P-selectin Targeting to Secretory Lysosomes of Rbl-2H3 Cells*

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The biogenesis of secretory lysosomes, which combine characteristics of both lysosomes and secretory granules, is currently of high interest. In particular, it is not clear whether delivery of membrane proteins to the secretory lysosome requires lysosomal, secretory granule, or some novel targeting determinants. Heterologous expression of P-selectin has established that this membrane protein contains targeting signals for both secretory granules and lysosomes. P-selectin is therefore an ideal probe with which to determine the signals required for targeting to secretory lysosomes. We have exploited subcellular fractionation and immunofluorescence microscopy to monitor targeting of transiently expressed wild-type and mutant horseradish peroxidase (HRP)-P-selectin chimeras to secretory lysosomes of Rbl-2H3 cells. The exposure of the HRP chimeras to intracellular proteolysis was also determined as a third monitor of secretory lysosome targeting. Our data show that HRP-P-selectin accumulates in secretory lysosomes of Rbl-2H3 cells using those cytoplasmic sequences previously found to be sufficient for targeting to conventional lysosomes. This work highlights the similar sorting signals used for targeting of membrane proteins to conventional lysosomes and secretory lysosomes.

Secretory lysosomes are a distinct class of regulated secretory organelle. Not only does this organelle serve as the final degradative compartment of the cell, but it also stores secretory molecules that are released in response to an extracellular trigger. This exocytic capacity clearly marks them from conventional lysosomes. Although conventional lysosomes can also fuse with the plasma membrane and release their soluble contents following stimulation (1), the extent of Ca2+-triggered secretion of lysosomal enzymes from cells such as fibroblasts and epithelial cells tends to be only 10–20% (2). In comparison, up to 80% of lysosomal markers are released upon a physiological trigger from cells that possess secretory lysosomes. Cells such as cytotoxic T lymphocytes, neutrophils, melanocytes, mast cells, and basophils use their secretory lysosomes to store specialized components such as granzymes, melanin, histamine, and serotonin, in addition to their usual lysosomal content (3–5).

Rbl-2H3 is a basophilic leukemia cell line that has been extensively used in studies of regulated exocytosis. Morphological and biochemical studies have revealed that Rbl-2H3 secretory granules possess an acidic pH, contain mature lysosomal enzymes, and are accessible to endocytic markers, all hallmarks of lysosomes, but they also contain secretory markers such as serotonin. Both “lysosomal” and “granule” markers can be released upon stimulation of the cells by aggregation of FceRI (6, 7). Thus the endocytic and exocytic apparatus are linked such that the lysosome has been modified to become the regulated secretory organelle of these cells.

Given the hybrid nature of the secretory lysosome, an interesting question is which cytoplasmic targeting signals (in this paper we define a targeting signal as a peptide sequence that is required for the accumulation of a protein within an organelle) direct membrane proteins to this organelle. The simplest possibility is that lysosomal targeting sequences operate to direct both lysosomal and secretory membrane proteins to the modified lysosome (3). Alternatively, secretory granule or entirely novel targeting signals that are specific to secretory lysosomes might be used.

When tyrosinase, the resident membrane protein of melanomes, is heterologously expressed in HeLa and Madin-Darby canine kidney cells, it localizes to lysosomes in a di-leucine signal-dependent manner (8, 9). In addition this di-leucine signal mediates targeting of tyrosinase to synaptic like microvesicles (SLMV)1 from the endocytic pathway of PC12 cells (10). Thus tyrosinase appears to possess the necessary sorting information for its localization to secretory lysosomes, conventional lysosomes, and SLMV.

In contrast, it has been suggested that Fas ligand is sorted to the secretory lysosome of cytotoxic T lymphocytes using novel signals in its cytoplasmic tail, specific to this class of organelle, because when heterologously expressed, Fas ligand localizes only to secretory lysosomes (Rbl-2H3 cells) but not to conventional lysosomes (HeLa cells) (11).

P-selectin resides in the membranes of α and δ granules of platelets (12–14) and also in Weibel-Palade bodies (WPB) (15, 16) and lysosomes (17) of endothelial cells. The short, 35-amino acid cytoplasmic tail of P-selectin is necessary for targeting to WPB, but surprisingly, deletion of this region does not affect sorting to the secretory lysosomal α granules of platelets (18). Importantly, heterologous expression of P-selectin has allowed for the definition of both granule and lysosomal targeting signals, as well as those for delivery to SLMV, in a variety of cell types (Fig. 1).

It is therefore an ideal candidate to investigate the following two questions: first, is the cytoplasmic tail of P-selectin sufficient for delivery to secretory lysosomes of Rbl-2H3 cells, and second, if so, then does delivery rely on granule, lysosomal, or novel targeting signals? We have combined subcellular frac-

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1 The abbreviations used are: SLMV, synaptic like microvesicles; WPB, Weibel-Palade bodies; DMEM, Dulbecco’s modified Eagle’s medium; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; PNS, post-nuclear supernatant; wt, wild type; TSA, tyramide signal amplification.

10498 This paper is available on line at http://www.jbc.org
tion coupled to transient transfection of HRP-P-selectin chimeras in Rbl-2H3 cells to answer these questions.

