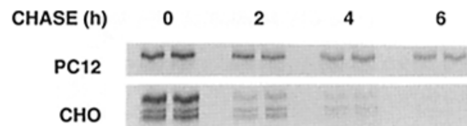

Figure 2. Turnover of P-selectin in PC12 and CHO cells. Transfected PC12 and CHO cells expressing P-selectin were pulse labeled with ^{35}S-amino acids in suspension, then aliquoted in chase medium onto plates. Cells were harvested from duplicate plates beginning 1 h after the end of the pulse (t = 0) to allow time for transport of labeled proteins through the Golgi apparatus, then at the indicated intervals. P-selectin was immunoprecipitated from detergent lysates of the cells and separated by SDS-PAGE. Autoradiographs of the duplicate samples are shown. All three bands immunoprecipitated from CHO cells expressing P-selectin were degraded at the same rate.

Table I. Turnover of P-Selectin in CHO and PC12 Cells in Suspension

	Half-lives in hours (and correlation coefficients) of P-selectin and LDL-R in transfected cells	
	LDL-R	P-Selectin
CHO cells	6.6 (0.99)	1.3 (0.97)
PC12 cells	20*	3.1 (0.97), 0-6 h
PC12 cells		9.7 (0.98), 7-23 h

Transfected cells expressing human LDL-R or P-selectin were pulse labeled with ^{35}S-amino acids in suspension, then aliquoted in chase medium onto plates. Cells were harvested and lysed beginning 1 h after the end of the pulse to allow time for transport of labeled proteins through the Golgi apparatus. P-selectin or LDL-R were immunoprecipitated and separated by SDS-PAGE. Autoradiographs of P-selectin from duplicate samples are shown in Fig. 2. Radioactivity in the bands was quantitated by phosphorimager analysis. Half-lives of P-selectin were determined from the average of duplicate samples in one experiment (shown), and confirmed using individual samples in an independent experiment (not shown). The half-life of LDL-R in CHO cells was determined from single experiments in two CHO clones, LDL-R 9 and LDL-R 20 (see Fig. 7), which yielded identical rates and correlation coefficients. LDL-R exhibited a single rate of turnover in both cell types. In PC12 cells, approximately two thirds of the labeled P-selectin was degraded with first order kinetics over the first 6 h, while the remainder exhibited a significantly longer half life, measured from 7 to 23 h of chase.

* Data from ref 17.

half-lives were significantly shorter than that of LDL-R, which was degraded with a single half time of 20 h in PC12 cells.

P-Selectin That Does Not Enter Secretory Granules in PC12 Cells Is Rapidly Degraded

The observation that P-selectin turnover was biphasic in PC12 cells but monophasic in CHO cells suggested that P-selectin that was not targeted to regulated secretory granules might have a shorter half-life than the molecules that are targeted to granules. Entering secretory granules would delay delivery of P-selectin to the cell surface by several hours relative to transport through the constitutive secretory pathway (26), and would delay transport to lysosomes. To determine whether targeting to secretory granules was an important factor in P-selectin turnover, organelles from PC12 A1 cells expressing P-selectin were fractionated on isosmotic sucrose/metrizamide linear density gradients to separate secretory granules from other membrane compartments that contain P-selectin. Markers for lysosomes (β-hexosaminidase activity), mitochondria (INT-succinate reductase activity), and secretory granules (secretogranin II, a soluble granule protein metabolically labeled overnight with $Na_2{}^{35}SO_4$) were concentrated in the dense fractions (Fig. 3 *A*). Western blot analysis showed that dopamine β-hydroxylase, a chromaffin granule marker, was also concentrated in dense fractions (Fig. 3 *B*), while transferrin receptor (plasma membrane and early endosomes), CI-MPR (late endosomes), synaptophysin (synaptic vesicles and endosomes), and TGN38 (TGN), were found primarily in lighter fractions.

P-selectin was found throughout the density gradient, with an accumulation in light fractions and another accumulation in the dense fractions corresponding to the peak of secretory granule markers (Fig. 3 *B*). At steady state P-selectin was most concentrated in secretory granules, as assessed by immunofluorescence microscopy (Fig. 1). Also, P-selectin did not colocalize with the lysosomal membrane protein lgpA by immunofluorescence microscopy in transfected PC12 A1 cells (not shown), indicating that the protein does not accumulate to a significant concentration in lysosomes (see below). Therefore, we conclude that P-selectin in the dense fractions (fractions 13–18) is mainly in secretory granules. The pool of P-selectin in light membrane fractions depleted in secretory granule markers presumably occupies a variety of organelles including plasma membrane and endosomes.

To determine the relative stability of P-selectin in light and dense organelles, PC12 A1 cells expressing P-selectin were labeled metabolically with ^{35}S-amino acids for 30 min, then chased for 2 or 11 h before homogenization and fractionation on the sucrose/metrizamide gradients. The fractions were merged into six pools of increasing density, diluted to lower densities, then centrifuged to recover the membranes. P-selectin was immunoprecipitated from detergent lysates of the membranes. After a 2 h chase, when the large rapidly degraded pool should predominate (Table I), labeled P-selectin was most concentrated in light membrane fractions (Fig. 3 *C*), with a smaller amount recovered from dense membrane fractions. In contrast, after an 11-h chase, when the slowly degraded pool should predominate, the fractions containing most of the secretory granule markers (Pool 5) also contained the most labeled P-selectin. Thus, rapid turnover of P-selectin in PC12 cells correlates with light membrane density, and slow turnover is associated with the secretory granule pool. This suggests that P-selectin that does not enter granules is degraded rapidly, which would prevent accumulation of P-selectin at the cell surface in the absence of a secretory stimulus. Since detailed analysis of P-selectin turnover in PC12 cells is complicated by the presence of a secretory granule pool and a light membrane pool, we chose to pursue this work using CHO cells, in which the turnover of P-selectin exhibited simple first order kinetics (Table I).

