Lysosomal Targeting of P-selectin Is Mediated by a Novel Sequence within Its Cytoplasmic Tail

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Signs controlling the intracellular targeting of many membrane proteins are present as short sequences within their cytoplasmic domains. P-selectin is a type I membrane protein receptor for leukocytes, acting during the inflammation response. Heterologous expression experiments have demonstrated that its 35-residue cytoplasmic tail contains signals for targeting to synaptic-like microvesicles, dense-cored granules, and lysosomes. We have examined the lysosomal targeting information present within the cytoplasmic tail by site-directed mutagenesis of horseradish peroxidase-P-selectin chimeras followed by transient transfection in H.Ep.2 cells. Assaying lysosomal targeting by subcellular fractionation as well as intracellular proteolysis, we have discovered a novel lysosomal targeting signal, KCPL, located within the C1 domain of the cytoplasmic tail. Alanine substitution of this tetrapeptide reduced lysosomal targeting to the level of a tailless horseradish peroxidase-P-selectin chimera, which was previously found to be deficient in both internalization and delivery to lysosomes. A proline residue within this lysosomal targeting signal makes a major contribution to the efficiency of lysosomal targeting. A diaminobenzidine density shift procedure established that chimeras with an inactivated KCPL sequence are present within transferrin-positive compartments. Such a mutant also displays an increased level of expression at the plasma membrane. Our results indicate that the sequence KCPL within the cytoplasmic tail of P-selectin is a structural element that mediates sorting from endosomes to lysosomes.

A substantial body of data indicates that post-Golgi sorting of integral membrane proteins is largely dependent upon information contained within the cytoplasmic domains of these proteins (for review, see References 1–4). This information is usually found in short stretches of amino acids, “sorting signals,” which serve to direct proteins to a variety of intracellular destinations. The most common signals can be classified into two groups: Tyr-based and di-Leu signals. Conformational modeling and two-dimensional nuclear magnetic resonance spectroscopy (NMR) of peptides encoding these motifs have revealed that the first group, typically conforming to Asn-Pro-Ø sequences (where Ø is a bulky hydrophobic amino acid), form a “tight turn” (5–7), whereas di-Leu or Leu-Ile motifs may display an extended, random coil conformation within the cytoplasmic domain of that protein (8). More recently, a novel category of targeting signals that lie within an α-helix (9, 10) has been defined. In contrast, other sorting signals are centered on strongly hydrophilic sequences, comprising clusters of acidic residues (Glu and Asp) functioning either as independent determinants or in concert with Tyr-based or di-Leu motifs (11, 12).

The type I membrane protein P-selectin belongs to a family of adhesion molecules. It initiates leukocyte recruitment during the inflammatory response and is involved in hemostasis (13–16). Found in the membrane of regulated secretory organelles of platelets and endothelial cells (16–18), it is redistributed to the plasma membrane after stimulation of these cells with agonists (14, 19, 20). When expressed in cells lacking a regulated secretory pathway, P-selectin is constitutively transported to the cell surface (21–23). After appearance at the plasma membrane, it is rapidly internalized (20–22, 24, 25) and passes through the endocytic pathway to its final destination (25, 26). When expressed in Chinese hamster ovary and PC12 cells, P-selectin is efficiently targeted to lysosomes, and its cytoplasmic tail comprising the C1 and C2 domains is both necessary and sufficient for this trafficking step mediated by the putative lysosomal targeting signal within the C1 domain (21).

Much data derived from a variety of systems suggest that LTS1 and internalization signals often overlap or are co-linear, both for proteins biosynthetically targeted to lysosomes such as resident lysosomal membrane proteins as well as for membrane proteins capable of efficient lysosomal targeting during receptor-mediated endocytosis (see Table I and references cited therein). One interesting exception is the EGFR, in which mutations inactivating lysosomal targeting were found not to affect internalization (27–29). Thus, although an overlap is often evident, there is no simple correlation between internalization and lysosomal targeting, implying that the structural requirements for interacting with the cytoplasmic machinery which facilitates these two trafficking events may be complex.

Since the short cytoplasmic tail of P-selectin has been found to contain a variety of signals for targeting to organelles along the regulated secretory pathway as well as on the endocytic pathway (21, 25, 26), distinguishing different signals from each other is a complex problem. We therefore chose to study targeting in a system that lacks some of the possible destinations. We have begun by analyzing P-selectin targeting to late endocytic compartments in cells in which the endocytic pathway has

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1 The abbreviations used are: LTS, lysosomal targeting signal; EGF, epidermal growth factor; EGFR, EGF receptor; HRP, horseradish peroxidase; Trn, transferrin; TrnR, Trn receptor; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid; PNS, postnuclear supernatant; Lys, lysosome; NAGA, N-acetyl-β-D-glucosaminidase; LTI, lysosomal targeting index; LE, late endosome; DAB, 3,3′-diaminobenzidine; MVB, multivesicular bodies.
been extensively characterized (30–34) and that lack regulated secretory organelles.

