A Balance of Opposing Signals within the Cytoplasmic Tail Controls the Lysosomal Targeting of P-selectin*

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Anastasia D. Blagoveshchenskaya, Eric W. Hewitt, and Daniel F. Cutler‡

From the MRC Laboratory for Molecular Cell Biology and Department of Biochemistry, University College London, Gower Street, London WC1E 6BT, United Kingdom

The 35-amino acid cytoplasmic tail of the adhesion receptor P-selectin is subdivided into stop transfer, C1 and C2 domains. It contains structural signals needed for targeting this protein to specialized secretory organelles and to lysosomes. Recently, using site-directed mutagenesis of horseradish peroxidase-P-selectin chimeras, we have uncovered a novel sequence within the C1 domain, KCPL, that mediates sorting from early, transferrin-positive endosomes to lysosomes and therefore operates as a positive lysosomal targeting signal (Blagoveshchenskaya, A. D., Norcott, J. P., and Cutler, D. F. (1998) J. Biol. Chem. 273, 2729–2737). In the current study, we examined lysosomal targeting by both subcellular fractionation and an intracellular proteolysis assay and found that a balance of positive and negative signals is required for proper lysosomal sorting of P-selectin. First, we have found that within the sequence KCPL, Cys-766 plays a major role along with Pro-767, whereas Lys-765 and Leu-768 make no contribution to promoting lysosomal targeting. In addition, horseradish peroxidase-P-selectin chimeras were capable of acylation in vivo with [3H]palmitic acid at Cys-766, since no labeling of a chimera in which Cys-766 was replaced with Ala was detected. Second, analysis of mutations within the C2 domain revealed that substitution of two sequences, YGVF and DPSP, causes an increase in both lysosomal targeting and intracellular proteolysis suggesting the presence of lysosomal avoidance signals. The inhibition or promotion of lysosomal targeting resulted from alterations in endosomal sorting since internalization was not changed in parallel with lysosomal delivery. Analysis of the double mutants KCPL/YGVF or KCPL/DPSP revealed that although the positive lysosomal targeting signal operates in the early/sorting transferrin-positive endosomes, the negative lysosomal targeting (lysosomal avoidance) signals act at later stages of the endocytic pathway, most likely in late endosomal compartments.

The endocytic trafficking of transmembrane proteins is controlled by sequences (sorting signals) within their cytoplasmic domains or within the cytoplasmic domains of associated proteins (for review see Ref. 1). The best documented signal-dependent sorting step is the recruitment of transmembrane proteins into clathrin-coated pits on the plasma membrane followed by internalization via clathrin-coated vesicles which, after uncoating, fuse with early/sorting endosomes (2, 3). This initial recruitment is mediated by sorting signals, most of which fall into one of the following two groups: tyrosine- or di-leucine-based motifs (4, 5). Early/sorting endosomes are thought to be a major sorting compartment along the endocytic pathway where the fate of internalized proteins is determined. From here, some proteins recycle back to the TGN (1) or to the plasma membrane, whereas others are directed to the late endosomes, morphologically identified as multivesicular bodies (MVB), and then to lysosomes for degradation. Moreover, in specialized cells, proteins can be transported to GLUT4-containing vesicles or may be transcytosed to an alternate plasma membrane (2, 6).

Sorting signals that mediate the delivery of proteins to lysosomes are also often members of the tyrosine- or di-leucine-based signal families. In most cases, internalization signals and lysosomal targeting signals (LTS) have been found to be identical (4). However, recently described novel LTS (7–10) provide evidence that the structural requirements for internalization and lysosomal trafficking can be different.

The selectivity of endosomal transport remains controversial. The earliest data showed that cross-linking of recycling proteins caused a re-direction to lysosomes, suggesting a default mechanism (11, 12). However, the existence of LTS supports the view that endosome-to-lysosome trafficking is a signal-dependent step. In addition, sorting within the late endosomal system is not well understood at a more than superficial level. First, it is still unclear whether late endosomes or MVB are involved in sorting to the same extent as early endosomes. Second, the mechanism of transfer between late endosomes and lysosomes also remains to be defined. In H.Ep.2 cells, where MVB were shown to be the dominant endocytic organelles (13–15), the removal of recycling proteins from early/sorting MVB gives rise to late MVB which gradually mature and then directly fuse with relatively stable pre-existing lysosomes (15). These findings have been extended with observations that the fusion of pre-existing lysosomes with late endosomes is followed by reformation of lysosomes from the resultant hybrid structures (16, 17).