Turnover of P-Selectin in CHO Cells Occurs in Lysosomes

Since 80% of the P-selectin in CHO-E4 cells is found on the cell surface at steady state (9), the observed rapid turnover of the protein was unexpected. This raised the possibility that P-selectin was released by proteolysis from the cell surface, although we have not been able to immunoprecipitate fragments of P-selectin from the medium of transfected cultures. To determine whether P-selectin is degraded in lysosomes, CHO-E4 cells metabolically labeled in suspension were incubated in the presence or absence of agents that inhibit lysosomal proteolysis. After a 90-min chase, approximately half of the labeled P-selectin present at the beginning of the chase was degraded (Fig. 4, *control*). Inclusion of 15 mM ammonium chloride in the chase medium, which raises the pH of acidic organelles and thereby potently inhibits lysosomal enzyme activity, protected most of the P-selectin from degradation during a 1.5-h chase (Fig. 4, *Amm. Chloride*). Since P-selectin binds to the tetrasaccharide sialyl Lewis x (30, 37), and since binding of polyvalent ligands (such as a serum glycoprotein with multiple glycosylation sites) can redirect rapidly internalized proteins from the recycling pathway into the lysosomal pathway (1, 21, 41, 60), the turnover of P-selectin was measured in the presence of medium containing 2% BSA instead of serum proteins. P-selectin was rapidly degraded in cells incubated in 2% BSA instead of serum (Fig. 4, *BSA, no serum*), and was protected from degra-

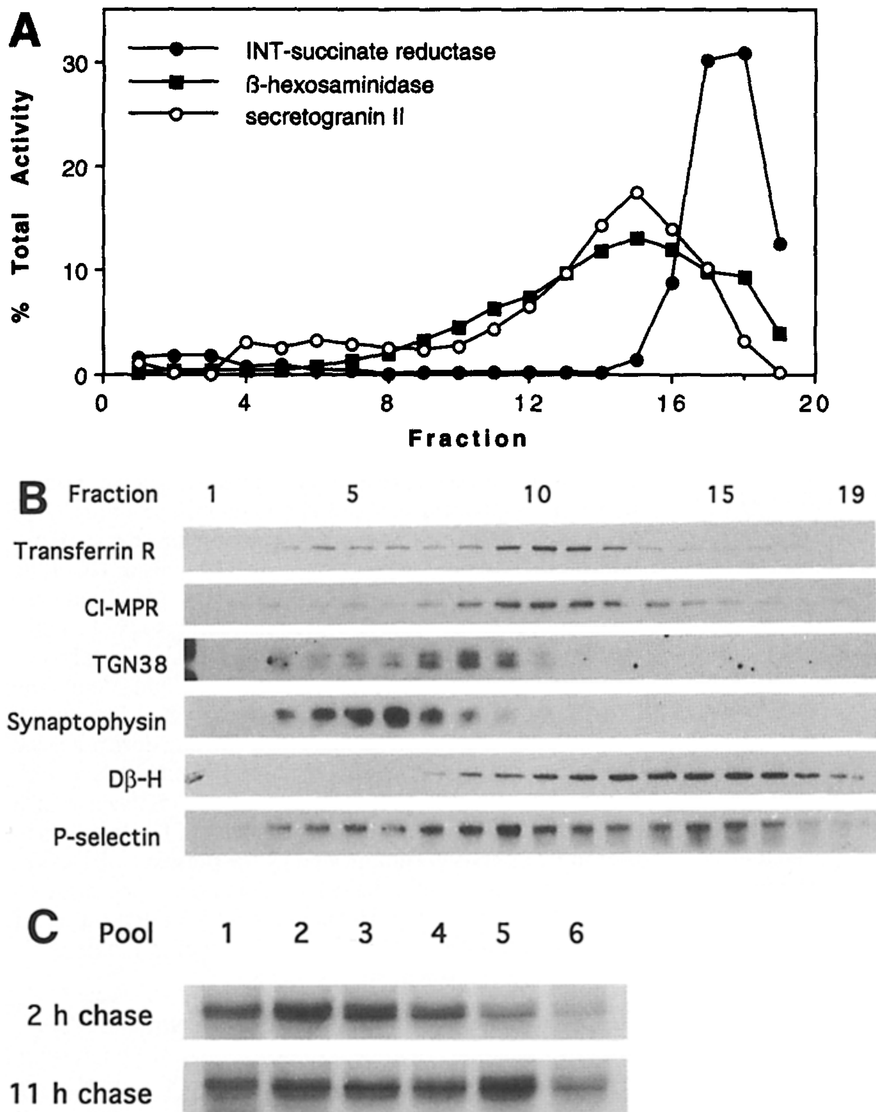

Figure 3. P-selectin in secretory granules has a longer half life than P-selectin in other membrane compartments. PC12 A1 cells expressing P-selectin were homogenized and fractionated on 10–23% metrizamide/sucrose gradients. Fractions were collected from the top of the gradient. (*A*) Activities of β-hexosaminidase (■) and INT-succinate reductase (●) were measured in the fractions. Secretory granules were identified by labeling cells for 16 h with $Na^{35}SO_4$ and chasing for 3 h before fractionation. Proteins in the fractions were precipitated with trichloroacetic acid, dissolved in urea and SDS, and separated by PAGE. Radioactivity in the secretory granule content protein secretogranin II, the major labeled species in the fractions, was quantitated by phosphorimager analysis (○). The same gradient profiles were obtained for all three markers in two independent trials. The enzyme activities shown in *A* and Western blots shown in *B* are from the same experiment. (*B*) Western blot analysis of the gradient fractions. Gradient fractions were mixed with concentrated SDS sample buffer, separated by SDS-PAGE and transferred to nitrocellulose filters. The filters were probed with antibodies against transferrin receptor (*Transferrin R*), CI-MPR, TGN38, synaptophysin, dopamine β-hydroxylase (*Dβ-H*) or P-selectin, followed by secondary antibodies coupled to HRP. Antibodies were detected by chemiluminescence. Fraction 1 did not contain detectable levels of any of the membrane proteins assayed. All gradient profiles were reproducible in at least two independent experiments. Data from a single gradient are shown. (*C*) Immunoprecipitation of metabolically labeled P-selectin from pooled gradient fractions. PC12 A1 cells expressing P-selectin were labeled with ^{35}S-amino acids for 30 min and chased for 2 h or 11 h before homogenization and fractionation. Fractions were pooled as follows: lane *1*, fractions 1–4; lane *2*, fractions 5–7; lane *3*, fractions 8–10; lane *4*, fractions 11–13; lane *5*, fractions 14–16; lane *6*, fractions 17–19. Pooled fractions were diluted sixfold with 0.32 M sucrose and centrifuged to recover the membranes. P-selectin was immunoprecipitated from the detergent-solubilized membranes and separated by SDS-PAGE. Autoradiographs of the gels from a single experiment are shown.

dation by inclusion of ammonium chloride (Fig. 4, *BSA, Amm. Chloride*), indicating that rapid turnover of the protein was not due to aggregation of the protein caused by binding of a soluble polyvalent ligand present in serum. Inclusion of the proteinase inhibitor leupeptin for the first hour of the chase incubation (until the control sample was harvested at $t = 0$) protected a fraction of the P-selectin from degradation (Fig. 4, *Leupeptin*). Addition of serum that had not been heat-inactivated to the chase medium did not promote degradation (Fig. 4, *Fresh serum*), suggesting that the protein was not cleaved from the cell surface by serum proteinases.

