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An Atypical Sorting Determinant in the Cytoplasmic Domain of P-Selectin Mediates Endosomal Sorting

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We previously identified the 11 amino acid C1 region of the cytoplasmic domain of P-selectin as essential for an endosomal sorting event that confers rapid turnover on P-selectin. The amino acid sequence of this region has no obvious similarity to other known sorting motifs. We have analyzed the sequence requirements for endosomal sorting by measuring the effects of site-specific mutations on the turnover of P-selectin and of the chimeric protein LLP, containing the lumenal and transmembrane domains of the low density lipoprotein receptor and the cytoplasmic domain of P-selectin. Endosomal sorting activity was remarkably tolerant of alanine substitutions within the C1 region. The activity was eliminated by alanine substitution of only one amino acid residue, leucine 768, where substitution with several other large side chains, hydrophobic and polar, maintained the sorting activity. The results indicate that the endosomal sorting determinant is not structurally related to previously reported sorting determinants. Rather, the results suggest that the structure of the sorting determinant is dependent on the tertiary structure of the cytoplasmic domain.

INTRODUCTION

The function of membrane-bounded organelles requires correct targeting of resident membrane proteins to each organelle and in many cases selective cycling of membrane proteins between organelles. Selective localization and targeting of membrane proteins to their appropriate destinations depend on structural features of these proteins, termed sorting determinants. Sorting determinants are recognized by sorting machinery that functions to concentrate proteins bearing the appropriate sorting determinants into specific transport vesicles, which can then carry their cargo vectorially to the correct destination. A number of sorting determinants have been characterized, including those that mediate localization to clathrin-coated pits in the trans-Golgi network (TGN)[11] or at the cell surface, sorting to the basolateral cell surface in polarized epithelial cells, and selective transport from endosomes to lysosomes (Sandoval and Bakke, 1994; Mellman, 1996; Kirchhausen *et al.*, 1997; Marks *et al.*, 1997).

Many sorting determinants are contained in short segments of amino acid sequence in the cytoplasmic domains of transmembrane proteins (Sandoval and Bakke, 1994; Marks *et al.*, 1997). These short sequences have been defined by mutagenesis experiments in which systematic substitution of amino acid residues identifies only a few mutations that disrupt sorting activity. To date, the most prevalent and most extensively studied determinants that operate in post-Golgi trafficking pathways are those that require a tyrosine residue and additional residues in specific contexts

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[1] Abbreviations used: CHO, Chinese hamster ovary; LDL, low density lipoprotein; LLP, chimeric protein containing the lumenal and transmembrane domains of LDL receptor and the cytoplasmic domain of P-selectin; NRK, normal rat kidney; PBS-G, PBS containing 1 mg/ml glucose; TGN, trans-Golgi network.

and those that include a dileucine (or dihydrophobic) motif. In some cases, sorting motifs are thought to adopt specific secondary structures (Ktistakis *et al.*, 1990; Bansal and Gierasch, 1991; Reich *et al.*, 1996) that interact with sorting machinery such as the adaptor complexes (Robinson, 1994). However, only one sorting motif, YXXΦ (where Φ has a large hydrophobic side chain), has been shown to be sufficient for binding to adaptor subunits independent of larger segments of the cytoplasmic domains that contain them (Ohno *et al.*, 1995, 1996; Boll *et al.*, 1996).

P-selectin is a type I transmembrane protein that is concentrated in the secretory granules of platelets and endothelial cells. It functions in the early stages of inflammation as a cell adhesion protein, recognizing a specific ligand on the surface of neutrophils and monocytes after delivery to the cell surface by fusion of the secretory granules (Bevilacqua and Nelson, 1993; McEver *et al.*, 1995; McEver and Cummings, 1997). P-selectin is targeted to secretory granules in neuroendocrine cells expressing P-selectin cDNA, and the cytoplasmic domain is sufficient to confer granule localization on other proteins (Disdier *et al.*, 1992; Koedam *et al.*, 1992). P-selectin is rapidly internalized after reaching the surface of endothelial cells (Hattori *et al.*, 1989), and the sequence requirements for the internalization activity of P-selectin have been examined in detail in transfected cell lines (Setiadi *et al.*, 1995). The internalization activity in P-selectin depends on amino acid residues located throughout the cytoplasmic domain and does not resemble previously identified signals, particularly tyrosine-containing signals or dileucine motifs. Rather, this mutagenesis study suggests that the internalization activity is dependent on correct folding of a substantial portion of the cytoplasmic domain (Setiadi *et al.*, 1995).

Previous work established the presence of an additional sorting activity that mediates sorting of P-selectin within endosomes, diverting it from the cell surface recycling pathway and toward lysosomes, where it is rapidly degraded (Green *et al.*, 1994). Endosomal sorting activity is lost when the 11 amino acid C1 region of the cytoplasmic domain of P-selectin is deleted (Green *et al.*, 1994), while internalization activity is maintained (Setiadi *et al.*, 1995), indicating that these two sorting activities are independent. This sorting phenotype may represent a constitutive equivalent of the ligand- and signal-dependent downregulation of epidermal growth factor receptor, in which endosomal sorting is also independent of rapid internalization (Opresko *et al.*, 1995).