In this study, we examined the effects of mutations within the cytoplasmic tail on lysosomal targeting of P-selectin in H.Ep.2 cells, which lack regulated secretory organelles. We have uncovered a novel short LTS, KCPL, which is located within the C1 domain and which has no obvious homology to LTSs identified in other proteins. The proline residue within this LTS significantly contributes to the efficiency of lysosomal sorting.

**EXPERIMENTAL PROCEDURES**

**Materials and Reagents**—Rabbit polyclonal anti-HRP was from DAKO, Glostrup, Denmark. Fab fragments of this antibody were generated using a commercial ImmunoPure® Fab preparation kit (Pierce) according to the manufacturer’s instructions. Mouse receptor-grade epidermal growth factor (EGF) and human, iron-saturated transferrin (Tfn) were purchased from Sigma. Iodination of Fab fragments, EGF, and Tfn has been performed according to the modified IODO-GEN method as described elsewhere (35). The specific activity of preparations was: 3 × 10⁴, 10⁴, and 2 × 10⁴ cpm/ng, respectively. Protein concentration was determined using Coomassie Plus protein assay reagent (Pierce) according to the manufacturer’s instructions. Other chemicals were from Sigma and Pharmacia Biotech Inc.

**Constructs**—A chimeric cDNA containing the human growth hormone hormone signal sequence, followed by HRP, and finally the transmembrane domain and cytoplasmic tail of P-selectin (Fig. 1) was generated as described previously (26). cDNAs encoding the deletion mutants, ssHRP-P-selectinNPHS and ssHRPP-selectin767, were constructed as detailed in this report. Both the point mutation of proline 767 and the tetra-alanine changes were made using the Strategene QuikChange site-directed mutagenesis kit. Briefly, coesium chloride-purified pRK34 plasmid containing the ssHRP-selectin was added to two complementary synthetic oligonucleotide primers which contained the change of interest. After temperature cycling with Pfu DNA polymerase, a mutated plasmid containing a staggered nick was generated. The parental DNA was then digested away with DpnI restriction endonuclease, which is active only on methylated and hemimethylated DNA. The nicked mutated vector was transformed into supercompetent Escherichia coli. The sequences generated were confirmed by sequencing. The sequences of the oligonucleotide primers used to generate the mutants are listed below. Only the sense primer is shown; the antisense primer is the exact complement.

The primers used are as follows: P-selectinP767, GATGGGAAATGCGCTTGGATCCTCACC; P-selectinP767A, GGTTTCAAGAGCAGGCTGCTTGG; P-selectinNPHS, AAATGCCCCTGTTTTCTCGC; and P-selectinP763, AAATGCCCCTGGGCTTGGAC. The primers used are as follows: P-selectinP767, GATGGGAAATGCGCTTGGATCCTCACC; P-selectinP767A, GGTTTCAAGAGCAGGCTGCTTGG; P-selectinNPHS, AAATGCCCCTGTTTTCTCGC; and P-selectinP763, AAATGCCCCTGGGCTTGGAC.

**Cell Culture and Transfection**—The human cell line H.Ep.2 was cultured and transiently transfected as described elsewhere (36). Cells were plated on 150- and 35-mm dishes and in some experiments were cultured and transiently transfected as described elsewhere (36). Cells on 35-mm dishes were washed twice with ice-cold binding medium, washed twice with HB (320 mM sucrose, 10 mM HEPES, pH 7.3), and subjected to subcellular fractionation as detailed below.

**Subcellular Fractionation and Quantitation of Data**—Following two rinses with ice-cold binding HB, cells were scraped into 1.5 ml of HB with a rubber policeman and passed 10 times through a ball bearing homogenizer with a 0.009-mm clearance (made at EMBL, Heidelberg, Germany). The cell homogenate was spun at 8,500 × g for 5 min and 1.3 ml of the supernatant was saved (NAGA). The supernatant was layered on an 11-ml 1-16% Ficoll gradient made in HB. The gradients were centrifuged for 45 min at 35,000 rpm in a SW40Ti rotor (Beckman Instruments, Palo Alto, CA) and then fractionated in 0.5-ml fractions from the top of the tube using an Autodensi-Flow IIC (Buchler Instruments, Kansas City, MO), and 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 (38).

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; 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-selectin. Accordingly, in all the experiments, the LTI for ssHRP-selectin 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 LE/Lys peaks was corrected for the amount of lysosomal activity or 125I-EGF radioactivity (NAGA,EGF) peak) within the LE/Lys fractions and for total HRP activity in the homogenate (HRP hmg). After simplifying the equation, the LTI was defined as follows.

\[
\text{LTI} = \frac{\text{mutant HRP peak/mutant NAGA (EGF) peak × mutant HRP hmg}}{\text{WT HRP peak/WT NAGA (EGF) peak × WT HRP hmg}}
\]

(1)

Typically, the LTI for tailless ssHRP-selectin763 was about 20% of that for ssHRP-selectin and was subtracted from those for the other chimeras in each experiment to provide a base-line value. Thus, the LTI for ssHRP-selectin763 was considered as 0. The LTIs of the mutants were therefore described on a scale within a range set by ssHRP-selectin (LTI = 1) and ssHRP-selectin763 (LTI = 0).