To confirm that P-selectin was rapidly delivered to lysosomes, CHO cells expressing P-selectin were incubated for 90 min with or without 15 mM ammonium chloride before processing for immunofluorescence microscopy. Since ammonium chloride treatment inhibited degradation of P-selectin (Fig. 4), rapid delivery of the protein to lysosomes in the presence of ammonium chloride should result in accumulation of P-selectin to high concentrations in lysosomes. Lysosomes were identified by labeling with monoclonal antibodies that recognize lgp-B (S. Pfeffer, personal communication), an abundant resident lysosomal membrane protein (28). P-selectin antibodies labeled numerous small structures throughout the cytoplasm, and were concentrated at one pole of the nucleus (Fig. 5, *A* and *C*). lgp-B antibodies labeled less numerous structures distributed randomly throughout the cytoplasm (Fig. 5, *B* and *D*). In the absence of ammonium chloride, some lgp-B-positive structures were also P-selectin-positive (Fig. 5, *A* and *B*, *small arrows*), but most were not (Fig. 5 *B*, *large arrows*). After 90 min in ammonium chloride, almost all lgp-B-positive structures were also P-selectin-positive (Fig. 5, *C* and *D*). These results show that

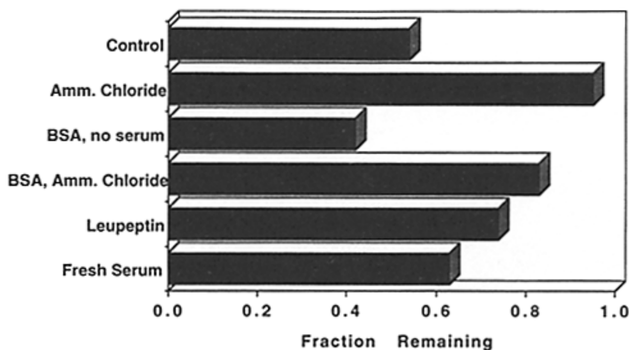

Figure 4. Rapid turnover of P-selectin is dependent on functional lysosomes. CHO cells expressing P-selectin were pulse labeled with ^{35}S-amino acids in suspension. Aliquots were plated in normal growth medium containing excess unlabeled methionine and cysteine (chase medium) for 1 h, at which time one plate was harvested. The remainder were incubated for an additional 90 min under the following conditions; normal growth medium (control); normal growth medium containing 15 mM ammonium chloride (*Amm. Chloride*); MEM α containing 2% BSA in place of serum (*BSA, no serum*), MEM α containing 2% BSA in place of serum and 15 mM ammonium chloride (*BSA, Amm. Chloride*); chase medium containing 10 μg/ml leupeptin for the first hour of chase only, then normal growth medium for 90 min (*Leupeptin*); and normal growth medium supplemented with an additional 5% (vol/vol) FBS that had not been heat-inactivated (*Fresh Serum*). After the chase incubation, P-selectin was immunoprecipitated from detergent lysates of the cells and separated by SDS-PAGE. The radioactivity in each P-selectin band was quantitated by phosphorimager analysis, and the radioactivity recovered after a 90-min chase was normalized to the radioactivity recovered at time zero. Results of a single experiment are shown. An independent experiment in which pre-treatment with leupeptin and incubation in fresh serum were omitted produced similar results.

when rapid degradation was inhibited by ammonium chloride, native P-selectin accumulated to a significant concentration in lysosomes within 90 min.

Expression of LDL-R and the Chimera LLP in CHO Cells

Delivery of resident lysosomal membrane proteins to lysosomes (19, 46, 62) and downregulation of plasma membrane proteins in response to signaling pathways (2, 53, 57) are dependent on cytoplasmic amino acid sequences. If the mechanism of targeting of P-selectin to lysosomes in the endocytic pathway is related to the targeting of other membrane proteins to lysosomes, the cytoplasmic domain of P-selectin would be expected to contain the necessary information. To determine whether this was the case, a cDNA was constructed encoding the COOH-terminal 35–amino acid cytoplasmic domain of P-selectin spliced precisely to the sequence encoding the lumenal and transmembrane domains of the human LDL-R. CHO clones exhibiting stable expressing of the chimeric protein, designated LLP, were generated. In addition, two deletion mutants of P-selectin, lacking different segments of the cytoplasmic domain were also analyzed (Fig. 6).[2] The rate of synthesis of the transfected proteins in each of these clones was compared by labeling equal numbers of each clone with the same mixture of ^{35}S-amino acids, chasing the label for a short time to allow oligosaccharide processing to occur, then immunoprecipitating the expressed proteins from detergent lysates. The immunoprecipitates were analyzed by SDS-PAGE and phosphorimager analysis of the gel bands. An autoradiograph of the gel is shown in Fig. 7. Minor high mobility bands varied in their relative abundance in different experiments, and likely represent less completely glycosylated versions of the predominant band. The radioactivity measured by phosphorimager analysis in each gel band relative to the other comparable constructs is indicated. P-selectin and the deletion mutants C1 and C2 were synthesized at similar rates. Two clones expressing significantly different levels of LDL-R (LDL-R 9 and LDL-R 20), and two clones expressing significantly different levels of the chimeric protein LLP (LLP 6 and LLP 31) were studied to determine whether expression level influenced the trafficking of these proteins (see below).

Steady-State Distribution of LLP and CI-MPR in CHO Cells

To determine whether LLP, P-selectin and LDL-R occupy the same endocytic compartments, the subcellular distribution of these proteins was compared to the distribution of CI-MPR, which is most concentrated in late endosomes (28). When examined by laser scanning confocal immunofluorescence microscopy, the chimeric protein LLP was found concentrated in small punctate structures distributed throughout the peripheral cytoplasm and in a concentration of small structures at one pole of the nucleus, and was present at low concentration on the cell surface (Fig. 8, *red*). CI-MPR (Fig. 8, *green*) was concentrated in structures that accumulated near the nucleus, and the receptor was not abundant in the peripheral cytoplasm. Although both proteins were concentrated in structures accumulating at one pole of the nucleus, close inspection showed that many of these structures were positive for one label or the other (Fig. 8 *B*), while some were positive for both (*yellow*). CHO cells expressing P-selectin or native LDL-R showed a pattern of labeling indistinguishable from the pattern seen for LLP (not shown). In CHO cells, the early endosome compartment includes a collection of tubules and vesicles termed recycling endosomes that accumulate at one pole of the nucleus, presumably at the microtubule organizing center (36). Since there was limited overlap between the distribution of P-selectin, LLP and LDL-R and the distribution of CI-MPR, we conclude that the transfected proteins are not concentrated in late endosomes.