**EXPERIMENTAL PROCEDURES**

**Materials and Reagents**—Micro-BCA Protein Assay Reagent kit was used according to the manufacturer’s instructions (Pierce). βH)-Serotonin (hydroxytryptamine bisnitylate-5), specific activity 28 Ci/mmol, was purchased from PerkinElmer Life Sciences. 125I-Diferric transferrin (human), specific activity 60 Ci/μm, was purchased from PerkinElmer Life Sciences. TSA Fluorescence Systems (cyanine 3) was purchased from PerkinElmer Life Sciences. All chemicals were purchased from Sigma, unless otherwise stated.

** Constructs **—HHR-P-selectin is a chimera comprising the human growth hormone signal sequence, followed by horseradish peroxidase (HRP) and the transmembrane and cytoplasmic domains of P-selectin in pHK8L(18). HHR-P-selectin is a truncation of the cytoplasmic tail (21), and KCPL, YGVF, DSMHHR-P-selectin are tetra-alanine mutations as described (24, 25) (see Fig. 3).

**Cell Culture and Transient Transfection**—Rbl-2H3 cells (kind gift from Mark Marsh, MRC Unit, LMCB, University College London) were grown as an adherent monolayer in DMEM (Life Technologies, Inc.), supplemented with 10% heat-inactivated fetal bovine serum, 50 μg/ml Gentamicin (Life Technologies, Inc.), at 37 °C, 5% CO2. For transient transfection studies, ~4 × 105 cells were electroporated (three pulses) at 125 microfarads, 250 V, infinity ohms. For wt, YGVF, DSMHHR-P-selectin constructs, 10 μg of DNA was used for transfection, whereas 3 μg of DNA was used for KCPL. HHR-P-selectin constructs to obtain similar levels of expression. Cells were replated in their usual growth medium and analyzed 3 days post-transfection.

**Stimulation of Cells**—Cells were primed overnight (day 2 post-transfection) with 0.5 μg/ml anti-2,4-dinitrophenol IgE (monoclonal, SPE-7, Sigma) and also incubated with 0.2 μCi/ml [3H]serotonin (hydroxytryptamine bisnitylate-5, PerkinElmer Life Sciences) in growth medium prior to stimulation the following day. Cells were rinsed twice and cultured for 1 h in normal media. Stimulation was induced by the addition of 50 ng/ml human serum albumin/2,4-dinitrophenol to cross-link the IgE bound to FceRI on the cell surface for 25 min at 37 °C. This took place in DMEM (without serum, 10 mM HEPES, 2 mg/ml bovine serum albumin) with no phenol red (to prevent interference of absorbance assays for β-hexosaminidase). Cells were then placed on ice, and the stimulation medium was removed and kept for analysis.

**125I-Transferrin Loading**—Three days post-transfection, cells were serum-starved (DMEM, 10 mM HEPES, 2 mg/ml bovine serum albumin) for 1 h and incubated with 0.6 μCi/ml 125I-transferrin for 1 h. Cells were then rinsed three times in cold DMEM. To locate the plasma membrane, cell surface 125I-transferrin was stripped by the following method. Following loading with 125I-transferrin, cells were transferred to ice, rinsed, and incubated for 15 min in 20 mM sodium acetate, pH 5, 2 mM CaCl2, 150 mM NaCl, and 50 μM deferoxamine mesylate. This was replaced with PBS- (PBS, 1 mM MgCl2, 0.1 mM CaCl2) and then further rinsed and incubated for 20 min with PBS+ and 50 μM deferoxamine mesylate.

**Subcellular Fractionation**—Single sucrose gradient subcellular fractionation was performed according to a procedure modified from that published by Baram et al. (27). Cells were placed on ice and rinsed twice with homogenization buffer (HB, pH 7.3, 0.25 M sucrose, 1 mM MgCl2, 10 mM HEPES). Samples were scraped (4 × 107) in 1.5-ml volume of HB (plus 1 mM phenylmethylsulfonyl fluoride and protease inhibitor mixture). The cell suspension was homogenized by five passages through a ball bearing homogenizer with 0.009 mm clearance (EMBL, Heidelberg, Germany). The nuclear fraction was spun down at 170 × g for 10 min at 4 °C and 800 units/ml DNase (type IV) added to the post-nuclear supernatant (PNS). The PNS was loaded onto a preformed continuous sucrose gradient as described (27). Gradients were collected in 25 fractions of 500 μl from the top of the tube using an Autodensi-Flow IIC (Buchler Instruments, Kansas City, MO) and analyzed when necessary for [3H]serotonin, 125I-transferrin, β-hexosaminidase, and HRP activities across the gradient. Recovery of β-hexosaminidase and HRP activities on the primary gradient was ~50% of total activity (homogenate + medium) in resting cells; however, for stimulated cells, recovery was reduced to around 40% of total activity. For experiments that required a further secondary gradient, fractions 14–20 were analyzed and the remainder pooled to 4 ml at 1.1 M sucrose. The pooled fractions were layered onto a preformed continuous sucrose gradient of 1.3–2.0 M sucrose (8 ml). This gradient was then run, fractionated, and analyzed as with the first gradient.