**DAB Cytochemistry**—A modification of the procedure, originally developed by Courtoy et al. (39) was used. A 4.5 mm solution of DAB in HB was prepared, adjusted to pH 7.3 with 1 N NaOH, and filtered through a 0.22-μm filter (Millipore Corp.). PNS or gradient fractions (700 μl) were mixed with 800 μl of DAB solution and 8 μl of 6% H₂O₂ and incubated for 0 min at room temperature in the dark. A 700-μl control sample from the same PNS or Ficoll fraction was incubated in parallel with 800 μl of HB alone. Subsequently, the mixtures (1.5 ml) were layered on Ficoll gradients made with HB supplemented with 1 μM imidazole. When the DAB reaction was performed with PNS, the samples were centrifuged on 1–16% Ficoll gradients. Ficoll gradients subjected to DAB reaction were centrifuged on secondary Ficoll gradients; 7–25% Ficoll gradients were used to analyze the LE/Lys fractions, and 3–16% Ficoll gradients were used for analysis of plasma-membrane vesicles labeled by 125I-EGF.

To quantitate the degree of the density shift of each ligand, the amount of radioactivity remaining within the peak of ligand distribu-
ssHRP-selectinP767A is the chimera in which proline 767 was replaced by alanine. The amino acids which have been replaced by alanine are shown. The tetrapeptide sequence at the end of each chimera’s name shows which amino acid has been deleted. The carboxyl-terminal end of the TM domain is boxed. Each chimera is listed to the left of the diagram. The number at the end of the chimera name indicates which amino acid has been changed to a stop signal (numbering is taken from the human P-selectin sequence). ssHRP-selectinC1 is the construct in which the C1 exon has been assigned to the stop transfer.

**RESULTS**

**HRP-P-selectin Chimeras Are Sorted to the Late Endosomes and Lysosomes in H.Ep.2 Cells**—It has been demonstrated previously that the C1 domain of the cytoplasmic tail of P-selectin is involved in the targeting of this protein to lysosomes (21). To further characterize the signal(s) involved in lysosomal targeting, a series of chimeras between P-selectin and the enzymatic reporter HRP (Fig. 1) were constructed, and their targeting, a series of chimeras between P-selectin and the horseradish peroxidase (HRP) were added during construction. H.RGP, human growth hormone signal sequence; HRP, horseradish peroxidase; P-selectin, 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 TM and cytoplasmic domains of the chimeras starting with the wild-type P-selectin tail, ssHRP-selectinC1, the carboxyl-terminal end of the TM domain is boxed. The name of each chimera is listed to the left of the diagram. The number at the end of the chimera name indicates which amino acid has been changed to a stop signal (numbering is taken from the human P-selectin sequence). ssHRP-selectinAC1 is the construct in which the C1 exon has been deleted. The tetrapeptide sequence at the end of each chimera’s name shows which amino acid has been replaced by alanine. ssHRP-selectinP767A is the chimera in which proline 767 was replaced by alanine.

The enzyme hGH was added during construction. hGH, human growth hormone signal sequence; HRP, horseradish peroxidase; P-selectin, 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 tail, ssHRP-selectinC1. The name of each chimera is listed to the left of the diagram. The number at the end of the chimera name indicates which amino acid has been changed to a stop signal (numbering is taken from the human P-selectin sequence). ssHRP-selectinAC1 is the construct in which the C1 exon has been deleted. The tetrapeptide sequence at the end of each chimera’s name shows which amino acid has been replaced by alanine. ssHRP-selectinP767A is the chimera in which proline 767 was replaced by alanine. ssHRP-selectinP767A is the chimera in which proline 767 was replaced by alanine.

**Fig. 1. Schematic illustration of HRP-P-selectin chimeras.** The top line shows the components used for construction: boxes represent the individual components; sequences outside boxes are added during construction. hRGP, human growth hormone signal sequence; HRP, horseradish peroxidase; P-selectin, 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 tail, ssHRP-selectinC1. The carboxyl-terminal end of the TM domain is boxed. The name of each chimera is listed to the left of the diagram. The number at the end of the chimera name indicates which amino acid has been changed to a stop signal (numbering is taken from the human P-selectin sequence). ssHRP-selectinAC1 is the construct in which the C1 exon has been deleted. The tetrapeptide sequence at the end of each chimera’s name shows which amino acid has been replaced by alanine. ssHRP-selectinP767A is the chimera in which proline 767 was replaced by alanine.