Internalization of LDL-R and LLP

To determine whether the rates of internalization were equal for LDL-R and LLP, internalization of these proteins was measured by cell surface biotinylation with the reducible probe NHS-SS-biotin. LDL-R 9 and LLP 31 cells were labeled at 0°C, recultured for varying intervals at 37°C, then returned to 0°C and incubated in reduced glutathione buffer to remove biotin from proteins remaining at the cell surface. LDL-R or LLP were immunoprecipitated from cell lysates, separated on non-reducing SDS-polyacrylamide gels, and transferred to nitrocellulose. Biotinylated proteins were detected with ^{125}I-streptavidin and quantitated by phosphor-

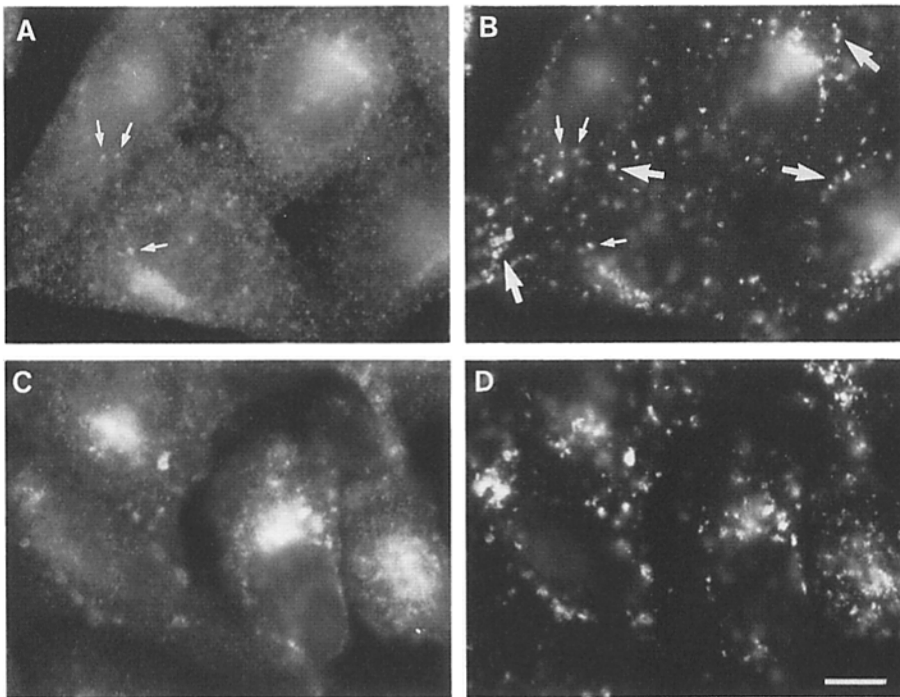

Figure 5. Rapid accumulation of P-selectin in lysosomes in the presence of ammonium chloride. CHO cells expressing P-selectin were incubated for 90 min in the absence (*A* and *B*) or presence (*C* and *D*) of 15 mM NH_4Cl before fixation and processing for indirect immunofluorescence microscopy. P-selectin was detected with a rabbit polyclonal anti-peptide antibody recognizing the cytoplasmic domain, and lgp-B, a resident lysosomal membrane protein, was localized with two mouse monoclonal antibodies recognizing distinct lumenal epitopes. Native P-selectin was detected in abundant small structures throughout the cytoplasm, and concentrated at one pole of the nucleus (*A*). lgp-B was found in less numerous, larger structures scattered throughout the cytoplasm (*B*). Some of the lgp-B–positive structures were also positive for P-selectin (*small arrows* in *A* and *B*), but many were not (*large arrows* in *B*). After ammonium chloride treatment for 90 min, which inhibits proteolysis in lysosomes, almost all of the structures containing lgp-B also contained detectable levels of P-selectin (*C* and *D*). Bar, 2 μm.

imager analysis. The cytoplasmic domains of P-selectin and LDL-R mediated internalization of LDL-R ectodomain and transmembrane domain with equal rate and efficiency (Fig. 9). Similar results were obtained using LDL-R 20 and LLP 6 (not shown). We conclude that the cytoplasmic internalization signal in P-selectin is equivalent to that found in LDL-R.

Turnover of P-Selectin, LDL-R, and LLP in CHO Cells

The half lives of P-selectin, LDL-R, and LLP chimera, and the deletion mutants C1 and C2 of P-selectin were measured in the transfected CHO cells by pulse-chase metabolic labeling. We found that the half-lives of these proteins were shorter when the cells were labeled metabolically in suspension (Table I) than when the entire experiment was performed on plated cells. Similar findings have been reported for the turnover of CI-MPR in CHO cells (50). Therefore, these turnover experiments were performed on plated cells, to be consistent with the immunofluorescence and internalization experiments. Turnover of the cell surface population of these proteins was also measured by derivatizing cell surface proteins with sulfo-NHS-biotin, and measuring loss of biotin from the immunoprecipitated proteins. In cells labeled on plates, the half-life of LDL-R was 9 h, and the half-life of P-selectin was 2.3 h, measured by both metabolic labeling and surface labeling (Table II). Since the half-life of the biosynthetically labeled pool was the same as the half-life of the cell surface pool in each case, we conclude that the rate-limiting step in turnover of these proteins occurs after internalization from the cell surface. The half-life of the chimeric protein LLP was similar to the half-life of P-selectin, indicating that the cytoplasmic domain of P-selectin is sufficient to mediate rapid delivery to lysosomes. The half-life of the P-selectin C2 deletion mutant was 9.5 h, similar to the half-life of LDL-R, demonstrating that the deleted region, C1, is necessary for rapid turnover (Table II). Turnover of the P-selectin C1 construct did not exhibit first order kinetics and therefore could not be compared directly with the other constructs. However, even though P-selectin C1 is internalized at less than half the rate of native P-selectin (Setiadi et al., submitted for publication), it appears to be turned over significantly faster than P-selectin C2.