**Quantification**—[3H]Serotonin incorporation was monitored by tritium radioactivity using liquid scintillation spectrometry. 125I-Transferrin loading was monitored by gamma counting using a Packard Cobra II auto-gamma counter. β-Hexosaminidase activity was determined using an absorbance assay in a microplate reader as follows. 50 μl of sample was mixed with 20 μl of subcellular fractionation buffer (1 mM NaHCO3, 1 mM EDTA, 0.01% Triton X-100). This was then incubated with 100 μl of substrate solution containing 4 mg/ml p-nitrophenyl-N-acetyl-β-glucosaminide in 0.1 M sodium citrate buffer, (pH 4.5, 0.2% Triton X-100) for 30 min at 37 °C in the dark. The reaction was stopped by the addition of 150 μl of pre-warmed (37 °C) stop buffer (0.25 M glycine, 0.2 mM NaCl, 4% SDS, pH 12.5). The samples were read at 405 nm in a Molecular Devices Thermo-max microplate reader. HRP activities were determined from aliquots of 75 μl as described previously (28).

**Immunofluorescence Microscopy of HRP Chimeras and Serotonin**—Transfected cells on glass coverslips 1 day post-transfection were paraformaldehyde (3%)-fixed, quenched with 50 mM NH4Cl, and rinsed with PBS. Coverslips were then placed in cyanine 3-tetramide signal amplification (TSA) reagent for 10 min. The reaction was stopped with 8 washes in PBS plus azide (0.2%). Samples were permabilized in 0.2% saponin and then transferred to PBS plus 0.02% saponin and 0.2% gelatin for subsequent antibody incubations. Cells were incubated with mouse monoclonal anti-serotonin (Biogenesis, Poole, UK), followed by goat anti-mouse fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories, West Grove, PA). After staining, cells were mounted in Mowiol and analyzed with the use of an Optiphot-2 microscope (Nikon, Tokyo, Japan) equipped with an MRC Bio-Rad 1024 confocal laser scanning system. Images were transferred to Adobe Photoshop (Adobe Systems, Mountain View, CA).

**Triton X-114 Assay**—The partitioning of membrane-associated HRP and soluble (clipped) HRP (28) was established using the Triton X-114 assay (29) with the addition of a protease inhibitor mixture upon cell lysis. Transfected and mock-transfected cells were separated into upper and lower phases, and HRP assays were carried out in triplicate. To normalize for differential cell number between samples, the protein
concentration (Micro BCA Protein Assay Reagent kit, Pierce) of each phase was determined. For transfected and mock-transfected cells, the HRP activity in each phase (upper or lower) was calculated per μg of total protein (upper plus lower). Levels of HRP activity/μg of protein in the two phases of mock-transfected cells was used to subtract background levels of endogenous peroxidase activity away from transfected cells. The extent of proteolysis of HRP from its P-selectin membrane-bound anchor was established by calculating the percentage of soluble (clipped) HRP in the upper phase to the total of both phases.

**RESULTS**

To determine which targeting signals are required by HRP-P-selectin for accumulation within Rbl-2H3 secretory lysosomes, we combined transient expression with subcellular fractionation, a strategy that has proved highly successful in previous analyses of targeting (21–25, 28). Because morphological co-localization of Rbl-2H3 endogenous markers such as the granule membrane protein 5G10 (30), serotonin, lgp120, and β-hexosaminidase has been reported within a single population of organelles (6, 7, 31), we expected to find a single peak containing these markers by fractionation.

We have chosen two markers for the secretory lysosome, the lysosomal enzyme β-hexosaminidase and the secretory monamine serotonin (5-hydroxytryptamine), to reflect the twin characteristics of the organelle. We assayed for endogenous activity of β-hexosaminidase and labeled cells with [3H]serotonin. We first established that the uptake of [3H]serotonin was specific by using reserpine, which blocks the vesicular uptake of catecholamines and serotonin (data not shown).

**Enrichment for the Secretory Lysosome**—Fig. 2A shows the subcellular distribution of the secretory lysosome marker β-hexosaminidase across a single sucrose gradient following centrifugation of the PNS from resting and cells stimulated by cross-linking the IgE receptor (FceRI) for 25 min. The lysosomal enzyme β-hexosaminidase distributes as a single peak of activity, highlighted in gray (fractions 14–20), and activity falls from this peak by 45% upon stimulation. This fractionation scheme was adapted from the work of Baram et al. (27), who enriched for secretory lysosomes from Rbl cells using this single sucrose gradient. We have observed the secretory marker [3H]serotonin to always co-distribute with β-hexosaminidase across the gradient in resting cells and to respond to stimulation in the same way as β-hexosaminidase in all experiments carried out to date. [3H]Serotonin, however, was not monitored at this early stage to retain material for subsequent analysis.