Having reached mature MVB, further transfer to the lysosomes, while apparently the default route, is neither obligatory nor a final destination. However, little is known about the molecular signals that divert proteins from delivery to lysosomes or to retrieve them thereafter. To date, two lysosome avoidance motifs (LAM) found in the cytoplasmic tail of the cation-dependent mannose 6-phosphate receptor (CD-M6PR)

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‡ To whom correspondence should be addressed. Tel.: 44-171-380-7808; Fax: 44-171-380-7805; E-mail: d.cutler@ucl.ac.uk.
are the only sequences identified. The first LAM, Cys-Arg-Ser-Lys-Pro-Arg, does not reveal any homology to other sorting signals known so far (18). Subsequent studies established that the palmitoylation of the Cys within this sequence facilitates the exposure of a second, upstream LAM, the di-aromatic motif Phe-Trp (19, 20). These data suggest a requirement for a positive retrieval signal to avoid transfer from late endosomes to lysosomes.

P-selectin is a type I membrane protein originally found within the secretory organelles of endothelial cells and platelets (21–23). Following the stimulation of these cells with thrombin or other agonists, it is redistributed to the plasma membrane (24–26) and then rapidly internalized (27–31). When heterologously expressed in cells lacking a regulated secretory pathway, P-selectin is constitutively transported to the cell surface and efficiently endocytosed to lysosomes for degradation (10, 28).

The cytoplasmic tail of P-selectin comprises three different domains as follows: ST (stop transfer), membrane-proximal C1, and membrane-distal C2 (32). The sequence KCPL within the C1 domain has been found to mediate transfer from early to late MVB and lysosomes, acting in a similar way to some other LTS (8, 9, 33). The cytoplasmic tail of P-selectin therefore provides a good model system with which to study the regulation of lysosomal targeting.

In the present work, we have exploited a series of HRP-P-selectin chimeras containing point mutations across the KCPL sequence as well as mutations within the C2 domain to examine traffic to late MVB and lysosomes in H.Ep.2 cells. We have found that Cys-766 (a palmitoylation site within the P-selectin cytoplasmic tail (34)) is a crucial amino acid within the positive LTS domain, YGVF and DPSP, which operate as negative LTS or modulating the activity of the positive LTS. Alanine substitution of these sequences resulted in increased lysosomal degradation (10, 28).

The internalization of HRP-P-selectin chimeras was demonstrated to be incapable of internalization and, consequently, of delivery to lysosomes instead accumulating on the plasma membrane (35).

**Subcellular Fractionation and Quantitation of Data**—Following two rinses with ice-cold HB (320 mM sucrose, 10 mM HEPES, pH 7.3), cells were scraped into 1.5 ml of HB with a rubber policeman and passed 10 times through a ball-bearing homogenizer with a 0.09-mm clearance (made in the EMBL, Heidelberg, Germany). The cell homogenate was spun at 8500 × g for 5 min, and 1.3 ml of postnuclear supernatant was layered on an 11-ml 1–16% preformed linear Ficoll gradient made in HB. The centrifugation was carried out for 45 min at 35,000 rpm in an SW40T rotor (Beckman Instruments, Palo Alto, CA), fractionated in 0.5-ml fractions from the top of the tube using an Autodensi-Flow IIC (Buchler Instruments, Kansas City, MO), and then radioactivity of fractions was counted. Positions of lysosomes and late endosomes were identified by measurement of activity of the lysosomal marker enzyme, N-acetyl-β-D-glucosaminidase (NAGA), as described previously (35).

The fractions containing most NAGA activity were further purified using a second centrifugation. Fractions 17–23 from the 1–16% Ficoll gradient were pooled together, and 2.5 ml of this material was diluted with HB to make 4 ml and layered on a 9-ml 7–25% Ficoll gradient. Centrifugation, fractionation, and measurement of NAGA activity were then carried out as described for initial Ficoll gradients.

Targeting data were described as a lysosomal targeting index (LTI), i.e. the amount of HRP activity present in LE/Lys fractions for each mutant normalized to that for ssHRP<sub>P-selectin</sub>. Accordingly, in all the experiments, the LTI for ssHRP<sub>P-selectin</sub> was set at 1. To take into account variations of expression level, number of cells and lysosomal yield, the amount of HRP activity present in the LE/Lys peak (HRP-peak) has been corrected for the amount of NAGA activity (NAGA peak) within the LE/Lys fractions and for total HRP activity in the homogenate (HRP hmg). After simplifying the original equation, the LTI was defined as follows:

$$\text{LTI} = \frac{\text{mutant HRP peak of ssHRP-P-selectin}}{\text{mutant NAGA peak} \times \text{WT HRP hmg}}$$

(1)

Typically, the LTI for tail-less ssHRP<sub>P-selectin</sub> was about 20% of that for ssHRP<sub>P-selectin</sub> and was subtracted from those for the other chimeras in each experiment to provide a base-line. Thus the LTI for ssHRP<sub>P-selectin</sub> was considered as 0. The LTI for the mutants of the chimeras were therefore described on a scale within a range set by ssHRP<sub>P-selectin</sub> (1) and ssHRP<sub>P-selectin</sub> (0).