The sequence of the C1 region of P-selectin has no obvious similarity to known sorting determinants. To gain some understanding of the endosomal sorting of P-selectin, we have attempted to identify the sequence requirements for endosomal sorting activity by site-directed mutagenesis of the C1 region and by analysis of the trafficking of the mutants in stably transfected cells. We have found that the endosomal sorting activity is extremely tolerant of alanine substitutions in the C1 region. Only one residue, L768, was found to be intolerant of some nonconservative substitutions. Our results suggest that, in contrast to most sorting determinants described to date, the endosomal sorting activity does not exist as a small primary or secondary structural feature and may not be contained in a discrete segment of the cytoplasmic domain. Rather, it appears more likely that the sorting determinant is a feature of the tertiary structure of the cytoplasmic domain.

MATERIALS AND METHODS

Recombinant DNA

Sequences of all oligonucleotides are available on request. All procedures were performed essentially as described in Sambrook *et al.* (1989). *Escherichia coli* CJ236 *dut−/ung−* cells were used to recover uridine-substituted single-stranded Bluescript plasmid using M13K07 helper phage (Stratagene, La Jolla, CA). DH10-α cells (Life Technologies–Bethesda Research Laboratories, Gaithersburg, MD) were used for growing all other plasmids. pBSLLP (Green *et al.*, 1994), a Bluescript plasmid (Stratagene) containing low density lipoprotein receptor cDNA spliced to cDNA encoding the cytoplasmic domain of P-selectin, was mutated to encode an *Xho*I site ~300 bp 5′ to the stop codon. The *Xho*I/*Hin*dIII fragment containing the 3′ end of the coding region of the chimeric protein containing the lumenal and transmembrane domains of low density lipoprotein (LDL) receptor and the cytoplasmic domain of P-selectin (LLP) was subcloned into Bluescript, creating pBSLXP. Oligonucleotide-directed mutagenesis was performed on this plasmid using single primers that encoded alanine substitutions at each of the 11 residues constituting the C1 region of P-selectin (Johnston *et al.*, 1989a, 1990) and either created or deleted a restriction site. Clones were screened first by restriction analysis, and those containing the desired sites were sequenced. Mutant sequences were subcloned into pCB6-LLP (Green *et al.*, 1994) containing the engineered *Xho*I site and were expressed in Chinese hamster ovary (CHO) cells as described (Green *et al.*, 1994). pIBI20-E4 (Disdier *et al.*, 1992), containing full-length human P-selectin cDNA, was digested with *Afl*II, filled with Klenow polymerase, ligated to *Bgl*II linkers (New England Biolabs, Beverly, MA), and digested with *Sal*I and *Bgl*II. The 2.5-kbp product of this digest was subcloned into pCDL-SR-α (Takebe *et al.*, 1988) using the *Xho*I and *Bgl*II sites. The 300-bp *Xba*I/*Bgl*II fragment from this construct was subcloned into Bluescript in which *Bgl*II linkers had been added at the *Eco*RV site, creating pBS-Psel3′B. The cDNA sequence encoding the native P-selectin cytoplasmic domain was replaced with the sequences encoding the alanine substitutions by overlap extension PCR mutagenesis (Ho *et al.*, 1989), using the pBSLXP mutants as templates for the cytoplasmic domains and pBS-Psel3′B as the template for the region from the internal *Xba*I site to the cytoplasmic domain. The inside mutagenic primers encoded sequences spanning the membrane–cytoplasmic junction. T3 and T7 primers were used as the outside primers. Subsequent mutagenesis of the P-selectin cytoplasmic domain was performed by overlap extension PCR mutagenesis using pBS-Psel3′B as the first template for both reactions. Second reaction PCR products were subcloned as *Xba*I/*Bgl*II fragments into Bluescript and sequenced and then subcloned into pCDL-SR-α containing full-length P-selectin cDNA.

Antibodies

Ascites fluid containing three monoclonal antibodies recognizing distinct epitopes in the lumenal domain of P-selectin, designated

S12, G5, and 2B8 (Johnston *et al.*, 1989b; Geng *et al.*, 1990), were pooled and used at 1:300 dilution for immunofluorescence microscopy and at a ratio of 6 μl per 6-cm plate of transfected cells for immunoprecipitation. IgG isolated from polyclonal goat serum against purified P-selectin (Lorant *et al.*, 1991) was also used for immunoprecipitation. Rabbit polyclonal antiserum recognizing whole human P-selectin was used for immunofluorescence microscopy. All of the above antibodies were generously supplied by Rodger McEver (Oklahoma Medical Research Foundation, Oklahoma City, OK). Rabbit polyclonal antibodies recognizing the 25 C-terminal amino acids of P-selectin were prepared as described (Green *et al.*, 1994). Affinity-purified anti-peptide antibody was biotinylated using NHS-LC-biotin (Pierce Chemical, Rockford, IL). Ascites containing mAb C7 (Beisiegel *et al.*, 1981) recognizing human LDL receptor was generated in pristane-primed BALB/c mice and was used unfractionated. IgG purified from polyclonal rabbit antiserum raised against rat cation-independent mannose 6-phosphate receptor was the generous gift of Dr. William Brown (Cornell University, Ithaca, NY). mAb H68.4 recognizing transferrin receptor (White *et al.*, 1992) was kindly provided by Dr. Ian Trowbridge (Salk Institute, La Jolla, CA). Goat anti-rabbit IgG, rabbit anti-mouse IgG and goat anti-mouse IgG conjugated to HRP, Texas Red- and FITC-conjugated goat anti-rabbit IgG, and FITC-conjugated goat anti-mouse IgG were from Cappel (ICN, Durham, NC).