**HRP-P-Selectin Localizes to the Secretory Lysosome**—These same resting and stimulated cells had been transiently transfected 3 days prior with wt HRP-P-selectin (21) to determine whether the cytoplasmic domain of P-selectin has the necessary sorting information required to direct it to the secretory lysosome of these cells. Fig. 2B shows that wt HRP-P-selectin peaks in the same fractions (15–19) that contain the marker β-hexosaminidase in resting cells and that this compartment also responds to stimulation of the cells caused by aggregation of the FceRI for 25 min. Thus HRP-P-selectin is targeted to the functionally active secretory lysosome. A more detailed stimulation profile was carried out by inducing exocytosis for 5 and 10 min to ascertain whether a significant proportion of HRP-P-selectin would shift out of the secretory lysosome peak into other organelar fractions, such as the plasma membrane, upon degranulation of the cells. HRP activity does appear as a shoulder to the main secretory lysosome peak from fractions 9–14, within which the HRP activity increases sequentially as we increase the stimulation period. However, the secretory lysosome peak remains the main peak of HRP activity. Following 5 min of stimulation, 29% HRP activity is lost from the secretory lysosome fractions (14–20); 10 min of stimulation results in a 35% fall, and after 25 min of stimulation there is a 38% fall in HRP activity from the secretory lysosome. This sequential loss of activity from the peak of the secretory lysosome upon stimulation coincides with the sequential increase in activity in fractions 9–14. Following the full 25-min stimulation period, we only observe a 38% fall in HRP activity within the secretory lysosome, whereas the extent of release of β-hexosaminidase following the same period was 45%.

**Further Resolution of the Secretory Lysosome Fractions**—To investigate the residual HRP activity within the secretory lysosome peak, we pooled fractions 14–20 (highlighted) and layered them onto a more shallow secondary sucrose gradient (1.3–2.0 M). Fig. 2C shows the distribution of β-hexosaminidase and [3H]serotonin from the pooled fractions of Fig. 2A. The profile of the two markers in resting cells reveals two peaks, the lighter one distributing between fractions 9 and 10 and the denser one between fractions 17 and 20, both of which are responsive when the cells are induced to exocytose for 25 min. Hence both peaks are secretory lysosomes. The traces for resting and stimulated [3H]serotonin show a significant number of counts in the load of the gradient (fractions 1–5) which probably reflect leakage of this tracer molecule from vesicular structures. This is also observed when [3H]serotonin is monitored on the primary gradient (data not shown).

Fig. 2D shows that the resting peaks of HRP-P-selectin activity co-distribute with those for the secretory lysosome markers (2C) and that they also both respond to stimulation for 25 min, HRP activity falling from the secretory lysosome peaks and redistributing to a new lighter peak at fraction 8. This peak is not observed upon stimulation for the secretory lysosome markers β-hexosaminidase and [3H]serotonin (Fig. 2C). The clear fall in activity from both peaks of the secretory lysosome markers upon stimulation (2C) is not as obvious as the fall in HRP activity, because of the overlap of the peak at fraction 8 with the lighter one of the two secretory lysosome peaks (9, 10). The shift of activity to fraction 8 is consecutive over time, HRP activity peaking at fraction 10 for resting and 5 min of stimulation, fraction 9 following 10 min and then to fraction 8 after 25 min of stimulation. This secondary gradient reveals that a large proportion of the residual HRP activity retained in the secretory lysosome peak following 25 min of stimulation in Fig. 2B has moved to a separate compartment from the markers β-hexosaminidase and [3H]serotonin.

**Release of Secretory Lysosome Markers**—In parallel, we quantified release of β-hexosaminidase and [3H]serotonin into the bathing medium. Fig. 2E shows that only 3.5% of both β-hexosaminidase and [3H]serotonin is constitutively secreted from resting cells, whereas 47% is released upon 25 min of stimulation. This 47% release is in good agreement with the 45% fall in β-hexosaminidase activity from the secretory lysosome peak in Fig. 2A.

**Effects of Mutations in the Cytoplasmic Tail of HRP-P-Selectin on Secretory Lysosomal Targeting**—We compared the targeting of wt HRP-P-selectin to secretory lysosomes with that of tetra-alanine mutant chimeras of the cytoplasmic sequences KCPL, YGVF, and DPSP and of the deletion mutant 763HRP-P-selectin, which lacks all three motifs (Fig. 3). These three motifs have been implicated in lysosomal, secretory granule, and SLMV targeting (Fig. 1). Rbl cells were transiently transfected with wt, KCPL, YGVF, DPSP, 763HRP-P-selectin, and cultured for 3 days. Fractionation was then carried out, and the subcellular profile across the primary sucrose gradient for each chimera is shown in Fig. 4A. The secretory lysosome fractions are highlighted in gray (14–20). The majority of wt, YGVF, DPSP-HRP-P-selectin activity is found in the secretory lysosome peak, all three having very similar profiles. However, much lower levels of HRP activity within the secretory
lysosome peak are found with the mutants KCPL and 763 HRP-P-selectin, which coincide with a significant accumulation of HRP activity elsewhere on the gradient. The profiles for 763 HRP-P-selectin and KCPL HRP-P-selectin differ in that there is a significant shoulder to the major peak for 763 HRP-P-selectin such that it covers fractions 6–15, whereas the peak for KCPL HRP-P-selectin is sharper (fractions 10–15). Mutating the sequence KCPL, which is implicated in lysosomal targeting (Fig. 1), to tetra-alanine has a similar effect on targeting to the secretory lysosome as the complete removal of the last 27 residues of the cytoplasmic domain.