**HRP Proteolysis Assay**—Cells on 35-mm dishes were plated on ice, washed twice with ice-cold PBS, and subjected to Triton X-114 partitioning as described (37) followed by a standard HRP assay (see below) performed with two phases. Amounts of HRP activity present in the upper, hydrophilic, and lower, hydrophobic, phases were used to determine the percentage of clipped chimeras as a ratio of HRP activity in the upper phase to the total activity in the lysate. To normalize for interexperimental variations, we subtracted the fraction of HRP proteolysis of tail-less ssHRP<sub>P-selectin</sub> chimera (typically about 25%) as background from each data set since this chimera has previously been demonstrated to be incapable of internalization and, consequently, of delivery to lysosomes instead accumulating on the plasma membrane (35).

**Internalization Assay**—The internalization of HRP-P-selectin chimeras was assessed using a surface biotinylation strategy. H.Ep.2 cells expressing HRP-P-selectin chimeras grown in 6-well plates were rinsed with ice-cold PBS, twice with PBS supplemented with 0.7 mM CaCl<sub>2</sub> and 0.25 mM MgSO<sub>4</sub> (PBS<sup>−</sup>), and then incubated with 0.5 mg/ml NHS-SBiotin in PBS<sup>−</sup> for 30 min. Biotinylation was stopped by washing once with 50 mM glycine in PBS<sup>−</sup> and twice with PBS<sup>−</sup>. Some of the cells were then incubated at 37 °C with prewarmed growth media containing 20 mM HEPES, pH 7.2, for different times (4, 8, and 12 min) in a water bath followed by transfer of the cells onto ice. To remove surface-exposed biotin, the cells were then treated with freshly prepared glutathione buffer (50 mM glutathione, 75 mM NaCl, 75 mM NaOH, pH 8.6, 10% fetal calf serum) three times for 15 min each. The excess glutathione was quenched with PBS<sup>−</sup> containing 5 mg/ml iodoacetamide. The cells were then washed twice with PBS<sup>−</sup>, lysed in 1 ml of NDE buffer (1% Nonidet P-40, 0.4% sodium deoxycholate, 66 mM EDTA, 10 mM Tris-HCl, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A). The lysates were spun for 15 min at 13,000 rpm in a microcentrifuge and 0.3% SDS was added.
FIG. 1. Schematic illustration of HRP-P-selectin chimera. The top line shows the components used for construction as follows: boxes represent the individual components; sequences outside boxes were added during construction. hGH, human growth hormone signal sequence; HRP, enzymatically active domain of horseradish peroxidase; P-selec-tin, transmembrane (TM) and cytoplasmic domains of P-selectin. The 35 residues of the wild type cytoplasmic domain have been assigned to the stop transfer (ST), C1 and C2 domains according to exon-intron boundaries. The bottom part shows the full amino acid sequences of the cytoplasmic domains of the chimeras starting with the wild type P-selectin, ssHRP*-selectin. The carboxyl-terminal end of the TM domain shown is boxed. The name of each chimera is listed to the left. The number at the end of the chimera name indicates which codon has been changed to a stop codon (numbering is taken from the human P-selectin sequence, Johnston et al. (32)). The tetrapeptide sequence or number of a single amino acid at the end of each chimera’s name show the amino acids that have been replaced by alanine.

to the resulting supernatants. Biotinylated proteins were collected by incubation with 30 μl of streptavidin-agarose at +4 °C for 2 h. Beads were then washed twice with NDEDSDS, one time with NDEDSDS containing 0.5 M NaCl, and finally one time with PBS. Samples were solubilized in 30 μl of reducing sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% [3H]palmitic acid, the transfected cells were grown on 90-mm dishes to subconfluency, incubated in Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum, 4 mM non-essential amino acids, and 5 mM sodium pyruvate (FA medium) for 1 h in the CO2 incubator, and labeled with [35S]methionine/cysteine, the cells grown on 60-mm dishes were rinsed twice with PBS and incubated in 2 ml of FA medium containing 2 mCi of [3H]palmitic acid for 6 h at 37 °C. For labeling with [3H]palmitic acid for 6 h at 37 °C. Metabolic Labeling and Immunoprecipitation—For labeling with [3H]palmitic acid, the transfected cells were grown on 90-mm dishes to subconfluency, incubated in Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum, 4 mM non-essential amino acids, and 5 mM sodium pyruvate (FA medium) for 1 h in the CO2 incubator, and labeled with [3H]palmitic acid for 6 h at 37 °C. Metabolic labeling and immunoprecipitation—For labeling with [3H]palmitic acid, the transfected cells were grown on 90-mm dishes to subconfluency, incubated in Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum, 4 mM non-essential amino acids, and 5 mM sodium pyruvate (FA medium) for 1 h in the CO2 incubator, and labeled with [3H]palmitic acid for 6 h at 37 °C. The tetrapeptide sequence or number of a single amino acid at the end of each chimera’s name show the amino acids that have been replaced by alanine.