P-selectin C2 Is Not Transported Efficiently to Lysosomes

The biochemical data presented above implicate the C1 region of the cytoplasmic domain as an element that is necessary for rapid delivery of P-selectin to lysosomes. To confirm that the P-selectin C2 construct, lacking the C1 region, is not delivered to lysosomes as rapidly as native P-selectin, CHO cells expressing P-selectin C2 were incubated for 90 min with or without 15 mM ammonium chloride before processing for immunofluorescence localization of P-selectin and lgp-B, under exactly the same conditions as shown for native P-selectin in Fig. 5. P-selectin antibodies labeled numerous small structures throughout the cytoplasm, and accumulated at one pole of the nucleus (Fig. 10, *A* and *C*), and lgp-B antibodies labeled less numerous structures distributed ran-

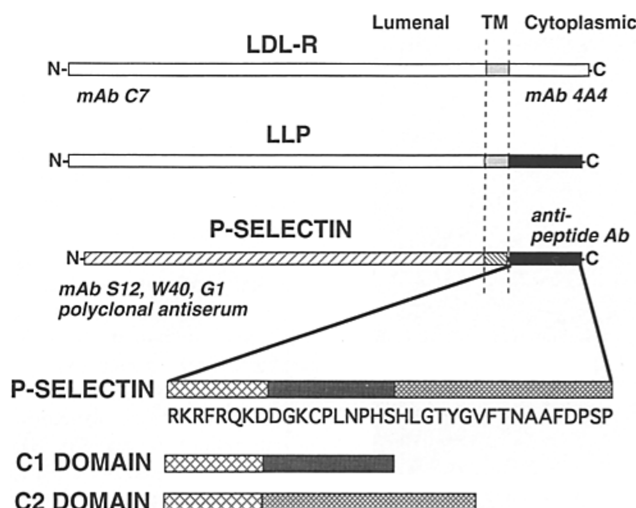

Figure 6. Constructs and antibodies used in this study. The following proteins were expressed by transfection of cDNA vectors; human LDL-R; the chimeric protein LLP, consisting of the lumenal and transmembrane domains of LDL-R and the cytoplasmic domain of P-selectin; human P-selectin; deletion mutants of P-selectin designated C1, lacking the C2 region of the cytoplasmic domain corresponding to the 17 carboxyl-terminal amino acid residues of native P-selectin; and C2, lacking the C1 region of the cytoplasmic domain corresponding to the 10-amino acid residues immediately amino-terminal to the C2 region. mAb C7 reacts with the amino-terminal region of the lumenal domain of LDL-R, and mAb 4A4 reacts with the cytoplasmic domain. mAb S12, mAb W40 and mAb G1 react with distinct epitopes in the lumenal domain of P-selectin, and an affinity purified rabbit polyclonal antibody raised against a peptide corresponding to the 25 COOH-terminal amino acids of P-selectin recognizes the cytoplasmic domain of P-selectin and the deletion mutant C2. A polyclonal goat antiserum raised against immunopurified native P-selectin was used to immunoprecipitate P-selectin constructs.

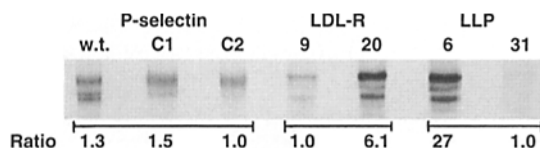

Figure 7. Comparison of rates of synthesis of P-selectin, P-selectin C1, P-selectin C2, LDL-R, and LLP. Equal numbers of CHO cells expressing native P-selectin or the deletion mutants C1 or C2, and two clones each expressing different levels of LDL-R or LLP were metabolically labeled with the same mixture of ^{35}S-amino acids for 45 min, then chased in medium containing excess unlabeled amino acids for 1 h. Labeled proteins were immunoprecipitated from detergent lysates, analyzed by SDS-PAGE and quantitated by phosphorimager analysis. The ratios of the phosphorimager values for comparable constructs are shown. P-selectin, P-selectin C1, and P-selectin C2 were synthesized at similar rates. Clones expressing significantly different levels of LDL-R (LDL-R 9 and LDL-R 20) or LLP (LLP 6 and LLP-31) were selected for analysis. This single experiment reflects the clonal differences in signal intensity obtained consistently in immunofluorescence, metabolic labeling and cell surface labeling experiments. Minor higher mobility bands seen in most of the samples varied in intensity relative to the main lower mobility band in different immunoprecipitations, and were not included in the analysis.

domly throughout the cytoplasm (Fig. 10, *B* and *D*). In contrast to cells expressing native P-selectin, in CHO cells expressing P-selectin C2 few if any lgp-B-positive structures could be clearly identified as P-selectin-positive, with (Fig. 10, *C* and *D*) or without (Fig. 10, *A* and *B*) ammonium chloride treatment. This demonstrates that delivery of P-selectin C2 to lysosomes was significantly less efficient than delivery of native P-selectin.

Discussion

P-selectin is targeted to regulated secretory granules in platelets and endothelial cells and is rapidly internalized from activated endothelial cells (38). The protein is targeted to regulated secretory granules in neuroendocrine AtT-20 (9, 27) and PC12 cells (this study), and is transported efficiently to the cell surface of transfected CHO cells where it is rapidly internalized (9).[2] We found that P-selectin, unlike LDL-R, was rapidly delivered to lysosomes and degraded after its internalization, both in PC12 an CHO cells. In PC12 cells, the fraction of P-selectin that failed to enter secretory granules was rapidly degraded. For each of the proteins we examined, the half-lives of the metabolic and surface pools were equal in CHO cells, indicating that the rate-limiting step in turnover of these proteins occurs after appearance at the cell surface. This means that for each construct, most of the protein passes through the plasma membrane en route to lysosomes, in contrast to resident lysosomal membrane proteins (8, 18, 19). Because the cytoplasmic domains of P-selectin and LDL-R supported internalization at equal rates, the observed 4–6-fold difference in the rate of turnover of P-selectin and LDL-R must be due to sorting of these proteins from each other in endosomes. The consequence of this sorting event is rapid delivery of P-selectin to lysosomes, confirmed by demonstrating accumulation of P-selectin in lysosomes when proteolysis was inhibited.