Identification of Peaks—Because expression of KCPL HRP-P-selectin in H.Ep.2 cells results in localization in transferrin-positive compartments (24), and 763 HRP-P-selectin localization at the plasma membrane (24, 25), we analyzed the distribution of 125I-transferrin across our sucrose gradients in addition to
Targeting to Secretory Lysosomes

In this work we have used a combination of transient transfection of HRP-P-selectin plus subcellular fractionation to analyze targeting to secretory lysosomes. We have discovered that in Rbl-2H3 cells, HRP-P-selectin accumulation in secretory lysosomes is dependent on the cytoplasmic tail of P-selectin and, in particular, on the sequence KCPL, elsewhere used for accumulation within conventional lysosomes (19, 21, 22).

There are multiple pathways for targeting of membrane proteins to lysosomes (31), and it is thought that certain components of this ubiquitous lysosomal targeting machinery are subtly rearranged or enhanced, leading to distinct tissue-specific secretory lysosomes. Trafficking of membrane proteins to the secretory lysosome would thus be dependent on one type of lysosomal sorting pathway, resulting in the differential sorting of proteins to the secretory lysosome, via the enhanced pathway, over conventional lysosomes. Cells that only possess secretory lysosomes would continue to use this enhanced lysosomal sorting pathway. This is exemplified in human genetic disorders and mouse models of diseases such as Hermansky-Pudlak syndrome and Griscelli syndromes, whereby components of ubiquitous cellular machinery are mutated such that the biogenesis of, sorting to, and secretion from certain secretory lysosomes is dramatically abrogated (32–38). Conventional lysosomes are affected to a lesser degree indicating that there are many redundant pathways for sorting to lysosomes.

DCG (YGVF) or SLMV (a combination of the sequences DCG (YGVF) or SLMV) targeting sequences do not abrogate targeting of HRP-P-selectin to secretory lysosomes of Rbl-2H3 cells (Fig. 4, A and C). Only when all three sequences are removed from the tail of P-selectin (765HRP-P-selectin) or the lysosomal targeting signal KCPL is replaced with tetra-alanine is targeting compromised. This supports the relationship between trafficking to conventional lysosomes and secretory lysosomes.

The 765HRP-P-selectin chimera is not able to internalize efficiently in H.Ep.2 and PC12 cells and has provided a base line of non-signal-mediated internalization and traffic through to the lysosome (22, 25). It has been found in H.Ep.2 and PC12 cells that the majority of 763HRP-P-selectin is localized to the

sent the biosynthetic traffic of HRP-P-selectin en route to the secretory lysosome. In contrast, KCPL-HRP-P-selectin localizes to very distinct structures from serotonin, being especially enriched at the processes of the cells. The localization of 763HRP-P-selectin is more diffuse, mainly at the plasma membrane, but it is also found within internal structures that do not co-localize with serotonin. Staining patterns for YGVF, DPSFHRP-P-selectin are similar to the wild-type protein (data not shown).

*Intracellular Proteolysis (Triton X-114 Assay)—Once HRP-P-selectin is delivered to and accumulates within protease-rich environments, the chimera becomes subject to proteolytic attack such that soluble HRP is released from the P-selectin membrane anchor. We made use of this phenomenon, determining the amount of soluble HRP compared with that which remains membrane-bound, as an independent measure of targeting to the secretory lysosome (22, 24, 25, 28). Partitioning of HRP activity between two phases of Triton X-114 reveals the extent of proteolysis for each of the HRP chimeras (Fig. 6), with 48.6% (±0.8) of wt HRP-P-selectin HRP in its soluble form and 24.2% (±3.1) proteolysis for 765HRP-P-selectin. KCPL-HRP-P-selectin has a very similar value to that of the 763HRP-P-selectin (27.4%, ±3.4), but the extent of proteolysis of YGVF and DPSFHRP-P-selectin was even higher than wt levels (73.4 ± 3.8 and 63.6 ± 0.9%, respectively). Thus, in the case of YGVF and DPSFHRP-P-selectin, more HRP is in its soluble, clipped form than its membrane-bound form.

**DISCUSSION**

Analyzing the secretory lysosome markers. Fig. 4B illustrates the distribution of 125I-transferrin following loading of transfected cells for 1 h and internal stores of 125I-transferrin after stripping transferrin bound at the plasma membrane. Internal transferrin peaks at fraction 12, whereas plasma membrane-bound transferrin peaks at fractions 5–6. 125I-Transferrin bound at the cell surface at 4 °C reveals this same peak (data not shown). A substantial proportion of HRP activity for the mutants KCPL, YGVF, and DPSP HRP-P-selectin co-distributes with the internal endosomal load of 125I-transferrin (fraction 12). The shoulders of HRP activity for 763HRP-P-selectin (fractions 6–15) co-distributes in part with the plasma membrane and mainly with endosomes. All the HRP chimeras in Fig. 4A show a peak of activity at fraction 4 that does not co-distribute with any marker we have assayed and may represent the newly synthesized material within the endoplasmic reticulum and Golgi. It is important to note that the redistribution of wt HRP-P-selectin upon stimulation of the cells from the secretory lysosome to the shoulder of activity between fractions 10 and 13 (see Fig. 2B) coincides with the peak of internal 125I-transferrin-positive compartments.