RESULTS

Contribution of Single Amino Acids within KCPL Sequence into Lysosomal Targeting of HRP-P-selectin Chimeras—We have previously shown that lysosomal targeting of P-selectin is dependent on the sequence KCPL located within the membrane-proximal C1 domain. Within this motif, Pro-767 was shown to make a major contribution to its function (10). To determine whether residues other than Pro-767 contribute to the efficiency of lysosomal targeting, we have engineered mutant HRP-P-selectin chimeras with alanine substitutions of Lys-765, Cys-766, and Leu-768 (Fig. 1). We have previously introduced two assays to characterize the lysosomal targeting of HRP-P-selectin chimeras in H.Ep.2 cells as follows: assay 1, quantitation of the efficiency of targeting to a mixed population
of late MVB and lysosomes (referred to below as lysosomal fractions) using subcellular fractionation to calculate the LTI, and assay 2, Triton X-114 partitioning to determine the extent of HRP released from its membrane anchor by proteolytic action (10). Accordingly, H.Ep.2 cells transiently transfected with cDNAs encoding ssHRP\(^{\text{P-selectinK765A}}\), ssHRP\(^{\text{P-selectinC766A}}\), or ssHRP\(^{\text{P-selectinL768A}}\) were grown for 2 days to allow for protein expression and then analyzed by subcellular fractionation or HRP proteolysis assay. In all experiments described, the difference between expression levels of the HRP-P-selectin chimeras, as determined by ratio of HRP activity to the total amount of protein, did not exceed 4-fold (data not shown).

As measured by LTI, the efficiency of lysosomal targeting for ssHRP\(^{\text{P-selectinK765A}}\) and ssHRP\(^{\text{P-selectinL768A}}\) was similar to that for ssHRP\(^{\text{P-selectin}}\) (1.19 ± 0.06; 0.94 ± 0.06 (±S.E.) and 1, respectively). In contrast, ssHRP\(^{\text{P-selectinC766A}}\) was incapable of targeting to lysosomal compartments since its LTI was barely above the basal level of tail-less ssHRP\(^{\text{P-selectin763}}\) or ssHRP\(^{\text{P-selectinKCP}}\) (Fig. 2A and Ref. 10). These results are in agreement with the HRP proteolysis data that indicate that HRP clipping for ssHRP\(^{\text{P-selectinK765A}}\) and ssHRP\(^{\text{P-selectinL768A}}\) is not significantly different to that of wild type protein (14.7 ± 1.3%, 13.6 ± 1.9%, and 16 ± 1.9%, respectively), whereas substitution of Cys-766 for Ala caused a fall in the amount of degraded HRP to 5 ± 1.5% (Fig. 2B). This decrease of LTI and HRP clipping in the latter case is not a consequence of either impaired internalization (see below) or accelerated removal of degraded HRP fragments from the cell. Indeed, following pretreatment of transfected cells with 100 μM mixture of pepstatin A and leupeptin, we detected only very small LTIs as follows: 0.12 ± 0.04 for non-treated cells and 0.28 ± 0.08 for treated cells expressing ssHRP\(^{\text{P-selectinC766A}}\) (Fig. 4). These inhibitors have been previously shown to dramatically reduce HRP clipping of chimeras (10). ssHRP\(^{\text{P-selectinP767A}}\) displayed both a slightly higher LTI and percentage of HRP proteolysis than did ssHRP\(^{\text{P-selectinC766A}}\) (Fig. 2, A and B) implying that Cys-766 is the most important amino acid residue within the KCPL motif with a lesser contribution from Pro-767. Mutation of the other two amino acids, Lys-765 and Leu768, had no effects on the efficiency of lysosomal targeting.

**Cys-766 within the ssHRP\(^{\text{P-selectin}}\) Is Acylated in H.Ep.2 Cells**—To determine whether HRP-P-selectin chimeras were acylated when heterologously expressed in H.Ep.2 cells, a series of metabolic labeling experiments were performed. H.Ep.2 cells transiently expressing ssHRP\(^{\text{P-selectinK765A}}\) or ssHRP\(^{\text{P-selectinC766A}}\) were labeled with either \(^{3}{\text{H}}\)palmitic acid or \(^{35}{\text{S}}\)methionine/cysteine, lysed, and immunoprecipitated with a polyclonal anti-HRP antibody. As revealed by SDS-PAGE, \(^{35}{\text{S}}\) label was equally detected in both chimeras, mainly within a protein with molecular mass of 65 kDa (Fig. 3A, lanes 1 and 2). No \(^{35}{\text{S}}\)-labeled 65-kDa protein was present in the MOCK-transfected H.Ep.2 cells (Fig. 3A, lane 3). \(^{3}{\text{H}}\) labeling was exclusively found in the cells expressing ssHRP\(^{\text{P-selectinK765A}}\) but not in ssHRP\(^{\text{P-selectinC766A}}\) nor MOCK-transfected cells (Fig. 3B, lanes 1–3). Since Cys-766 is the only cysteine within the transmembrane and cytoplasmic domains (32), our data show that HRP-P-selectin chimera is acylated at Cys-766 when expressed in H.Ep.2 cells.