The rate of turnover of these proteins is determined by the cytoplasmic domains, since replacing the cytoplasmic domain of LDL-R with the cytoplasmic domain of P-selectin conferred rapid turnover on the chimeric protein. A 10-amino acid deletion mutant of P-selectin exhibited the same half-life as LDL-R, and the same subcellular distribution even when lysosomal proteolysis was inhibited. Thus, the cytoplasmic domain is sufficient, and the deleted region necessary, for rapid turnover. These results indicate that the cytoplasmic domain of P-selectin contains a determinant for lysosomal targeting, in addition to the previously identified determinants for targeting to regulated secretory granules in the biosynthetic pathway (9) and for rapid internalization from the cell surface.[2] Our results indicate that rapid delivery of plasma membrane proteins to lysosomes does not represent random missorting from endosomes, but is signal mediated.

The exact sequence requirements for the sorting determinant mediating lysosomal targeting of P-selectin remain to be determined. Since the P-selectin deletion mutant missing the C1 region of the cytoplasmic domain exhibited the same half life as LDL-R, the C1 region is the most likely candidate for this sorting determinant, although it is possible that sequences outside of, or overlapping with, the C1 region also participate in lysosomal targeting. The information required

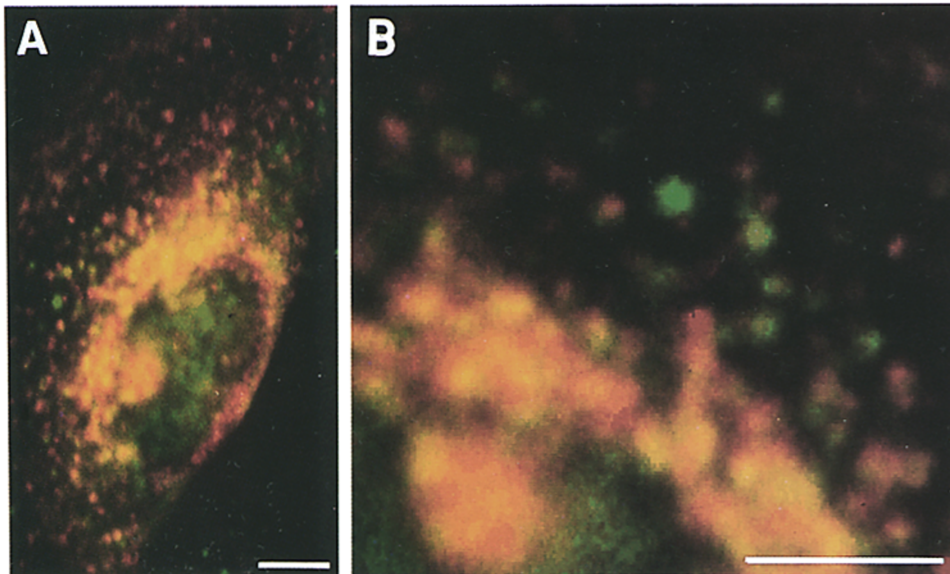

Figure 8. Simultaneous localization of LLP and CI-MPR in CHO clone LLP 6. Indirect immunofluorescence labeling of the chimeric protein LLP simultaneously with the cation-independent mannose 6-phosphate receptor, which is most concentrated in late endosomes, was analyzed by laser scanning confocal microscopy. LLP (*red*), localized with mAb C7, was found in numerous small structures throughout the peripheral cytoplasm and in an accumulation of small structures at one pole of the nucleus (*A*). Structures enriched in CI-MPR (*green*), localized using a polyclonal rabbit antiserum, accumulated in the same juxtanuclear region, and were sparse in the peripheral cytoplasm. Confocal microscopy demonstrated that many of the labeled structures are enriched either in CI-MPR or in LLP, with some enriched in both (*yellow*). *B* shows an enlargement of the juxtanuclear region enriched in both markers. Equivalent experiments using CHO cells expressing P-selectin and CHO LDL-R 20 produced the same labeling patterns (not shown). Bars, 1 μm.

for lysosomal targeting is independent of a rapid internalization signal in the C2 region of the cytoplasmic domain.[2] The sequence of the C1 domain, DGKCPLNPHS, does not resemble any of the sorting signals known to operate in the TGN or endosomes, including those that target resident lysosomal membrane proteins (19, 62), CD3 chains (32) or lysosomal acid phosphatase (46) to lysosomes. It also does not resemble internalization motifs, most of which contain aromatic residues (58) or the signals that target mannose 6-phosphate receptors from the TGN to endosomes (22, 23). C1 does contain a sequence resembling the internalization signal DXKS found on yeast alpha factor receptor (49), and supports internalization, albeit at a significantly reduced rate, independently of the C2 domain (Setiadi et al., submitted for publication).

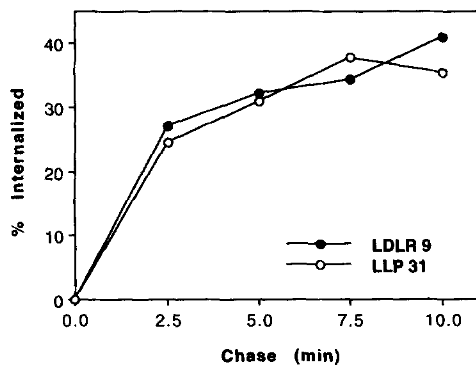

Figure 9. The cytoplasmic domains of P-selectin and LDL-R contain equivalent internalization signals. Internalization was measured by derivatizing LDL-R 9 or LLP 31 cell surface proteins with disulfide-linked biotin, warming for the indicated time intervals, and removing biotin remaining on the cell surface by reduction with glutathione. LDL-R (●) or LLP (○) were immunoprecipitated from detergent lysates, separated on non-reducing SDS-polyacrylamide gels, and transferred to nitrocellulose. Biotin was detected with radioiodinated streptavidin, and quantitated using a phosphorimager. Signals obtained from cells that were not warmed prior to reduction (3–4% of the total incorporated) were subtracted from all samples. Remaining signals were compared to the signal obtained from cells that were not warmed or reduced (100%). The data shown are from one experiment. Similar results were obtained using clones LDL-R 20 and LLP 6. The cytoplasmic domain of P-selectin mediated internalization of LLP with the same rate and efficiency as native LDL-R.