**Fig. 2. Distribution of internalized 125I-EGF and NAGA after centrifugation on a 1–16% Ficoll gradient.** H.Ep.2 cells expressing ssHRP-selectin were incubated with 5 ng/ml 125I-EGF at 4 °C for 60 min and then for 30 min at 37 °C without ligand. Surface-bound 125I-EGF was removed by a mild acid wash. Cells were homogenized in HB, and the PNS was centrifuged on a preformed 1–16% Ficoll gradient. The radioactivity of fractions was counted and expressed as a percentage of the total radioactivity along the gradient (●). The activity of the lysosomal marker enzyme NAGA was determined in each fraction as described under “Experimental Procedures” and shown as OD420 nm (○).

125I-EGF as a tracer, which is internalized from the plasma membrane through endosomal compartments en route to lysosomes. To establish conditions for the internalization of 125I-EGF so that it is within late endocytic compartments, the kinetics of degradation and the dynamics of compartmentalization of internalized ligand were followed by centrifugation of PNS on linear 1–16% Ficoll gradients. 125I-EGF was bound to the cells at 4 °C for 60 min and internalized at 37 °C for various times, and the cells were treated with acidic buffer to remove the plasma membrane-bound ligand. Kinetic experiments showed that after 30 min of internalization, 90% of the cell-associated radioactivity was acid-resistant, i.e. intracellular, but no significant degradation had occurred, as judged by a trichloroacetic acid precipitation of the chase medium (data not shown). When PNS obtained from these cells was centrifuged on a 1–16% Ficoll gradient, most of the NAGA and 125I-EGF sedimented in the same highest buoyant density fractions (fractions 16–20 and 21–23) (Fig. 2). In contrast, at earlier internalization times, 125I-EGF was found exclusively in the low density fractions (fractions 4–10), in which internalized 125I-Trn, a marker for early/recycling endosomes (40), was detected (Fig. 8, −DAB traces). Together, these observations strongly support the notion that the peaks of 125I-EGF in the heavy fractions of a 1–16% Ficoll gradient comprise LE/Lys.

To further purify late endocytic organelles, a secondary centrifugation of the fractions containing most of the NAGA and 125I-EGF was performed. As shown in Fig. 3A, 125I-EGF and NAGA co-sedimented within the same single symmetrical peak with a low surrounding background after this treatment. An estimation of organelle yield within this peak was carried out using the endogenous lysosomal marker NAGA. Thus, the ratio of NAGA to total protein within the equilibrium peak divided by that for PNS demonstrated a 90–100-fold enrichment for the marker over starting material while still recovering 26% of the NAGA activity from total activity present in PNS. This two-gradient procedure therefore provides an appropriate preparation for quantitation of targeting of our chimeras to LE/Lys.

To determine whether significant levels of ssHRP-selectin and ssHRP-selectinAC1 were present in the LE/Lys equilbrium peak, cells were transfected either with ssHRP-selectin or with ssHRP-selectinAC1 and

**NAGA activity, OD420 nm**
subjected to subcellular fractionation according to the two-gradient protocol. As shown in Fig. 3B, significant amounts of the ssHRP-selectin chimera were recovered in the purified LE/Lys peak. Approximately 21% of cellular HRP activity was found within the LE/Lys fractions. Given the high levels of P-selectin biosynthesis 2 days after transfection on the one hand and the expected clearance of degraded HRP from lysosomal compartments on the other (Fig. 6), this percentage shows that targeting to the degradative compartments is a major pathway for HRP-P-selectin chimeras. Although detectable, much less targeting to LE/Lys of ssHRP-selectin C1 was seen, in line with expectations.

One advantage of using HRP chimeras is that the vulcanizing effect of the DAB reaction product within organelles containing HRP can be exploited to determine whether co-distribution on gradients is matched by co-occupation of the same membrane-bound organelle. If the chimeras are within the same membrane-bound organelle where the marker is present, then a shift in density of the marker following the DAB reaction will occur. In previous studies, this approach was successfully applied to establishing the sorting kinetics of two distinct endocytic tracers at various time points after internalization and, in particular, determining the duration of two tracers remaining within the same compartment (41, 42). Accordingly, cells were transfected to express ssHRP-P-selectin or ssHRP-P-selectin C1 labeled with 125I-EGF (see above and “Experimental Procedures”), followed by centrifugation of PNS on 1–16% Ficoll gradients. Fractions 17–23 were pooled and then subjected to centrifugation on secondary 7–25% Ficoll gradients. Fractionation was followed by γ-counting and assaying for HRP and NAGA activities. Radioactivity of fractions is expressed in percentages (●), calculated as for Fig. 2. NAGA activity is shown in the absolute values of OD420 nm (□) and HRP activity (OD450 nm) for ssHRP-P-selectin and ssHRP-P-selectin C1 (× and ■, respectively) is expressed in arbitrary units calculated as a ratio of HRP activities present in each fraction to that of the total homogenate. A, distribution of 125I-EGF and NAGA; B, distribution of HRP activity.