**Effects of Mutations in the C2 Domain of the Cystolic Tail on the Lysosomal Targeting**—Green et al. (28) concluded that the membrane-distal C2 domain of the cytoplasmic tail of P-selectin does not affect lysosomal sorting since its deletion did not extend the half-life of this protein when expressed in Chinese hamster ovary or PC12 cells. However, given the presence both of major phosphorylation sites and of potential targeting sequences, (e.g. YGVF), we examined whether mutations within the C2 domain might reveal effects on sorting processes within the endosomal/lysosomal system in H.Ep.2 cells. We therefore constructed a series of HRP-P-selectin chimeras with either alanine substitutions or with progressive truncations of the C2 domain (Fig. 1). To characterize the lysosomal targeting of these mutants, H.Ep.2 cells were transiently transfected with cDNAs encoding the various chimeras, grown for 2 days to allow for protein expression, and then analyzed by subcellular fractionation or HRP proteolysis assay as described (10).

As shown in Fig. 2A, all the HRP-P-selectin chimeras...
with mutations in the C2 domain (ssHRP-P-selectinHLGT, ssHRP-P-selectin763, ssHRP-P-selectinTNAAF, ssHRP-P-selectinDPSP, ssHRP-P-selectin786, ssHRP-P-selectin782, and ssHRP-P-selectin776) were present within the lysosomal fractions in amounts comparable to ssHRP-P-selectin. However, the percentage of clipped HRP for ssHRP-P-selectinYGVF, ssHRP-P-selectinDPSP, ssHRP-P-selectin786, and ssHRP-P-selectin782 was about 2-fold higher than that found for other mutations within the C2 domain or for wild type protein (Fig. 2B). These results indicate that there is not necessarily a linear correlation between proteolytic clipping and accumulation of the chimeras in the lysosomal fractions as determined by LTI. One possible explanation is that ssHRP-P-selectinYGVF, ssHRP-P-selectinDPSP, ssHRP-P-selectin786, and ssHRP-P-selectin782 are sorted to the late MVB and lysosomes more efficiently than ssHRP-P-selectin and subsequently are more subject to proteolysis. As soon as they degrade, they might rapidly disappear from the late endocytic and lysosomal compartments. Alternatively, proteolysis of these chimeras might continue until HRP activity is reduced; both possibilities would lead to an underestimation in the fractionation assay. Analysis of lysosomal targeting in the presence of protease inhibitors might reveal if this is indeed the case.

Those chimeras most sensitive to proteolytic action, i.e. ssHRP-P-selectinYGVF and ssHRP-P-selectinDPSP, were therefore analyzed by subcellular fractionation following the treatment of cells with a mixture of pepstatin A and leupeptin. As shown in Fig. 5, the amount of internalized chimera protein recovered within lysosomal compartments for ssHRP-P-selectinYGVF and ssHRP-P-selectinDPSP rose up to 2.8 times compared with ssHRP-P-selectin expressed in treated cells and up to 6-fold compared with untreated control cells expressing ssHRP-P-selectin. Following the same pretreatment, ssHRP-P-selectinTNAAF, a chimera with alanine substitution of the five amino acids located in between YGVF and DPSP behaved similarly to the wild type chimera demonstrating a high selectivity of this phenomenon for YGVF and DPSP. The phenotypes observed by substitution of YGVF or DPSP might be accounted for by two different possibilities. The first is that increased lysosomal targeting at steady state relative to that for wild type protein correlates with an accelerated internalization of the mutated proteins. Alternatively, YGVF and DPSP might be responsible for reducing the delivery of P-selectin to lysosomes.