Table II. Turnover of P-Selectin, LDL-R, LLP, and P-Selectin Deletion Mutants C1 and C2 in Plated CHO Cells

Construct	Half-lives in hours (and correlation coefficients) for each protein	
	Metabolic label	Surface label
P-selectin	2.3 (0.98)	2.3 (0.95)
LDL-R 9	8.8 (0.93)	9.0 (0.87)
LDL-R 20	8.5 (0.95)	9.3 (0.97)
LLP 6	3.5 (0.99)	3.1 (0.93)
LLP 31	2.7 (0.99)	2.3 (0.99)
P-selectin-C2	9.5 (0.97)	9.1 (0.87)
P-selectin-C1	1–2 (*)	3–6 (*)

Turnover was measured after metabolic labeling with ^{35}S-amino acids or after cell surface biotinylation, using plated cells. Cells were harvested at intervals, and proteins were immunoprecipitated from detergent lysates and separated by SDS-PAGE. Metabolic label was quantitated by phosphorimager analysis of the gel bands. Biotin-derivatized cell surface proteins were transferred to nitrocellulose and localized by probing with ^{125}I-streptavidin. Radioactive bands on the blots were quantitated by phosphorimager analysis. The log of the phosphorimager values vs. chase times were plotted. Half-lives were calculated from the slope of the plot. All half-lives were determined in two independent experiments, which yielded similar values, except metabolic labeling of LDL-R 20 (n = 3), and metabolic labeling of LLP 31 and P-selectin C1 (n = 1). The correlation coefficients obtained from at least four time points in a single experiment are shown.

* Turnover of the deletion mutant P-selectin-C1 did not display first order kinetics.

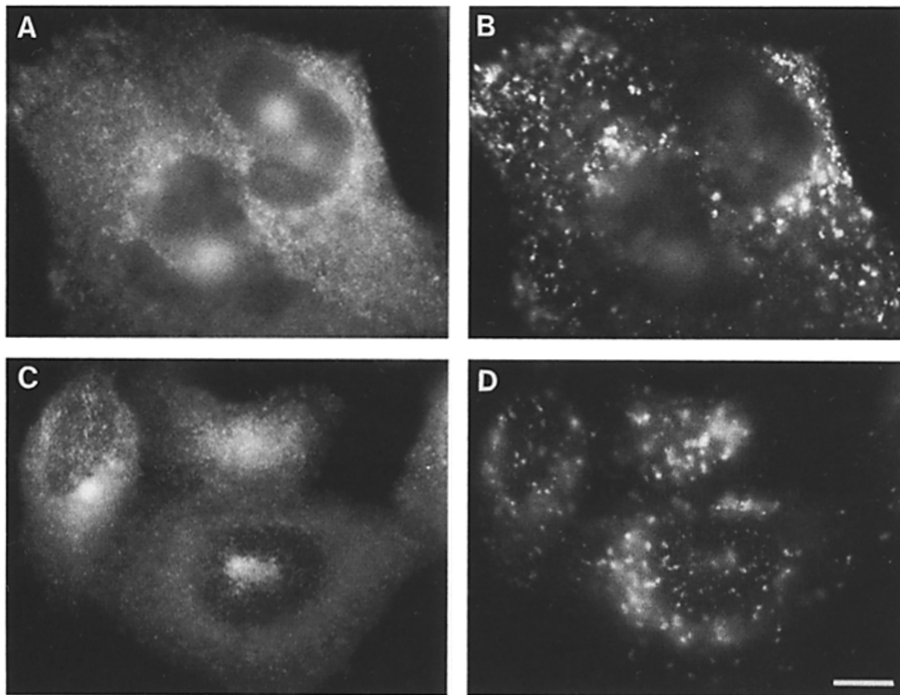

Figure 10. P-selectin C2 does not accumulate rapidly in lysosomes. CHO cells expressing P-selectin C2 were incubated for 90 min in the absence (*A* and *C*) or presence (*B* and *D*) of 15 mM NH_4Cl before fixation and processing for indirect immunofluorescence microscopy. P-selectin was detected with a rabbit polyclonal anti-peptide antibody recognizing the cytoplasmic domain, and lgp-B, a resident lysosomal membrane protein, was localized with two mouse monoclonal antibodies recognizing distinct lumenal epitopes. As shown for native P-selectin in Fig. 5, P-selectin C2 was found in abundant small structures throughout the cytoplasm, and concentrated at one pole of the nucleus (*A*), and lgp-B was found in less numerous, larger structures scattered throughout the cytoplasm (*B*). Unlike cells expressing native P-selectin, cells expressing the C2 construct had virtually no lgp-B-positive structures that were clearly P-selectin-positive, without (*A* and *B*) or with (*C* and *D*) ammonium chloride treatment. Bar, 2 μm.

Aggregation of the lumenal domains of membrane proteins by polyvalent ligands or cross-linking antibodies can divert these proteins from the recycling pathway to the lysosomal route (1, 21, 41, 60). Beyond the physical restraints it imposes, it is not clear how aggregation re-directs recycling proteins to lysosomes. The observation that the chimeric protein LLP is delivered to lysosomes as rapidly as P-selectin indicates that rapid transport of P-selectin to lysosomes is independent of aggregation of the lumenal domains. It is possible that the cytoplasmic domain of P-selectin causes rapid lysosomal degradation by mediating aggregation in the plane of the membrane.

The transport events we have described for P-selectin in CHO cells may parallel the biosynthetic transport of lysosomal acid phosphatase. Lysosomal acid phosphatase is synthesized as a transmembrane protein, and, unlike other resident lysosomal membrane proteins (8, 18, 19) is transported through the constitutive secretory pathway to the cell surface at a rate that is comparable with plasma membrane proteins (6). It is internalized from the surface at a rate similar to LDL-R, and recycles between endosomes and the cell surface for a short time after internalization. Comparing the rates of delivery of nascent lysosomal acid phosphatase to the cell surface ($t_{1/2}$ = 45 min) (6) and to lysosomes ($t_{1/2}$ = 5–6 h) (15) suggests that the half time for transport of lysosomal acid phosphatase from the surface to lysosomes is ~3–4 h in fibroblasts. This would suggest that lysosomal acid phosphatase is delivered from endosomes to lysosomes much faster than LDL-R. The 18-amino acid cytoplasmic domain of lysosomal acid phosphatase also appears to contain all of the information necessary for rapid delivery to lysosomes, and rapid internalization of the protein is required for lysosomal targeting (46).

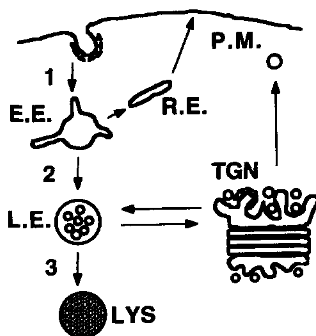

Figure 11. Possible sites of sorting of P-selectin into the lysosomal pathway. The organelles involved in endocytosis and recycling of cell surface receptors are shown. Diverting P-selectin from the recycling pathways to lysosomes could occur either by selective delivery from early endosomes (*E.E.*) to late endosomes (*L.E.*) (Step *2*), from late endosomes to lysosomes (*Lys.*) (Step *3*), or both (see Discussion). *P.M.*, plasma membrane; *R.E.*, recycling endosome; *TGN*, *trans*-Golgi network.