**Secondary Gradient of the Secretory Lysosome Fractions—**To investigate the targeting of each mutant to the two peaks of the secretory lysosome identified on secondary gradients, we pooled fractions 14–20 and ran them on a 1.3–2.0 M sucrose gradient as in Fig. 2, D and E. It is clear from Fig. 4C, that YGVF, DPSFHRP-P-selectin show a similar distribution as the wt chimera, localizing to the two peaks that also contain β-hexosaminidase and 3H-serotonin. Hence there is no differential targeting of the mutant chimeras between the two peaks. The HRP activity that appeared to be within the secretory lysosome peak for KCPL, 765HRP-P-selectin in Fig. 4B is now seen to partially co-distribute with the 125I-transferrin positive peak (fraction 8) rather than with the secretory lysosome markers (Fig. 4D). However, the peaks for KCPL, 765HRP-P-selectin are broader than that of 125I-transferrin, also showing a partial overlap with the lighter of the two peaks containing β-hexosaminidase and 3H-serotonin.

**Immunofluorescence Microscopy of HRP Chimeras and Serotonin—**To confirm the subcellular localization of the HRP chimeras, we visualized HRP using Tyramide Signal Amplification (TSA), which uses HRP activity to catalyze the deposition of cyanine 3-tyramide immediately adjacent to the immobilized HRP-P-selectin chimeras. We also stained the cells with an antibody to the secretory lysosome marker serotonin, and Fig. 5 shows the fluorescence pattern of HRP and serotonin in cells transfected with wt, KCPL and 765HRP-P-selectin. The great majority of wt HRP-P-selectin is found to co-localize with serotonin-positive structures, as highlighted by the magnification of one of the processes of the cell. A few structures stain only for serotonin and not HRP, and these most likely reflect secretory lysosomes made before the transient expression of wt HRP-P-selectin. Also present are a small number of HRP-positive structures that appear devoid of serotonin, which may repre-
plasma membrane and that \textit{KCPL}HRP-P-selectin accumulates in early endosomes. In Rbl-2H3 cells, however, the majority of \textit{763}HRP-P-selectin co-distributes with internal stores of \textit{125I}-transferrin with a shoulder of activity that partially overlaps with the plasma membrane (Fig. 4, \textit{A} and \textit{B}). This is confirmed by immunofluorescence microscopy of \textit{763}HRP-P-selectin and serotonin (Fig. 5) that shows internal vesicular structures as well as labeling the plasma membrane. This contrasting localization of \textit{763}HRP-P-selectin between PC12 cells, H.Ep.2 cells, and the Rbl-2H3 cells in this study may reflect a highly active endocytic pathway within Rbl cells. The high ratio of internal to cell surface \textit{125I}-transferrin shown in Fig. 4\textit{B}, indicated by the cell surface strip, would be consistent with this. However, a full analysis of the endocytic pathway within Rbl-2H3 cells is beyond the scope of this paper.

The proteolysis data of Fig. 6 reflect the reduced exposure of \textit{KCPL}HRP-P-selectin and \textit{763}HRP-P-selectin to proteolytic cleavage within late endosomal and lysosomal compartments in comparison with the wt chimera. This is in accordance with the subcellular targeting profiles of Fig. 4, \textit{A} and \textit{B}, further confirming that mutation of the sequence \textit{KCPL} results in reducing accumulation within secretory lysosomes to base-line levels.

It has been shown that in addition to the lysosomal targeting signal \textit{KCPL}, negative targeting or lysosomal avoidance signals (YGVF and DPSP) also operate in the accumulation of HRP-P-selectin to lysosomes of H.Ep.2 and PC12 cells (22, 25). Mutation of these avoidance motifs leads to an increase above wt levels (49%) of proteolysis for \textit{YGVF}HRP-P-selectin (73%) and \textit{DPSP}HRP-P-selectin (64%), as is the case for targeting to conventional lysosomes in H.Ep.2 and PC12 cells. This further reinforces the similarity of the targeting behavior of HRP-P-selectin to lysosomes and secretory lysosomes.

Upon stimulation of cells transfected with wt HRP-P-selectin, the HRP activity shifts out of the secretory lysosome peak and into a shoulder of activity from fractions 9 to 14 (Fig. 2\textit{B}). This shoulder co-distributes with \textit{125I}-transferrin-rich compartments. P-selectin reaching the plasma membrane has been shown to internalize (39) and subsequently pass through endosomal compartments (22, 23, 26, 40), yet our time course of stimulation (Fig. 2\textit{B}) does not reveal a simple bulk transfer of HRP activity to the plasma membrane. This might reflect an asynchronous fusion of secretory lysosomes with the plasma membrane, especially if compound exocytosis, common to mast cells and other hematopoietic cells is taking place (41). Further
Targeting to Secretory Lysosomes

dase with pro-cathepsin D (53 kDa), which is a form usually associated with the Golgi in Rbl-2H3 cells (6, 42). In our hands, β-hexosaminidase and the secretory marker serotonin have always co-distributed across the first gradient within a single dense peak, which when further analyzed on secondary gradient reveals two peaks (Fig. 2C) with similar profiles for the two markers. When we stimulate our cells, there is a 45% fall in the peak for β-hexosaminidase on the primary gradient (Fig. 2A) and a fall in both peaks for β-hexosaminidase and [3H]serotonin on the secondary gradient. Because both peaks respond to stimulation, and both contain our markers, they must be secretory lysosomes. The most likely explanation is that these peaks represent immature and mature secretory lysosomes, with increasing density reflecting maturation. However, in the absence of a thorough analysis of the make-up of the peaks, this remains speculation. Certainly, for the purposes of this work, we see no differential targeting of our HRP chimeras between the two peaks (Fig. 4C) and so we conclude that they are closely related secretory lysosomes. The residual β-hexosaminidase activity and [3H]serotonin within the secretory lysosome may represent a complete (100%) degranulation of a proportion of the cell population, a 47% release from the entire population of the cells, or a situation somewhere between these two extremes.