Alterations of Lysosomal Targeting Are Independent of Internalization of HRP-P-selectin Chimeras—To ascertain whether the mutations having the most profound effects on lysosomal targeting also affect internalization, the kinetics of internalization for ssHRP-P-selectin, ssHRP-P-selectin763, ssHRP-P-selectinC766A, ssHRP-P-selectinYGVF, and ssHRP-P-selectinDPSP were determined using a cell-surface biotinylation procedure. As shown in Fig. 5, the amount of internalized chimera progressively increased with time at 37 °C for all the mutants, except for the tail-less ssHRP-P-selectin763 which was previously shown to be deficient in internalization (27, 35). First order internalization rates have been calculated from data collected after the first 4 min of endocytosis at 37 °C (Table I). Thus, ssHRP-P-selectinYGVF and ssHRP-P-selectinC766A were efficiently internalized (on average, 2.5%/min), whereas the basal level of ssHRP-P-selectin763 was only 0.12%/min. The internalization rates of ssHRP-P-selectinRCPL and ssHRP-P-selectinDPSP were reduced by 40% and 46%, correspondingly (1.5%/min and 1.35%/min). This inhibition for ssHRP-P-selectinRCPL is unlikely to be the cause of the impairment of its lysosomal targeting, since ssHRP-P-selectinC766A, also incapable of delivery to lysosomes, was internalized slightly more efficiently than wild type protein. Moreover, there is an inverse correlation between the internalization rate and lysosomal targeting for ssHRP-P-selectinRCPL, the former was inhibited by 46%, and the latter, as judged by LTI calculated in the presence of protease inhibitors, was 2.6 times higher than that for ssHRP-P-selectin763. If the 46% inhibition of internalization is taken into account, a re-estimation of the efficiency of lysosomal targeting for ssHRP-P-selectinRCPL would lead to an even more pronounced rise of LTI; up to 4.8 times above the level of ssHRP-P-selectin. Together, these data indicate that both the inhibition and activation of lysosomal sorting are independent of the internalization of HRP-P-selectin chimeras and therefore are most likely regulated at the endosomal level.

YGVF and DPSP Operate Downstream of the CP Motif Along the Endosomal Pathway—The positive LTS within the P-selectin

![Fig. 3.](image-url) **Opposing Signals for Lysosomal Delivery of P-selectin.** H.Ep.2 cells were transiently transfected with cDNAs encoding chimeras as indicated beneath each pair of bars, grown for 24 h without inhibitors, and then for 24 h in growth medium supplemented with 100 μg each of pepstatin A and leupeptin (filled bars) or without inhibitors (dotted bars). Each bar represents the mean ± S.E. of three independent determinations. Cells have then been subjected to subcellular fractionation using the two-step procedure, and LTI have been calculated as indicated in the legend for Fig. 2. * † or ‡ above bars indicates control or inhibitor-treated cells, respectively.

![Fig. 4.](image-url) **The effect of protease inhibitors on lysosomal targeting of HRP-P-selectin chimeras.** H.Ep.2 cells were transiently transfected with cDNAs encoding chimeras as indicated beneath each pair of bars, grown for 24 h without inhibitors, and then for 24 h in growth medium supplemented with 100 μg each of pepstatin A and leupeptin (filled bars) or without inhibitors (dotted bars). Each bar represents the mean ± S.E. of three independent determinations. Cells have then been subjected to subcellular fractionation using the two-step procedure, and LTI have been calculated as indicated in the legend for Fig. 2. * † or ‡ above bars indicates control or inhibitor-treated cells, respectively.
Internalization rates of different HRP-P-selectin chimeras

**TABLE I**

| Chimera                      | Initial internalization rate (%/min) |
|------------------------------|--------------------------------------|
| ssHRP<sup>P</sup>-selectin    | 2.48 ± 0.19                          |
| ssHRP<sup>P</sup>-selectin763 | 0.12 ± 0.12                          |
| ssHRP<sup>P</sup>-selectinKCPI| 1.50 ± 0.28                          |
| ssHRP<sup>P</sup>-selectinYGVF| 2.88 ± 0.38                          |
| ssHRP<sup>P</sup>-selectinDPSP| 2.10 ± 0.32                          |
| ssHRP<sup>P</sup>-selectinC766A| 1.35 ± 0.23                          |

Opposing Signals for Lysosomal Delivery of P-selectin

![Graph](image)

**Fig. 5. Internalization of HRP-P-selectin chimeras in H.Ep.2 cells.** H.Ep.2 cells expressing ssHRP<sup>P</sup>-selectin, ssHRP<sup>P</sup>-selectin763, ssHRP<sup>P</sup>-selectinKCPI, ssHRP<sup>P</sup>-selectinYGVF, ssHRP<sup>P</sup>-selectinDPSP, or ssHRP<sup>P</sup>-selectinC766A were biotinylated using NHS-SS-biotin, transferred at 37°C for the indicated time, and chilled on ice followed by glutathione wash to remove the biotin groups remaining at the cell surface. The cells were lysed, biotinylated proteins were collected on streptavidin-agarose beads and resolved by SDS-PAGE. After transfer onto Immobilon-P membrane, biotinylated HRP-P-selectin chimeras were detected with a polyclonal anti-HRP antibody followed by incubation with 125I-biotin-P membrane, biotinylated HRP-P-selectin chimeras were detected for ssHRP<sup>P</sup>-selectinKCPL or ssHRP<sup>P</sup>-selectinC766A. Alternatively, if lysosomal fractions were analyzed, then different phenotypes were observed for ssHRP<sup>P</sup>-selectinKCPL or ssHRP<sup>P</sup>-selectinC766A. The finding that acylated Cys-766 of P-selectin is located 12 amino acids from the lipid bilayer is also consistent with these studies. Palmitoylation has been found to be required for tight membrane binding of Ras proteins (42, 43), we speculate that palmitoylation of Cys-766 may modify the conformation of the cytoplasmic tail by anchoring the juxtamembrane region to the lipid bilayer thus facilitating the lateral redistribution of P-selectin along the endosomal membrane to those parts of endosome that give rise to the inner vesicles of MVB. Such recruitment into inner vesicles was shown to be important for lysosomal degradation in the case of epidermal growth factor receptor where this process is controlled by the receptor tyrosine kinase domain (44).