FIG. 3. Purification of LE/Lys-containing fractions by centrifugation on a secondary 7–25% Ficoll gradient. Cells expressing ssHRP-P-selectin or ssHRP-P-selectin C1 were labeled with 125I-EGF and then subjected to centrifugation on initial 1–16% Ficoll gradients as described in the legend for Fig. 2. Fractions 17–23 were pooled and recentrifuged on secondary 7–25% Ficoll gradients. Fractionation was followed by γ-counting and assaying for HRP and NAGA activities. Radioactivity of fractions is expressed in percentages (●), calculated as for Fig. 2. NAGA activity is shown in the absolute values of OD420 nm (□) and HRP activity (OD450 nm) for ssHRP-P-selectin and ssHRP-P-selectin C1 (× and ■, respectively) is expressed in arbitrary units calculated as a ratio of HRP activities present in each fraction to that of the total homogenate. A, distribution of 125I-EGF and NAGA; B, distribution of HRP activity.

FIG. 4. DAB-induced density shift of 125I-EGF-containing organelles by HRP-P-selectin chimeras. A and B, cells expressing ssHRP-P-selectin and ssHRP-P-selectin C1 were homogenized and centrifuged on secondary 7–25% Ficoll gradients and the radioactivity of fractions was counted and expressed in percentages of total radioactivity along the gradient. C, cells expressing ssHRP-P-selectin were incubated with 5 ng/ml 125I-EGF at 4 °C for 60 min, homogenized in HB, and centrifuged on a 1–16% Ficoll gradient. The peak (fractions 7–10) was collected and split into two parts. One aliquot was incubated with DAB/H2O2 (●), and the other with HB alone (○). Recentrifugation was performed on secondary 3–16% Ficoll gradients, radioactivity of fractions was measured and expressed in percentages as described in Fig. 2.
at 4 °C for 60 min, followed by centrifugation of PNS on 1–16% Ficoll gradients. Only one peak of 125I-EGF (fractions 7–10) was found (data not shown), which was then pooled, incubated with or without DAB plus H2O2, and subjected to subcellular fractionation using 3–16% Ficoll gradients. As seen in Fig. 4C, no shift of 125I-EGF after the DAB reaction was detected. These experiments indicate that targeting of HRP-P-selectin chimeras is expressed relative to that for ssHRPP-selectin, which was set at 1. B, lysosomal targeting indexes. Cells were treated and fractionated as described in the legend for Fig. 3. Targeting to LE/Lys was quantitated by calculating LTIs allowing for HRP activity in the LE/Lys peak to be normalized by the chimera expression level, the number of cells, and the organelle recovery as independently judged by NAGA activity (filled bars) and 125I-EGF radioactivity (hatched bars). Each bar represents the mean ± S.E. of 3–10 independent determinations with or without DAB plus H2O2, and subjected to subcellular fractionation using 3–16% Ficoll gradients. As seen in Fig. 4C, no shift of 125I-EGF after the DAB reaction was detected. These experiments indicate that targeting of HRP-P-selectin chimeras to the LE/Lys compartment of H.Ep.2 cells can be quantified by subcellular fractionation.

**Effect of Different Mutations within the C1 Domain of the Cytoplasmic Tail on Targeting to LE/Lys—**To quantitate the targeting to LE/Lys of wild-type and mutant ssHRP-P-selectin chimeras, cDNAs encoding the various chimeras (Fig. 1) were transiently transfected into H.Ep.2 cells. To make sure that the data on targeting for different mutants were comparable, we determined the expression level for each chimera as a ratio of HRP activity to milligrams of total protein. In two independent experiments. For comparative purposes, the ratio of HRP activity to milligrams of protein for each chimera is expressed relative to that for ssHRPP-selectin, which was set at 1.

**Transfected cells were subjected to subcellular fractionation and targeting indexes to LE/Lys for each chimera were then calculated as described under “Experimental Procedures.”** Fig. 5A shows that the efficiency of accumulation of HRP activity within LE/Lys varied significantly between different chimeras. Two of the tetrapeptide alanine substitutions within the C1 domain (ssHRPP-selectinKDDG and ssHRPP-selectinNPHS) and the C2-truncated chimera (ssHRPP-selectin776) displayed lysosomal targeting indexes similar to that of ssHRPP-selectin. In contrast, the ability of ssHRPP-selectinM3 to reach lysosomes was about 7-fold less than that for wild type. The targeting of ssHRPP-selectinM3 and ssHRPP-selectinKDDG was barely above the level of ssHRPP-selectin763. Since the internalization of P-selectin lacking either the C1 or the C2 domains is not abrogated (21, 24, 26), one could envisage two scenarios accounting for the failure of ssHRPP-selectinM3 and ssHRPP-selectinKDDG to accumulate in LE/Lys. The first is that these chimeras are capable of trafficking to lysosomes but could not be detected there due to rapid degradation, causing a loss of HRP activity from this compartment. Alternatively, sorting of these mutants to lysosomes does not occur at all, which would presumably result in rerouting to the recycling pathway coupled to a transient appearance of protein at the plasma membrane (see below). A similar situation has been described previously for mutants of the EGF-receptor (27, 29), influenza virus hemagglutinin (43), and β-chain of the interleukin-2 receptor (9), which have had their degradation signals disrupted.