Cell Culture

CHO cells were grown in α-modified MEM containing 5% heat-inactivated FBS (Hyclone, Logan, UT). Normal rat kidney (NRK) fibroblasts were grown in DMEM containing 7% heat-inactivated FBS. Growth of CHO cells overexpressing LDL receptor ectodomain has been described previously (Green *et al.*, 1994).

Transfection and Screening

Cells were transfected in 6-cm plates using Lipofectin reagent (Life Technologies–Bethesda Research Laboratories) according to the method of Muller *et al.* (1990). CHO cells were transfected with 6 μg of pCB6 plasmids containing LLP constructs. NRK cells and CHO cells were cotransfected with pSV2neo and with pCDL-SRalpha containing cDNA inserts, using these plasmids at a ratio of 1:120, with a total of 6 μg of plasmid DNA. Transfected cells were passaged into selection medium containing 400 μg/ml G418 3 d after transfection. Clones were picked directly from the plates using 200-μl pipets after washing once with HBSS lacking divalent cations and containing 1 mM EDTA and 5 mM HEPES. CHO transfectants expressing LLP constructs were screened by immunofluorescence microscopy using the C7 anti-LDL receptor mAb. CHO cells expressing the LLP chimeric protein were subcloned to obtain clones with uniform expression levels. NRK cells or CHO cells expressing P-selectin and mutants thereof were screened by immunofluorescence microscopy using a mixture of ascites containing S12, G5, and 2B8 monoclonal antibodies.

Immunofluorescence Labeling and Microscopy

Cells were processed for immunofluorescence microscopy at room temperature. Cells grown on 12-mm glass coverslips coated with poly-D-lysine were washed with PBS and fixed in 3% formaldehyde and 100 mM sodium phosphate, pH 7.4, for 15 min. After washing three times in PBS, cells were permeabilized in 2% BSA, 0.5% fish skin gelatin (Sigma, St. Louis, MO), 0.02% saponin (Sigma), 150 mM NaCl, and 10 mM HEPES, pH 7.4 (blocking buffer), for 30 min. Antibodies were applied for 1–1.5 h in blocking buffer. Ascites fluid and antisera were diluted 1:300, and purified antibodies were used at 20 μg/ml. Secondary antibodies were diluted 1:300. For epifluorescence microscopy, mouse monoclonal antibodies were detected with FITC-conjugated secondary antibodies, and rabbit polyclonal antibodies were detected with Texas Red-conjugated secondary antibodies. Biotin-labeled antibodies were detected with Texas Red-Neutralite (Molecular Probes, Eugene, OR). Each antibody incubation was followed by three 5 min washes in blocking buffer. The cells were then washed three times in PBS and once in distilled water and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Samples were viewed and photographed on Kodak T-Max 400 film using a Zeiss Axioplan epifluorescence microscope with a 63× oil immersion planapochromatic objective lens. Negatives were scanned on a Nikon LS3510AF Film Scanner. Images were processed using Adobe Photoshop and were printed on a Kodak XLS 8600 printer.

Metabolic Labeling

For pulse metabolic labeling with [^{35}S]amino acids, cells were washed with PBS and then incubated for 20 min in DME lacking methionine and cysteine and containing 10% dialyzed FBS or LDL-depleted serum. Cells were labeled in the same medium containing 300–800 μCi/ml [^{35}S]amino acid mixture (EXPRE^{35}S^{35}S Protein Labeling Mix; New England Nuclear, Boston, MA). Chase incubations were performed by washing the cells once in complete growth medium and then culturing in growth medium supplemented with 3 mM methionine and 3 mM cysteine.

Cell Surface Biotinylation

For irreversible cell surface biotinylation to measure turnover of cell surface proteins, cells were grown to 70–90% confluence on 6-cm dishes. Cells were washed three times with ice-cold PBS containing 1 mg/ml glucose (PBS-G) and then labeled for 20 min on ice with 0.5–1 mg/ml sulfo-NHS-biotin or NHS-LC-biotin (Pierce Chemical) dissolved just before use in PBS-G. Labeling was stopped by washing once with ice-cold PBS-G containing 1 mg/ml lysine and once with PBS-G. Cells were then recultured at 37°C in complete growth medium. Reversible biotinylation with NHS-SS-biotin (Pierce Chemical) to measure the rate of internalization was performed as described previously (Le Bivic *et al.*, 1990).

Immunoprecipitation

Cells expressing P-selectin on 6-cm plates were washed twice with PBS and once with PBS lacking divalent cations and containing 5 mg/ml iodoacetamide and were lysed in 300 μl of IP buffer (1% Triton X-100, 0.5% sodium deoxycholate, 200 mM NaCl, 10 mM Tris-HCl, pH 7.8, 1 mM EDTA, 5 mg/ml iodoacetamide, and Protease Inhibitor Cocktail [Sigma] containing AEBSF, pepstatin A, E-64, bestatin, leupeptin, and aprotinin, used at 1:1000 dilution) for 5 min on ice. The lysate was recovered, and the plate was washed with an additional 100 μl of buffer. Nuclei and insoluble material were pelleted at 15,000 × *g* for 10 min, and the supernatant was adjusted to 0.1% SDS and 2 mg/ml BSA. Polyclonal goat anti-P-selectin antibodies were added in the ratio of 6 μg of IgG for each 5×10^5 cells. Alternatively, 6–8 μl of a mixture of equal volumes of ascites fluid containing S12, G5, and 2B8 monoclonal antibodies was used, followed by 4 μl of rabbit anti-mouse IgG (Cappel). Lysates were incubated overnight after addition of primary antibody and for 1 h after addition of secondary antibody. Twenty-five microliters of a 50% slurry of Protein G-Sepharose (Pharmacia, Piscataway, NJ) in 1% Triton X-100, 1% BSA, 150 mM NaCl, and 10 mM Tris-HCl, pH 7.8, were added for recovery of goat antibodies; 25 μl of Protein A-Sepharose (Pharmacia) in the same buffer was used for recovery of monoclonals; and the mixtures were incubated with constant gentle mixing for 60 min. The sepharose beads were washed five times with 300 mM NaCl, 10 mM Tris, pH 8.0, 0.2% SDS, and 0.1% Triton X-100 and once with 50 mM NaCl and 5 mM Tris-HCl, pH 7.4. Bound proteins were eluted into electrophoresis sample buffer (Laemmli, 1970) containing 4% SDS. CHO cells expressing LLP constructs were lysed on the plates in 400 μl of IP buffer containing 1 mg/ml BSA. Precipitations were performed with 6 μl of C7 ascites