In addition to the positive LTS within the C1 domain, we also show that lysosomal targeting of HRP-P-selectin chimeras is modulated by sequences within the membrane-distal C2 domain, YGVF and DPSP sequences within the C2 domain function as negative LTS or LAM. One of the questions that arises from this observation is where in the cell with respect to the positive LTS do these LAM operate? Since the initial internalization rates of chimeras with inactivated YGVF or DPSP sequences have not been increased, then it seems reasonable that (a) either the LAM function simultaneously with the positive LTS, i.e., in the transferrin-positive endosomes, or (b) further down the endocytic route, most likely at the level of the maturing late MVB. If double mutants, having both the positive LTS and LAM replaced were to be analyzed, then different phenotypes would be predicted depending on which model is correct. If the positive LTS and LAM operate simultaneously, then the resulting phenotype of each double mutant, although dependent on the relative strength of each signal, most likely would reveal a higher level of lysosomal targeting and HRP proteolysis than for ssHRP<sup>P</sup>-selectinKCPL or ssHRP<sup>P</sup>-selectinC766A. Alternatively, if the LAM operate downstream of the positive LTS along the endocytic pathway, then the double mutants would exhibit levels of lysosomal targeting and HRP proteolysis similar to the chimeras with an inactivated positive LTS. To address this issue, the double mutants with both KCPL plus DPSP or KCPL plus YGVF substituted by Ala were constructed (Fig. 1) and analyzed for efficiency of lysosomal targeting and HRP proteolysis. As shown in Fig. 2, A and B, both the LTI and percent of HRP proteolysis for these chimeras were as low as those for the tail-less ssHRP<sup>P</sup>-selectin763 suggesting that YGVF and DPSP function after KCPL, most likely at the level of maturing MVB.

**DISCUSSION**

We previously described a short positive LTS, KCPL, located within the membrane-proximal C1 domain of P-selectin and found that Pro-767 within it significantly contributes to the efficiency of lysosomal sorting (10). In this report, we have analyzed mutations of the other three amino acids within KCPL and showed that Cys-766 makes an even greater contribution than Pro-767, whereas Lys-765 and Leu-768 did not reveal any effects (Fig. 2) implying that the LTS comprises the di-amino acid motif CP. We have also confirmed that when heterologously expressed in H.Ep.2 cells, HRP-P-selectin chimeras undergo acylation with [3H]palmitic acid at Cys-766 (Fig. 3). This is in agreement with other data which established that acylation occurred at this amino acid residue both within endogenous P-selectin in platelets and within P-selectin expressed in COS-7 cells (34). Although it is most likely that P-selectin is palmitoylated, the [3H]palmitate could potentially be metabolized to other fatty acids such as stearic acid (34). An interesting feature of transmembrane proteins known to acquire this post-translational modification is the relatively close proximity of acylation sites within their cytoplasmic tails to the plasma membrane, typically from 2 to 16 amino acids (38–41). The finding that acylated Cys-766 of P-selectin is located 12 amino acids from the lipid bilayer is also consistent with these studies. Palmitoylation has been found to be required for tight membrane binding of Ras proteins (42, 43), we speculate that acylation of Cys-766 may modify the conformation of the cytoplasmic tail by anchoring the juxtamembrane region to the lipid bilayer thus facilitating the lateral redistribution of P-selectin along the endosomal membrane to those parts of endosome that give rise to the inner vesicles of MVB. Such recruitment into inner vesicles was shown to be important for lysosomal degradation in the case of epidermal growth factor receptor where this process is controlled by the receptor tyrosine kinase domain (44).
A significant increase in targeting to late MVB and lysosomes can therefore be caused by small specific changes to the cytoplasmic tail, strongly implying further controls on endosomal/lysosomal trafficking of P-selectin than hitherto appreciated. One possible way in which this regulation might occur is that the C2 domain contains sequences involved in promoting the avoidance of lysosomal targeting of P-selectin, i.e. functioning as LAM. The operation of the multiple signals that we have now described may depend on a relatively intact cytoplasmic domain, since a deletion of the entire C2 domain (ssHRP<sub>P-selectin</sub>776) enhanced neither lysosomal targeting nor HRP proteolysis of the chimera (Fig. 2). Green et al. (28) have also shown that the half-life of P-selectin with a truncated C2 domain was similar to that for wild type protein. One plausible explanation for this apparent discrepancy is that compared with the substitution of short sequences within the C2 domain, deletion of the entire C2 domain causes a structural deformation of the whole cytoplasmic tail thereby not only removing the LAM (which might have led to an increase in lysosomal targeting) but also adversely affecting the functioning of the positive LTS.