In contrast to our findings with P-selectin (this work) and the behavior of tyrosinase (8, 9, 10, 43), the targeting of Fas ligand to secretory lysosomes seems to use a novel sorting pathway, specific to these organelles (11). Fas ligand targeting is dependent on a proline-rich domain, contrasting with more conventional short contiguous sequences, as used by P-selectin and tyrosinase, suggesting a different sorting mechanism for targeting of Fas ligand to secretory lysosomes.

The recovery of the cytoplasmic tail of P-selectin is not required for targeting to α granules of platelets (18) in contrast to our finding that it is sufficient for accumulation in secretory lysosomes of Rbl-2H3 cells. It is not clear whether the proposed variations in biogenesis and trafficking between different secretory lysosomes is sufficient to explain this discrepancy. Further examination of the targeting of P-selectin to the secretory lysosomes within platelets, the α granule (44) and δ granule (45–47), and to lysosomes (17), and WBP (15, 16) of endothelial cells may reveal key differences in their biogenesis. By identifying the sorting and targeting machinery used by P-selectin to reach these organelles, we could subdivide secretory lysosomes into further classes, mapping which lysosomal sorting pathways are enhanced for which secretory lysosome.

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REFERENCES
1. Andrews, N. (2000) Trends Cell Biol. 10, 316–321
2. Rodríguez, A., Webster, P., Ortego, J., and Andrews, N. (1997) J. Cell Biol. 137, 93–104
3. Dell’Angelica, E. C., Mullins, C., Caplan, S., and Bonifacino, J. S. (2000) FASEB J. 14, 1265–1278
4. Stinchcombe, J. C., and Griffiths, G. M. (1999) J. Cell Biol. 147, 1–5
5. Griffiths, G. M. (1996) Trends Cell Biol. 6, 329–332
6. Dragonetti, A., Baldassarre, M., Castino, R., Demoz, M., Luini, A., Bucalone, R., and Isidoro, C. (2000) J. Cell Sci. 113, 3289–3298
7. Xu, K., Williams, R. M., Holowka, D., and Baird, B. (1998) J. Cell Sci. 111, 2385–2390
8. Calvin, P. A., Frank, D. W., Bieler, B. M., Berson, J. F., and Marks, M. S. (1999) J. Biol. Chem. 274, 12780–12789
9. Simmen, T., Schmidt, A., Hunziker, W., and Beermann, F. (1999) J. Cell Sci. 112, 45–53
10. Blagoveshchenskaya, A. D., Hewitt, E. W., and Cutler, D. F. (1999) Mol. Biol. Cell 10, 3979–3990
11. Roost, G., and Griffiths, G. M. (1999) Nat. Med. 5, 90–96
12. Berman, C. L., Yeo, E. L., Wenzel-Drake, J. D., Furie, B. C., Ginsberg, M. H., and Furie, B. (1986) J. Clin. Invest. 78, 130–137
13. Stenberg, P. E., McEver, R. P., Shuman, M. A., Jacques, Y. V., and Bainton, D. F. (1985) J. Cell Biol. 101, 880–886

Fig. 5. Immunofluorescence microscopy of HRP-P-selectin chimeras and endogenous serotonin. Transfected Rbl cells grown on coverslips were fixed, incubated with TSA reagent for 10 min, and subsequently permeabilized and stained for serotonin (see “Materials and Methods”). Wt, KCPL, HRP-P-selectin and 763HRP-P-selectin were visualized by cyanine 3-tyramide (red channel) and endogenous serotonin in the green channel. The insert in the wt panel contains a 2-fold magnification of the boxed area. Bar represents 50 μm.

Fig. 6. Proteolysis of HRP-P-selectin chimeras in Rbl cells. HRP activities in the upper and lower phases of Triton X-114 were determined for wt, KCPL, YGVF, DSFP, 763HRP-P-selectin and mock-transfected cells. The protein concentration as a total of the two phases was also established to normalize the data for cell number. The values for mock-transfected cells were used to subtract background levels of peroxidase activity from the HRP chimeric mutants. Percentage proteolysis is defined as the fraction of HRP activity in the upper aqueous phase of the partition. Results are means of four independent experiments ± S.E.
Targeting to Secretory Lysosomes