plus 2 μg of anti-P-selectin peptide antibody for each 5×10^5 cells (one 6-cm dish).

Electrophoresis, Western Blotting, and PhosphorImager Analysis

Immunoprecipitates were separated on SDS-polyacrylamide gels containing 7.5% acrylamide (Laemmli, 1970). Prestained molecular weight markers (Sigma) were used, and biotinylated markers (Sigma) were included where appropriate. Gels containing ^{35}S-labeled proteins were fixed in 30% methanol and 10% acetic acid in water and dried. Radioactivity in the gel bands was quantitated using a Molecular Dynamics PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Gels were subsequently exposed to Kodak XAR-5 or Fuji x-ray film at −80°C. For Western blotting, proteins were transferred to nitrocellulose filter paper (MSI, Westborough, MA); blocked in PBS, 0.1% Tween 20, and 5% nonfat milk solids; and probed with antibodies diluted in the same buffer. After unbound HRP-conjugated secondary antibodies were washed off, the filters were washed twice with PBS, impregnated with ECL substrate (Amersham, Arlington Heights, IL), and exposed to x-ray film. Biotinylated proteins transferred to Immobilon (Millipore, Bedford, MA) were detected using ^{125}I-streptavidin as described (Lisanti *et al.*, 1988; Green and Kelly, 1992), and radioactive streptavidin bound to the bands was quantitated using the PhosphorImager.

Analysis of Half-Lives

Turnover experiments were analyzed by plotting the log of the PhosphorImager volume for each gel band (with background from a blank lane on the gel subtracted) versus chase time and by obtaining a slope m for the plot by regression analysis. All measurements presented were obtained from at least four time points for LLP constructs and from at least five time points for P-selectin constructs. Turnover was assumed to be a first-order process, with $t_{1/2} = 0.693/k$, where $k = -2.3\ m$. All half-life measurements are from at least two independent experiments yielding correlation coefficients ≥0.9.

RESULTS

Alanine Scan of the C1 Region in the LLP Chimeric Protein

We began this study by extending our previous observations on the trafficking of P-selectin and the chimeric protein LLP in CHO cells (Green *et al.*, 1994). Because deletion of the C1 region of the cytoplasmic domain (Figure 1) prevents endosomal sorting without affecting internalization activity, we assessed the role of each residue in this region by alanine substitution in the context of the LLP chimeric protein. Three of the alanine substitutions analyzed, D763A,[2] L768A (Figure 2A), and P767A (Hockenson and Green, unpublished observations), exhibited increased half-lives, similar to wild-type LDL receptor. The LLP-D763A construct showed a significant decrease in the rate of internalization, proportional to the increased half-life (approximately fivefold) (Figure 2B). Similar results were obtained for the P767A mutant. Because reducing the internalization rate significantly could reduce the rate of delivery of the protein to lysosomes independently of any sorting that occurs in endosomes, it is unclear from these results whether D763 or P767 are involved in endosomal sorting. LLP-L768A exhibited a long half-life, comparable with the LDL

[2] Numbering of residues corresponds to the position of residues in native human P-selectin (Johnston *et al.*, 1989a).

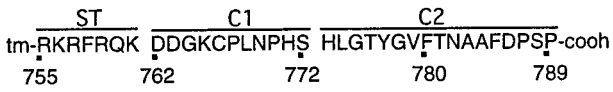

Figure 1. The cytoplasmic domain of P-selectin. Numbers correspond to the positions of residues in the human sequence (Johnston *et al.*, 1989a).