To test the efficiency of proteolysis of the chimeras, Triton X-114 partitioning (44) was exploited for separation of membrane-bound and soluble HRP activity. The extent of HRP proteolysis of ssHRPP-selectinM3 and ssHRPP-selectinKDDG (2.97 ± 0.9% and 0.5 ± 0.2%, correspondingly) was significantly lower than that for ssHRPP-selectinM3 and ssHRPP-selectinKDDG, ssHRPP-selectinNPHS, and ssHRPP-selectin776 (on average, 12–17%), although ssHRPP-selectinM3 displayed a slightly higher percent than ssHRPP-selectinKDDG (Fig. 5C). The latter observation presumably reflects the effect(s) of the larger mutation.

**Absence of HRP activity of ssHRPP-selectinKDDG within the LE/Lys fractions or in the soluble phase in the proteolysis assay might be explained by either a failure of targeting to LE/Lys or increased proteolytic degradation within those fractions followed by rapid loss of the enzyme activity.** We therefore quantified the lysosomal targeting indexes in cells pretreated with a mixture of 0.1 mm pepstatin A and leupeptin for 24 h before fractionation. These inhibitors, which block the enzymatic activity of major lysosomal hydrolases, have been shown previously to prevent the degradation of proteins delivered to lysosomes.
been identified as a key amino acid within NP
targeting of P-selectin to the LE/Lys fractions of H.Ep.2 cells.

Importantly, the selective action of these inhibitors does not interfere with membrane traffic, as is the case for other agents such as weak bases and proton ionophores. Thus, following pretreatment with a mixture of pepstatin A and leupeptin, the extent of HRP proteolysis in cells expressing ssHRP P-selectin was reduced more than 4-fold (data not shown), strongly suggesting that degradation was indeed lysosomal. We therefore employed the same treatment to examine the lysosomal fate of chimeras through subcellular fractionation. The amount of HRP activity recovered in the LE/Lys fraction of cells expressing ssHRP P-selectin rose by up to 2.2 times compared with control untreated cells, as would be expected when degradation is blocked (Fig. 6). The other mutants (ssHRP P-selectinKDDG and ssHRP P-selectinNPHS), which behaved similarly to ssHRP P-selectin as judged by lysosomal targeting indexes and proteolysis data in nontreated cells, also exhibited an elevated level of lysosomal HRP activity after treatment. Thus, ssHRP P-selectinKDDG and ssHRP P-selectinNPHS are delivered to LE/Lys and degraded as efficiently as ssHRP P-selectin.

In contrast, we could not detect any increase in the level of HRP activity for ssHRP P-selectinKCLP: 0.2 ± 0.08 for treated cells and 0.13 ± 0.03 for nontreated cells, indicating that ssHRP P-selectinKCLP is neither routed to LE/Lys nor degraded. Altogether, these results support the contention that KCPL is a novel LTS within the C1 domain that is required for efficient internalization, e.g. ssHRP P-selectin763 and a protein that fails to be internalized, e.g. ssHRP P-selectin763. To determine whether this is the case, we employed an 125I-Fab anti-HRP binding assay to detect the chimeras on the cell surface. As seen in Fig. 7, ssHRP P-selectin763 showed the highest level at the plasma membrane, twice that of ssHRP P-selectin. Analysis of the other chimeras revealed that ssHRP P-selectin763, ssHRP P-selectinKDDG, and ssHRP P-selectinKCLP displayed an intermediate level between ssHRP P-selectin and ssHRP P-selectin763, whereas ssHRP P-selectinNPHS and ssHRP P-selectinKDDG were present at the same level as ssHRP P-selectin. Since substitution of KCPL, deletion of the C1 domain, or substitution for proline 767 did not affect or had only a little effect on internalization rates
(24), the appearance of these chimeras at the plasma membrane strongly suggests an increased pool of recycling protein. ssHRP<sup>P-selectin</sup><sub>C1</sub> and ssHRP<sup>P-selectin</sup><sub>KCPL</sub> Are Localized in Trn-positive Endosomes—The recycling pathway of H.Ep.2 cells has previously been characterized in detail, and Trn has been found to be the best marker of this itinerary (31, 32, 41). To determine whether ssHRP<sup>P-selectin</sup><sub>C1</sub>, ssHRP<sup>P-selectin</sup><sub>KCPL</sub>, and Trn are present within the same endosomes, cells expressing ssHRP<sup>P-selectin</sup><sub>C1</sub>, ssHRP<sup>P-selectin</sup><sub>KCPL</sub>, or ssHRP<sup>P-selectin</sup><sub>KCPL</sub> were loaded with 125I-Trn, treated with MES buffer to remove the plasma membrane-bound ligand, and then the co-localization of HRP activity and iodinated ligand was examined by exploiting the DAB-induced density shift procedure. PNS obtained from these cells was split in two equal parts. One of them was incubated with DAB and H2O2, and the another one retained from these cells was split in two equal parts. One of these was incubated with DAB and H2O2, and the another one with HB alone. Samples were layered on 1–16% Ficoll gradients and centrifuged to equilibrium as described under “Experimental Procedures.” In control cells expressing ssHRP<sup>P-selectin</sup><sub>C1</sub> the DAB reaction shifted 24% of the total 125I-Trn radioactivity, whereas in cells expressing ssHRP<sup>P-selectin</sup><sub>C1</sub> or ssHRP<sup>P-selectin</sup><sub>KCPL</sub>, significantly larger amounts of ligand were shifted: 54% and 52%, respectively. Based on these data, we assume that ssHRP<sup>P-selectin</sup><sub>C1</sub> moves transiently through the Trn-positive endosomes en route to the lysosome. Importantly, both ssHRP<sup>P-selectin</sup><sub>C1</sub> and ssHRP<sup>P-selectin</sup><sub>KCPL</sub> reveal significant co-localization with Trn, strongly suggesting a recycling itinerary for these chimeras.