Where does sorting of recycling receptors from lysosomally targeted proteins occur? Since LDL-R and LLP are internalized at equal rates, the difference in their rates of turnover cannot be explained by sorting at the cell surface (Fig. 11, step *1*). The results presented here demonstrate that P-selectin and LDL-R are sorted from each other in endosomes, but they do not distinguish between sorting in early endosomes, sorting in late endosomes, or both. The steady-state concentration of P-selectin in early endosomes does not address the location of the sorting event, as illustrated briefly in the following two extreme scenarios. If P-selectin and LDL-R were sorted from each other exclusively in early endosomes such that P-selectin was delivered from early endosomes to late endosomes (Fig. 11, step *2*) 10 times more efficiently than LDL-R, ~20–30% of the P-selectin molecules entering early endosomes would be diverted to late endosomes, as opposed to 2–3% of the LDL-R molecules.

Therefore, ~70–80% of the P-selectin molecules entering early endosomes would recycle to the cell surface in each cycle of endocytosis, as opposed to recycling of ~98% of the LDL-R molecules. The observed steady-state distribution of P-selectin and LLP in early endosomes (Fig. 8) does not prove or preclude this model. Since the cycle between the surface and early endosomes is many times faster than transport to lysosomes (1, 21, 36), recycling of P-selectin to the cell surface could predominate over lysosomal targeting even at the site of sorting from LDL-R. Since LDL-R that reaches late endosomes recycles to the TGN more efficiently than it is transported to lysosomes (17), this model predicts that P-selectin would recycle very efficiently to the TGN where, in regulated secretory cells, it could be re-incorporated into nascent secretory granules.

At the other extreme, P-selectin and LDL-R could recycle with the same efficiency from early endosomes to the cell surface, and be sorted from each other only upon reaching late endosomes. LDL-R would then recycle efficiently to the TGN and P-selectin would be efficiently retained and delivered to lysosomes (Fig. 11, step *3*). This scenario is consistent with the measured rates of transport between the endocytic compartments in PC12 cells; the rate of transport of LDL-R from the cell surface to the TGN, reflecting passage through late endosomes, is 2.5–3 h (17), which is similar to the rapid rate of turnover of P-selectin in these cells (Table II). This model predicts that very little P-selectin would recycle to the TGN after exocytosis. Evidence that recycling membrane proteins can be sorted from lysosomally-directed membrane proteins in late endosomes comes from the study of epidermal growth factor receptor. This protein normally undergoes down regulation upon binding high concentrations of ligand (56). During transport from the cell surface to lysosomes normal epidermal growth factor receptors accumulate in the internal vesicles of multivesicular bodies, while mutant receptors that lack tyrosine kinase activity, and recycle much more efficiently than native receptor, and are found on the limiting membrane of multivesicular bodies (11). Additional experiments will be required to determine the relative contributions of early and late endosomes to lysosomal targeting of P-selectin.

The observed rapid transport of P-selectin from the cell surface to lysosomes in transfected cells suggests that P-selectin is rapidly removed from the recycling pathway after internalization in endothelial cells, where it is normally expressed. P-selectin is highly concentrated in the Weibel-Palade bodies (regulated secretory granules) of endothelial cells and the alpha granules of platelets (39). Upon activation of these cells by thrombin, the granules fuse rapidly with the cell surface, exposing P-selectin to the circulation (30, 37). P-selectin then binds to a glycoprotein ligand expressed on myeloid cells (43). This is one of multiple binding interactions between circulating leukocytes and endothelial cells leading to the escape of the leukocytes from the circulation and delivery to sites of injury. Rapid internalization of P-selectin from the surface of activated cultured endothelial cells has been demonstrated (20). However, turnover and recycling of the protein have not been examined quantitatively in this system. If P-selectin were recycled from endosomes to the cell surface with the same efficiency as LDL-R, clearance of P-selectin from the surface of activated endothelial cells would occur with a long halftime equal to the turnover of LDL-R. Furthermore, if packaging of P-selectin into nascent secretory granules is not 100% efficient, the molecules that fail to enter granules would be transported through the constitutive secretory pathway to the cell surface, as they are in CHO cells, and could accumulate at the cell surface even in the absence of a secretory stimulus. However, if P-selectin is targeted to lysosomes rapidly in endothelial cells as it is in PC12 and CHO cells, no accumulation at the cell surface would occur due to constitutive exocytosis, and clearance from the cell surface following activation would take at most a few hours. This could be an important regulatory mechanism for controlling the activity of P-selectin in activated endothelial cells. As discussed above, it is not clear from our results whether P-selectin would recycle to the TGN to be re-incorporated into nascent secretory granules.

E-selectin, a cell adhesion molecule related to P-selectin, also functions as a leukocyte receptor in activated endothelial cells (5, 30, 38). Unlike P-selectin, E-selectin is not targeted to secretory granules. Rather, its expression is transiently induced in endothelial cells exposed to cytokines, resulting in its transient appearance at the cell surface. E-selectin is degraded rapidly in cultured endothelial cells (54). This suggests that, although the mechanisms for inducing expression of P-selectin and E-selectin at the surface of activated endothelial cells are entirely different, they may share a common mechanism, lysosomal targeting, for limiting cell surface expression. The cytoplasmic domain of E-selectin, while highly conserved between human and mouse (3), bears no obvious resemblance to the cytoplasmic domain of P-selectin.

An important and well-studied example of rapid transport of membrane proteins to lysosomes is receptor downregulation. Downregulation is clearly dependent on cytoplasmic domains of the receptor proteins. This is true of receptors that are themselves ligand-activated signaling molecules, such as receptors for epidermal growth factor or platelet derived growth factor (56), and of membrane proteins that are substrates of activated kinases, such as CD4 (2, 53). When receptors are downregulated after phosphorylation, they interact with the same internalization machinery, clathrin-coated pits, that is used for ligand-independent internalization of LDL-R (56). Similarly, the downregulated receptors may be targeted to lysosomes after activation of the signaling pathway by interacting with the same machinery that constitutively targets P-selectin to lysosomes.

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