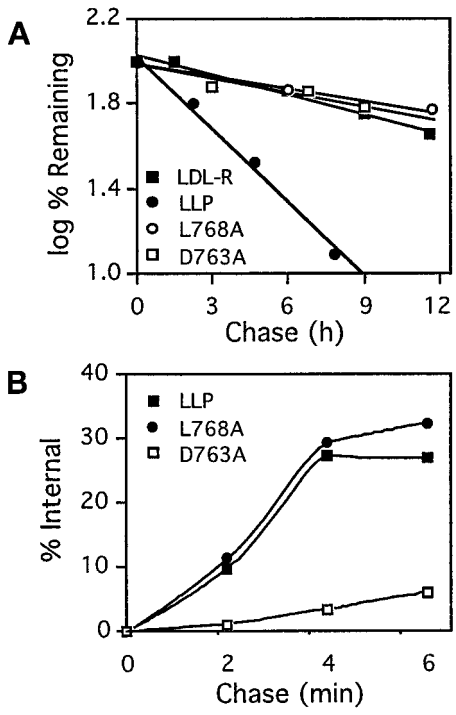

Figure 2. Turnover and internalization of LLP chimeric proteins containing the native or mutant cytoplasmic domain of P-selectin. (A) Turnover of LLP constructs. CHO cells expressing the indicated LLP constructs were labeled with biotin at 0°C and then recultured at 37°C for the indicated times before cell lysis and immunoprecipitation of the chimeric proteins. Immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with ^{125}I-streptavidin. Radioactivity in the bands was quantitated using the PhosphorImager. Data for native LDL receptor (LDL-R) are from Green *et al.* (1994). (B) Internalization of LLP constructs. Cells were labeled with disulfide-linked biotin at 0°C, warmed to 37°C for the indicated intervals, and then incubated at 0°C with glutathione to reduce exposed disulfide bonds. Proteins were immunoprecipitated from detergent lysates, separated on nonreducing SDS-PAGE, and transferred to nitrocellulose. Biotin was detected with ^{125}I-streptavidin and quantitated by PhosphorImager analysis. Background, defined as the signal obtained from cells that were labeled but not warmed to 37°C before glutathione treatment (0 chase), was 3–5% of the total label (no glutathione treatment) and was subtracted from all data points.

receptor, in these cells, and its internalization rate was normal, suggesting an important role for L768 in endosomal sorting.

In contrast to the results obtained with the LLP chimeric protein, a previous study analyzing the internalization activity in P-selectin showed that the L768A substitution in the context of native P-selectin was internalized at ~40% of the wild-type rate, while D763A or P767A substitutions were internalized at ~60% of the wild-type rate (Setiadi *et al.*, 1995). The difference in internalization activity between the native protein and the LLP chimera containing the same mutations expressed in the same cell type suggested that the cytoplasmic domain was not folded or oriented identically in the native and chimeric proteins. This raised the possibility that analysis of the endosomal sorting activity in chimeric proteins could be misleading. We therefore repeated the alanine scan in the context of native P-selectin.

Expression and Localization of P-Selectin in NRK Cells

Because expression of transfected proteins was quite unstable in CHO cells, we began experiments in NRK fibroblasts, used successfully in other mutagenesis studies (Marks *et al.*, 1996). After isolating cell lines expressing wild-type P-selectin in these cells, we analyzed the distribution of the protein in comparison with known markers of the endocytic pathway. Immunofluorescence microscopy showed that P-selectin was concentrated primarily in small punctate structures that showed partial overlap with the distribution of transferrin receptor, a marker of sorting and recycling endosomes (Mayor *et al.*, 1993), in these cells (Figure 3, A–C). Some overlap was also seen with the distribution of cation-independent mannose 6-phosphate receptor (Figure 3, D–F), which is concentrated in late endosomes (Griffiths *et al.*, 1988); and limited overlap was seen with lgpA, a resident lysosomal membrane protein (Lewis *et al.*, 1985) (Figure 3, G–I). As seen in CHO cells (Green *et al.*, 1994), P-selectin in NRK cells was concentrated primarily in early endocytic compartments, with less present in late compartments at steady state.

Rapid Transport of P-Selectin to Lysosomes in NRK Cells

The half-life of P-selectin was measured in NRK cells by metabolic labeling and by cell surface biotinylation. In both experiments, the half-life was ~3 h (Figure 4), indicating that most or all of the P-selectin reaches the cell surface before delivery to lysosomes (Green *et al.*, 1994). The half-life of transferrin receptor in the same cells was 9 h (Figure 4). These observations are similar to those seen in CHO cells for wild-type P-selectin and LDL receptor (Green *et al.*, 1994). To determine whether P-selectin degradation represented transport to lysosomes, we labeled cells expressing wild-type P-selectin with biotin, incubated the cells in the presence or absence of a proteinase inhibitor cocktail for 3 h, and then analyzed the cells by immunoprecipitation of P-selectin as described for the turnover experiment. In untreated cells, approximately one-half of the labeled P-selectin was degraded, whereas in cells exposed to proteinase inhibitors very little P-selectin had been degraded (Figure 5). Lumenal fragments of P-selectin were not detectable by immunoprecipitation from the culture media under any of these conditions (Daugherty, Aeder, and Green, unpublished observations). These results suggest that P-selectin was degraded primarily in lysosomes in NRK cells. In CHO cells, inhibition of lysosomal proteinases by incubation with ammonium chloride results in rapid accumulation of P-selectin in lysosomes to levels detectable by immunofluorescence microscopy (Green *et al.*, 1994). We conclude that the half-life of the protein reflects the rate of transport to lysosomes.

Alanine Scanning of the C1 Region in Native P-Selectin

To determine whether the results of mutagenesis of the LLP chimera were valid for the cytoplasmic domain in its native context, we repeated the alanine scan through the C1 region of the cytoplasmic domain in native P-selectin. Half-lives of the constructs were measured using biotin labeling of cell surface proteins. In contrast to the results obtained with LLP, the only alanine substitution that increased the half-life of native P-selectin significantly was L768A. P-selectin mutants containing alanine substitutions at all other positions in the C1 region, including D763A and P767A, had short half-lives (Figure 6).

In a very small number of clones, expression levels were high enough to yield intense labeling of the entire cell surface in addition to vesicular staining. Constructs expressed at this level were found to exhibit somewhat longer half-lives than did the same constructs expressed at levels at which cell surface staining was not readily detected, a result consistent with our earlier observations of LLP turnover in CHO cells (Green *et al.*, 1994). We interpret this result to mean that excessively high expression levels saturate the sorting machinery, either at the cell surface, in endosomes, or in both. Data from such clones were not included in the analysis.