**DISCUSSION**

In the present work, we have identified a novel LTS in the cytoplasmic tail of P-selectin. Green et al. (21) had found previously that a putative LTS of P-selectin is located within the membrane-proximal C1 domain of the cytoplasmic portion of this protein, the amino acid sequence of which is shown in Fig. 1. Using site-directed mutagenesis, we have engineered a series of tetrapeptide substitutions within HRP-P-selectin chimeras to further characterize lysosomal targeting information within the C1 domain. The assays introduced in the present study have allowed us to quantify even minor differences in endosomal/lysosomal sorting. Summarizing the results of both lysosomal targeting and HRP proteolysis experiments, we have uncovered a novel determinant within the C1 domain of P-selectin: KCPL. This LTS appears to be the sole element of the cytoplasmic tail necessary for lysosomal targeting since neither the disruption of the two other tetrapeptides within the C1 domain, KDDG and NPHS, nor the removal of the C2 domain affected lysosomal targeting. Whether this sequence is also sufficient for lysosomal targeting has not yet been directly established. Carrying out such experiments may not provide simple answers, since by placing it into another context, KCPL may well operate differently, as has been observed elsewhere.

For example, the transfer of the Leu-Ile signal of LIMP2 to CD36 and CD8 plasma membrane proteins did not induce diversion of these proteins to lysosomes (8). In addition, the spacing of an LTS relative to the transmembrane domain in proteins possessing a short cytoplasmic tail has been found to be critical for proper functioning of the signal (45).

The HRP proteolysis data are more complex than those obtained by subcellular fractionation, since we observed some difference between ssHRP<sup>P-selectin</sup><sub>C1</sub> and mutants in which KCPL has not been inactivated. We suspect that this may reflect some differential targeting between subcompartments of the late endocytic pathway. It is striking, however, that mutation of KCPL to tetra-alanine resulted in levels of HRP proteolysis similar to those seen for tailless ssHRP<sup>P-selectin</sup><sub>763</sub>, strongly suggesting that KCPL contains all the information needed to accomplish the lysosomal delivery of P-selectin.

The effect of tetra-alanine substitution of KCPL was very marked: lysosomal targeting and HRP clipping were reduced by 7- and 15-fold, respectively, of the levels found for ssHRP<sup>P-selectin</sup><sub>C1</sub> (Fig. 3). By contrast, inactivation of the LTSs of other transmembrane proteins and receptors caused no more than a 3-fold reduction of targeting compared with that of the intact protein, suggesting that additional sequences may be involved in those cases (9, 27, 29, 43). The existence of multiple lysosomal sorting signals has indeed been demonstrated for γ and δ chains of CD3 (48), for the invariant chain (II) of class II major histocompatibility complex (49) and EGFR (27–29). In principal, by acting in concert, multiple LTSs could provide for a targeting activity equivalent to that of KCPL within P-selectin.

Many LTSs also operate to mediate internalization at the plasma membrane, as is the case for the interleukin-6 signal transducer gp130 (50), LAMP1 (45, 51, 52), II (49), lysosomal acid phosphatase (8, 53, 54), LIMP2 (8), and CD36 (55) chains (8). These data indicate that in most cases the LTS and internalization signal are co-linear (see Table I). The relationship between internalization and lysosomal targeting is generally acknowledged, although its functional significance remains to be investigated. One of the sources of confusion is that there is no simple correlation between internalization and degradation rates for proteins targeting to lysosomes via the plasma membrane (9, 27–29, 43, 45). Thus, whether the mul-

**Table I**

An alignment of the LTS in P-selectin with other lysosomal targeting determinants