14. Israels, S. J., Gerrard, Y. V., Jacques, Y. V., McNicol, A., Cham, B., Nishibori, M., and Bainton, D. F. (1992) Blood 80, 143–152
15. Bonfanti, R., Furie, B. C., Furie, B., and Wagner, D. D. (1989) Blood 73, 1109–1112
16. McEver, R. P., Beckstead, J. H., Moore, K. L., Marshall-Carlson, L., and Bainton, D. F. (1998) J. Clin. Invest. 84, 92–99
17. Arribas, M., and Cutler, D. F. (2000) Traffic 1, 783–793
18. Hartwell, D. W., Mayadas, T. N., Berger, G., Frenette, P. S., Rayburn, H., Hynes, R. O., and Wagner, D. D. (1998) J. Cell Biol. 143, 1129–1141
19. Disdier, M., Morrissey, J. H., Fugate, R. D., Bainton, D. F., and McEver, R. P. (1992) Mol. Biol. Cell 3, 309–321
20. Modderman, P. W., Beuling, E. A., Gevers, L. A. T., Calafat, J., Jansen, H., Von Dem Borne, A. E., and Sonnenberg, A. (1998) Biochem. J. 336, 153–161
21. Norcott, J. P., Solari, R., and Cutler, D. F. (1996) J. Cell Biol. 134, 1229–1240
22. Blagoveshchenskaya, A. D., Hewitt, E. W., and Cutler, D. F. (1999) J. Biol. Chem. 273, 1801–1814
23. Blagoveshchenskaya, A. D., and Cutler, D. F. (2000) Mol. Biol. Cell 11, 1801–1814
24. Blagoveshchenskaya, A. D., Norcott, J. P., and Cutler, D. F. (1998) J. Biol. Chem. 273, 2729–2737
25. Blagoveshchenskaya, A. D., Hewitt, E. W., and Cutler, D. F. (1998) J. Biol. Chem. 273, 27896–27903
26. Green, S. A., Setiadi, H., McEver, R. P., and Kelly, R. B. (1994) J. Cell Biol. 124, 435–448
27. Baram, D., Adachi, R., Medalia, O., Tuim, M., Dickey, B. F., Mekori, Y. A., and Sagi-Eisenberg, R. (1999) J. Exp. Med. 189, 1649–1657
28. Blagoveshchenskaya, A. D., and Cutler, D. F. (2000) Methods Enzymol. 327, 45–60
29. Masterton, W. J., and Magee, A. I. (1992) in Protein Targeting, A Practical Approach (Magee, A. I., and Wileman, T., eds) p. 242, Oxford University Press, New York
30. Bonifacino, J. S., Yuan, L., and Sandoval, I. V. (1989) J. Cell Sci. 92, 701–712
31. Dell’Angelica, E. C., and Payne, G. S. (2001) Cell 106, 395–398
32. Better, J. C., Zhang, Q., Mules, E. H., Novak, E. K., Mishra, V. S., Li, W., McMurtrie, E. B., Tchernev, V. T., Wallace, M. R., Seabra, M. C., Swank, R. T., and Kingsmore, S. F. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 4144–4149
33. Hume, A. N., Collinson, L. M., Rapak, A., Gomes, A. Q., Hopkins, C. R., and Seabra, M. C. (2001) J. Cell Biol. 152, 795–808
34. Kantheti, P., Qiao, X., Diaz, M. E., Feden, A. A., Meyer, G. E., Carsskaden, S. L., Kaphamer, D., Sufalko, D., Robinson, M. S., Neubels, J. L., and Burmeister, M. (1998) Neuron 21, 111–122
35. Pastural, E., Barrat, F. J., Dufourcq-Lagelouse, R., Certain, S., Sanal, O., Jabado, N., Seger, R., Griscelli, C., Fischer, A., and de Saint Basile, G. (1997) Nat. Genet. 16, 289–292
36. Stinchcombe, J. C., Barral, D. C., Mules, E. H., Booth, S., Hume, A. N., Machsky, L. M., Seabra, M. C., and Griffiths, G. M. (2001) J. Cell Biol. 152, 825–833
37. Wilson, S. M., Yip, R., Swing, D. A., O’Sullivan, N., Zhang, Y., Novak, E. K., Swank, R. T., Russell, L. B., Copeland, N. G., and Jenkins, N. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7933–7938
38. Marks, M. S., and Seabra, M. C. (2001) Nat. Rev. Mol. Cell. Biol. 2, 738–748
39. Setiadi, H., Disdier, M., Green, S. A., Canfield, W. M., and McEver, R. P. (1995) J. Biol. Chem. 270, 26818–26826
40. Straley, K. S., Daugherty, B. L., Aeder, S. E., Hockenson, A. L., Kim, K., and Green, S. A. (1998) Mol. Biol. Cell 9, 1683–1694
41. Guo, Z., Turner, C., and Castle, D. (1998) Cell 94, 537–548
42. Baldassarre, M., Dragonetti, A., Marra, F., Laini, A., Iaduno, C., and Buccione, R. (2000) J. Cell Sci. 113, 741–748
43. Vijayasaradhi, S., Xu, Y., Bouchard, B., and Houghton, A. N. (1995) J. Cell Biol. 130, 807–820
44. Heijnen, H. F. G., Dehili, N., Vainchenker, W., Breton-Gorius, J., Geuze, H. J., and Sixma, J. J. (1998) Blood 91, 2313–2325
45. Youssoufiyan, P., and Cramer, E. M. (2000) Blood 95, 4004–4007
46. Israels, S. J., McMillan, E. M., Robertson, C., Singhroy, S., and McNicol, A. (1996) Thromb. Haemostasis 75, 623–629
47. Nishibori, M., Cham, B., McNeill, A., Shalev, A., Jain, N., and Gerrard, J. M. (1993) J. Clin. Invest. 91, 1775–1782