Other Substitutions

Because the C1 region contains one glycine, for which alanine could be a conservative substitution in some contexts, G764 was substituted with valine. P-selectin G764V also exhibited a short half-life (Figure 6). In

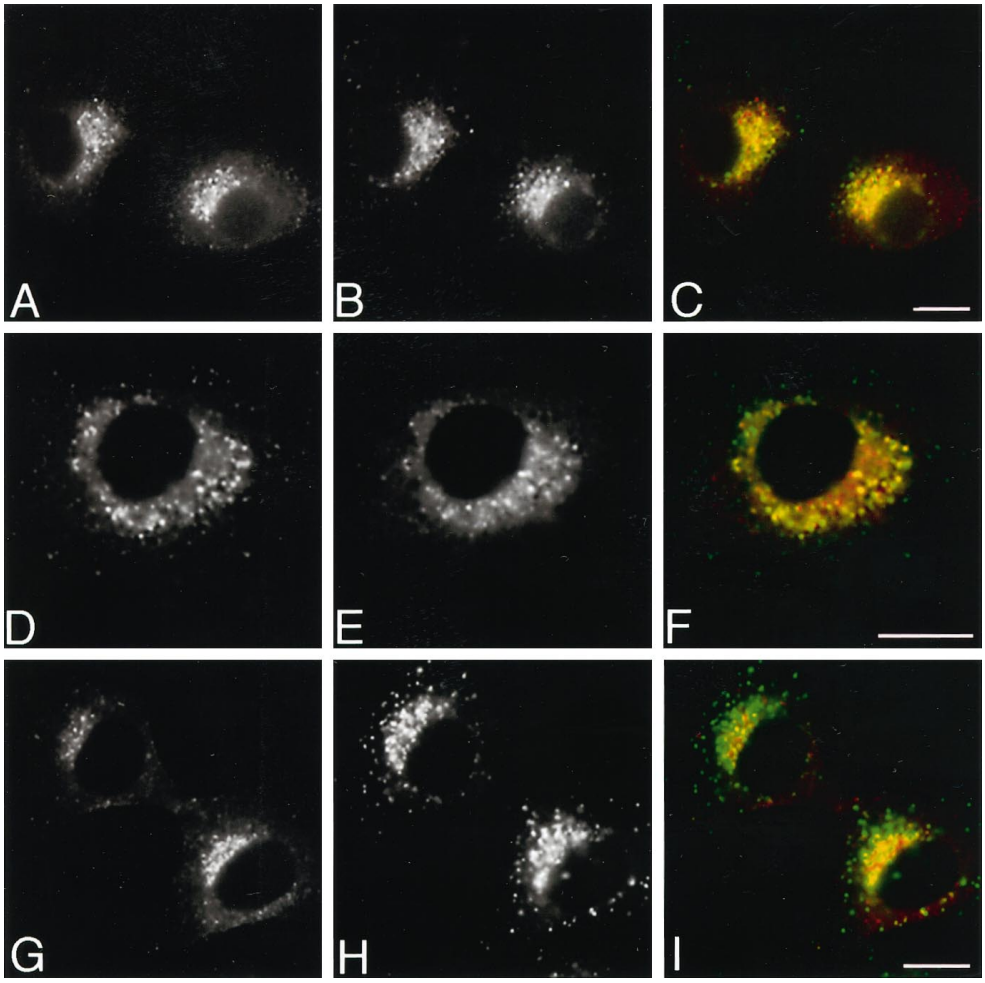

Figure 3. Immunofluorescence localization of P-selectin to endosomes in NRK cells. NRK cells expressing native P-selectin were processed for immunofluorescence microscopy and double labeled with antibodies to P-selectin (A, D, and G) and either transferrin receptor (B), cation-independent mannose 6-phosphate receptor (E), or lgpA (H) followed by FITC–goat anti-mouse IgG and Texas Red–goat anti-rabbit IgG. The combined images for each double label are shown in C, F, and I. P-selectin was detected in a significant fraction of transferrin receptor-positive structures (A–C) and in some mannose 6-phosphate receptor-positive structures (D–F) and was not detected in lgpA-positive structures (G–I). Bars, 8 μm.

addition, because L768 is flanked by two prolines at 767 and 770, we tested the effect of substituting both prolines with alanines to determine whether the prolines participated in creating local secondary structure that might be required for sorting. The P767A/P770A double mutant was also found to have a short half-life (Figure 6).

Because changes in half-life could result from changes in internalization rates, the internalization rates of P-selectin and several of the alanine mutants were measured in NRK cells. As shown in Figure 7, internalization rates of the L768A, D763A, and P767A mutants were approximately one-half that of wild-type P-selectin, similar to the results obtained in CHO cells (Setiadi *et al.*, 1995). Several other alanine substitutions were also found to have little or no effect on internalization activity (Straley, Hockenson, and Green, unpublished observations). This is in contrast to the results obtained for the LLP chimera, in which the D763A and P767A mutants were severely impaired in internalization activity and L768A was internalized as rapidly as wild-type P-selectin (Figure 2B).

Summarizing the results of the alanine scan, we found that only the L768A mutation yielded an increase in the half-life of the protein without a corresponding decrease in the rate of internalization, both in the context of the LLP chimeric protein and in the context of native P-selectin. We concluded that L768 is the only amino acid residue in the C1 region required for endosomal sorting; it is not required for rapid internalization of P-selectin.