<table>
<thead>
<tr>
<th>Protein</th>
<th>Targeting sequence</th>
<th>Targeting activity present for</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-P-selectin</td>
<td>KCPL</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD3γ</td>
<td>YQPLR</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD3δ</td>
<td>YQPLR</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LTP</td>
<td>PGRIV</td>
<td>+</td>
<td>(53,54)</td>
</tr>
<tr>
<td>LAMP1</td>
<td>GYRT</td>
<td>+</td>
<td>(52,59)</td>
</tr>
<tr>
<td>LAMP2</td>
<td>GYEQF</td>
<td>+</td>
<td>(60,61)</td>
</tr>
<tr>
<td>LAMP3</td>
<td>GYEV</td>
<td>+</td>
<td>(62)</td>
</tr>
<tr>
<td>LIMP2</td>
<td>LIR</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HA±8</td>
<td>YKSF</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EGFR</td>
<td>a) YLYL</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>b) TK domain</td>
<td>-</td>
<td>-</td>
<td>(27)</td>
</tr>
<tr>
<td>c) 1002–1123</td>
<td>-</td>
<td>-</td>
<td>(29)</td>
</tr>
<tr>
<td>HLA-DMβ</td>
<td>YTPL</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL-2β</td>
<td>PDRKFFSQL</td>
<td>+</td>
<td>+ (9)</td>
</tr>
<tr>
<td>IL-6 gp130</td>
<td>STQPLL</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LII</td>
<td>a) LI</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>b) PML</td>
<td>+</td>
<td>+</td>
<td>(12,63)</td>
</tr>
</tbody>
</table>

- A. D. Blagoveshchenskaya and D. F. Cutler, unpublished observations.
tiple signals function to increase the overall efficiency of targeting by acting together at one rate-limiting step or operate at different stages is not clear.

Does KCPL operate at more than one step in directing the protein to late endocytic compartments? A detailed search for internalization signals in the cytoplasmic tail of P-selectin did not succeed in identifying any potential motifs despite the presence of an obvious candidate sequence within the C2 domain (YGVFTNAAF) that provides a good match with known internalization signals. Instead, mutation of most amino acids throughout the cytoplasmic tail affects the efficiency of internalization (24). This finding, coupled to internalization experiments using HRP-P-selectin chimeras firmly supports the view that KCPL is a signal that mediates endosome-to-lysosome trafficking without affecting internalization.

As illustrated in Table I, the LTS of P-selectin, KCPL, has no obvious similarity to a large number of lysosomal targeting determinants, most of which also act as internalization signals in other proteins. Our data also indicate that the proline 767 provides a major contribution to the LTS. Instead, mutation of most amino acids throughout the cytoplasmic tail affects the efficiency of internalization (24). This finding, coupled to internalization experiments using HRP-P-selectin chimera2 firmly supports the view that KCPL is a signal that mediates endosome-to-lysosome trafficking without affecting internalization.

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Our findings suggest that KCPL is most likely to operate where the segregation of recycling and degradative pathways occurs. Electron microscopy revealed that, during the maturation of MVBs, EGFR are recruited from the outer membrane of MVB onto internal vesicles while TrnR remains on the perimembrane (34, 56). This recruitment onto inner vesicles is critical for the lysosomal degradation of the EGFR. Felder et al. (27) have documented that the tyrosine kinase of the EGFR controls sorting of internalized receptor through spatial segregation within the MVB. Thus, wild-type EGFR was shown to be recruited to internal vesicles of MVBs and subsequently degraded, whereas kinase-negative receptor underwent increased recycling and was localized to the outer membrane of the MVBs followed by removal to small tubulovesicles (27).

We speculate therefore that KCPL promotes the retention of P-selectin within the maturing MVBs, possibly by promoting inclusion in inwardly budding vesicles along with the EGFR. When KCPL-dependent retention within MVBs is abolished, then P-selectin normally destined for degradation is likely to be diverted to the recycling pathway which may occur by default (4, 57). This scheme is supported by experiments (Fig. 8) showing that the level of ssHRP-P-selectinKCPL on the plasma membrane is greater than that of ssHRP-P-selectin, as would be ex-
Lysosomal Targeting Signal of P-selectin

predicted if the mutant protein efficiently recycles. Moreover, DAB cytochemistry experiments in the present work demonstrate that, although the wild-type HRP-P-selectin chimera is found within 125I-EGF-containing endosomes and lysomes, the distribution of ssHRP-P-selectinKCPL and ssHRP-P-selectinKC1 overlaps with 125I-TrnR-containing compartments. Other studies have also revealed increased levels of recycling for an EGFR lacking a degradation signal (29), as well as for a LAMP1 mutant in which the LTS was displaced within the cytoplasmic tail (45).

The data obtained in the present study coupled with published observations from other laboratories demonstrate an abundance of different sorting signals involved in lysosomal targeting. What are the reasons for such a heterogeneity? One possibility is the need to provide for the different physiological and signaling functions of proteins routed to lysosomes. For example, some receptors of hormones, growth factors, and cytokines utilize the degradative pathway to desensitize the cell during down-regulation. In these cases, rapid clearance of membrane to their final destination, may not have the same need for rapid clearance or degradation. Such different trafficking requirements would explain the variety of signals, but, clearly, further studies will be needed to investigate the basis for LTS diversity.

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