One of the LAM of P-selectin, YGVF, is similar to the Tyr-based family of targeting signals described by the consensus XXXφ (where X is any amino acid while φ is an amino acid with a bulky hydrophobic group) that is predicted to form a “tight turn” (for review see Refs. 1, 5, and 45). A large body of data indicates that Tyr-based signals are involved in the internalization and lysosomal targeting of endocytic receptors as well as lysosomal membrane proteins (1, 4, 5). Other sorting processes that require Tyr-based signals include sorting from early endosomes to late endosomes (for review see Ref. 46), delivery to the specialized endosomal/lysosomal organelles of the major histocompatibility complex II antigen-processing compartments (47), sorting to the TGN (48, 49), and basolateral targeting in polarized epithelial cells (for review see Refs. 6 and 50). Thus, Tyr-based signals mediate more than one sorting step. One novel finding of the present work is that avoiding delivery of P-selectin to lysosomes also relies on a Tyr-based signal.

The second LAM of P-selectin, DPSP, is different from other sorting signals. However, it does contain a serine residue (in position 788) that was found to be a principal site of phosphorylation of P-selectin both in platelets and endothelial cells as well as when expressed in Chinese hamster ovary cells (51). Serine phosphorylation was shown to play a diverse role in modulating the sorting of membrane receptors (52–55). It was of interest to determine whether phosphorylation of serine 788 facilitates the functioning of DPSP.

A large body of evidence suggests that lysosomes and late endosomes represent different subcompartments (for review see Refs. 6 and 56). However, sorting of proteins from late endosomes to lysosomes is poorly understood. Thus it is still unclear whether transfer of proteins from late endosomes to lysosomes occurs by maturation or is a discontinuous process. Studies on H.Ep.2 cells suggest that late or mature MVF fuse with a relatively stable set of lysosomes. This fusion is thought to be preceded by a continuous maturation of the early MVF accompanied by the removal of recycling proteins, giving rise to the late MVF (13–15). Our current data, which reveal a balance between a positive LTS operating to facilitate exit from the early MVF and the negative LTS or LAM acting to promote retrieval from the lysosomal pathway at late endocytic stage, suggest a complex regulation of the late stages of endocytosis.

The LAM of CD-M6PR do not reveal any significant homologies to either DPSP or YGVF. However, the CD-M6PR is a recycling receptor that moves constitutively between the TGN, endosomes, and the plasma membrane, not trafficking to lysosomes (57). When expressed in Chinese hamster ovary, PC12, or HEP2 cells, P-selectin is efficiently delivered to lysosomes for degradation (10, 28), implying very different itineraries for these two proteins. Importantly, the disruption of YGVF or DPSP did not increase the internalization rate of P-selectin thus indicating that the efficient delivery of mutant proteins to lysosomes is not a consequence of accelerated internalization. If internalization is not increased then how might DPSP and YGVF be functioning? The most likely explanation is that in the presence of intact LAM, P-selectin is transferred along tubular extensions of the MVB followed by exit to recycling compartments. Mutation of LAM would then cause the retention of P-selectin in a Cys/Pro-dependent manner in the endosomal domains which become late MVF and finally fuse with lysosomes.

Whereas recycling of CD-M6PR to the TGN to bind newly synthesized hydrolases provides a physiological reason for the presence of a lysosomal avoidance signal, why would P-selectin possess sequences that similarly modulate its lysosomal targeting? In the cells in which P-selectin was originally found, it is stored in specialized regulated secretory compartments, such as the Weibel-Palade bodies and α-granules (22, 58). Following exocytosis and retrieval, P-selectin recycling back to these granules was recently shown to progress through the endosomal intermediates morphologically identified as MVB (59). How sequences controlling this itinerary would be interpreted in cells such as H.Ep.2, which lack endothelial-specific compartment(s) as well as the corresponding sorting machinery is unknown, but it is tempting to speculate that this may explain such complex trafficking.

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Note Added in Proof—We have now constructed the double mutant ssHRP<sub>P-selectin</sub>YGVF<sub>-selectin</sub> and tested its lysosomal trafficking by LTI and by proteolysis. We find that it behaves indistinguishably from either ssHRP<sub>P-selectinDPSP</sub> or ssHRP<sub>P-selectinYGVF</sub>. The effect of altering both these sequences together is therefore not additive.

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