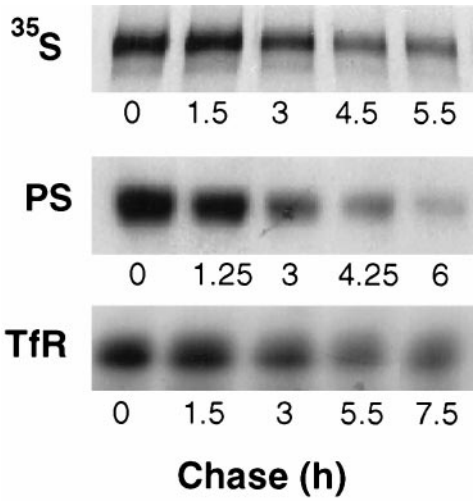

Figure 4. Turnover of P-selectin and transferrin receptor in NRK cells. Top, metabolic labeling of P-selectin. NRK cells expressing native P-selectin were labeled with [^{35}S]amino acids for 1 h and then chased for 1 h to allow for transport through the Golgi apparatus (t = 0). Plates were then harvested at the indicated intervals. P-selectin was immunoprecipitated from detergent lysates of the cells and separated by SDS-PAGE. Radioactivity was quantitated by PhosphorImager analysis. A radiograph of the gel is shown. Middle and bottom, cell surface labeling. NRK cells expressing native P-selectin were labeled with biotin at 0°C and then recultured for the indicated times before detergent lysis and immunoprecipitation of P-selectin (PS, middle) or endogenous transferrin receptor (TfR, bottom). Precipitates were separated by SDS-PAGE, transferred to Immobilon membranes, and probed with ^{125}I-streptavidin. Autoradiographs of the blots are shown. Radioactivity was quantitated by PhosphorImager analysis. Half-lives were calculated as described in MATERIALS AND METHODS. In these experiments, the half-life of P-selectin was 2.5 h by metabolic labeling and 3.0 h by cell surface labeling, and the half-life of transferrin receptor was 9.0 h.

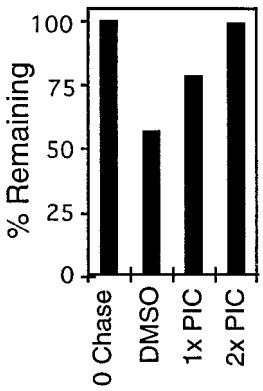

Figure 5. Degradation of P-selectin is prevented by a proteinase inhibitor cocktail. NRK cells expressing native P-selectin were labeled with biotin and then either harvested (0 chase) or recultured for 3 h in the presence of a proteinase inhibitor cocktail (containing AEBSF, aprotinin, bestatin, E64, leupeptin, and pepstatin A) diluted 1:1000 (1× PIC) or 1:500 (2× PIC) or in the presence of DMSO alone diluted 1:500 (DMSO). P-selectin was immunoprecipitated from detergent lysates of the cells, and the remaining biotin was measured as described for the turnover experiment in Figure 4. Numbers represent the average of duplicate samples, expressed as the percentage of P-selectin remaining, normalized to 0 chase. The proteinase inhibitor cocktail potently inhibited degradation of P-selectin.

Other Substitutions of L768

To explore the possible role of L768 in endosomal sorting, we made other amino acid substitutions at this position. Substitution of L768 with isoleucine, valine, or methionine increased the half-life of P-selectin only slightly (Figure 8A). Because all of these large hydrophobic side chains functioned well in endosomal sorting, we tested the activity of the isosteric polar substitution L768N. Multiple attempts to isolate stable clones of NRK cells expressing this construct were unsuccessful, so the L768N mutant and other selected mutants were expressed in CHO cells. Although native P-selectin has a half-life of 2.3 h in CHO cells (Green *et al.*, 1994), the L768A mutant exhibited a half-life of 8.4 h (Figure 8B), similar to the half-life of P-selectin C2 (lacking the C1 domain) and LDL receptor in these cells (Green *et al.*, 1994) and similar to the half-life of this mutant in NRK cells (Figure 8A). Surprisingly, the L768N mutant was degraded rapidly ($t_{1/2}$ = 3.0 h) in CHO cells (Figure 8B). Thus, several large side chains, hydrophobic or polar, can substitute for leucine at position 768, whereas substitution with the small hydrophobic side chain of alanine did not support endosomal sorting. We conclude that the specificity of the sorting interaction cannot reside in the leucine side chain itself, because even nonconservative (polar) substitutions maintain endosomal sorting activity.

DISCUSSION

We have examined sequence requirements for the endosomal sorting of P-selectin away from the recycling pathway and its delivery to lysosomes. Pursuing our earlier observation that deletion of the C1 region eliminates the endosomal sorting activity without interfering with internalization activity (Green *et al.*, 1994), we have made single amino acid substitutions through this region. Surprisingly, only one residue, L768, was important for endosomal sorting, and other large residues, both hydrophobic and polar, substituted for leucine at this position. Nonconservative substitutions at all other positions in the C1 region had no significant effect on the half-life of P-selectin. Because the results indicate that the sorting determinant does not correspond to known motifs and they preclude defining a precise role for residue 768 in endosomal sorting (see below), we concluded that testing the effects of the many other possible mutations at this position would be unlikely to provide additional insight into the structure of the sorting determinant.

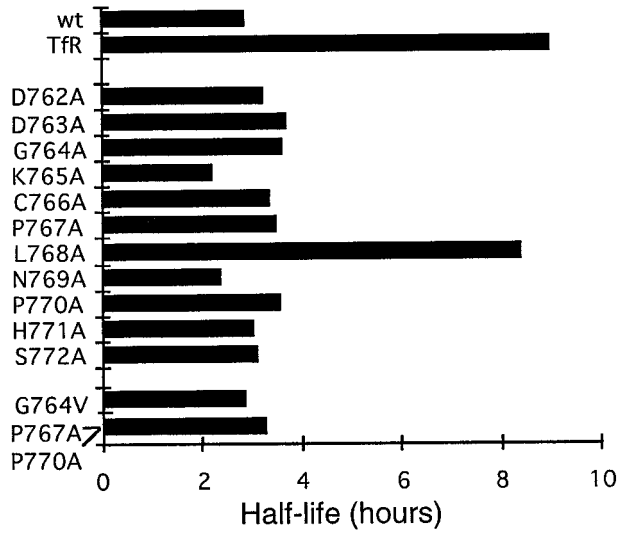

Figure 6. Analysis of the endosomal sorting activity in P-selectin by alanine scanning mutagenesis of the C1 region. Each of the amino acid residues in the C1 region of P-selectin was substituted with alanine by site-directed mutagenesis and expressed in NRK cells. In addition, the glycine at position 764 was substituted with valine (G764V), and the prolines at positions 767 and 770 were substituted with alanine concomitantly (P767A/P770A). Half-lives of the mutants and of transferrin receptor (TfR) and of wild-type P-selectin (wt) were measured after biotin labeling of the cell surface as described in the legend of Figure 4. Only substitution of L768 with alanine caused a significant increase in the half-life of P-selectin.

Certain mutations in P-selectin, e.g., D763A and P767A, resulted in significantly reduced rates of internalization in the context of the LLP chimeric protein but not in the context of native P-selectin (Setiadi *et al.*, 1995; this study). This was unexpected, especially in light of the observation that the LLP chimeric protein containing the wild-type cytoplasmic domain is internalized just as rapidly as LDL receptor, whereas native P-selectin is internalized at approximately one-half the rate of LDL receptor and LLP (Setiadi *et al.*, 1995). These findings suggest that the cytoplasmic domain of P-selectin is either not oriented or not folded identically in the native and chimeric proteins. Although our results with both proteins lead us to the same conclusions regarding the endosomal sorting activity in P-selectin, these observations present a potentially significant caution in view of the many other mutagenesis studies performed using chimeric proteins as reporters.

In particular, a recent report (Blagoveshchenskaya *et al.*, 1998) concluded that P767 is the critical residue within a signal for endosomal sorting of P-selectin comprising KCPL (residues 765–768). These authors did not test the role of L768 specifically, although the mutant that included the L768A substitution appeared to be deficient in endosomal sorting in their system.

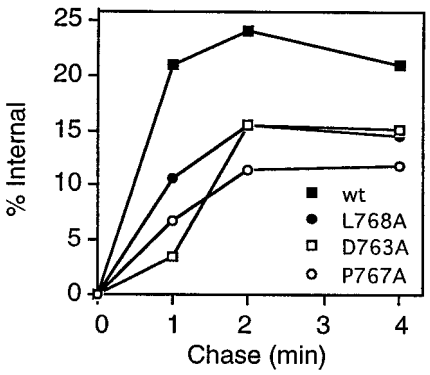

Figure 7. Internalization of P-selectin and P-selectin mutants in NRK cells. NRK cells expressing the indicated P-selectin cDNAs were labeled at 0°C with disulfide-linked biotin and were warmed to 37°C for the indicated intervals before removal of the biotin remaining on the cell surface by reduction with glutathione at 0°C. P-selectin was immunoprecipitated from detergent lysates, separated on nonreducing SDS-PAGE, and transferred to Immobilon membranes. Biotin was detected with ^{125}I-streptavidin and quantitated using the PhosphorImager. The D763A, P767A, and L768A mutants were internalized more slowly than native P-selectin (wt) but significantly faster than the same mutations expressed in the context of the LLP chimeric protein (Figure 2) (Setiadi *et al.*, 1995).

We have found that residues K765, C766, and P767 can all be substituted with alanine without increasing the half-life of P-selectin. We believe that the most likely reason for the discrepancy between the results of the two studies is that Blagoveshchenskaya *et al.* (1998) studied chimeric proteins containing horseradish peroxidase fused to the transmembrane and cytoplasmic domains of P-selectin. We found some differences in

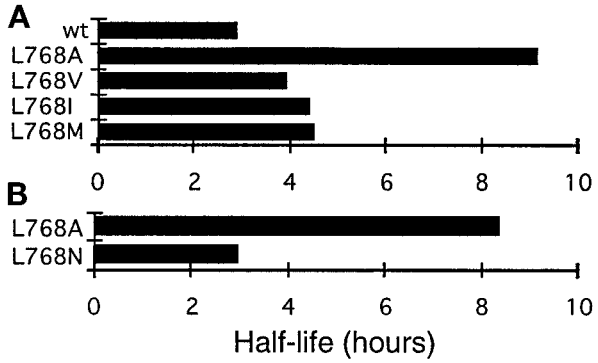

Figure 8. Half-lives of P-selectins containing conservative and nonconservative amino acid substitutions at position 768. (A) P-selectin cDNAs encoding the indicated amino acid substitutions were expressed in NRK cells. Half-lives were measured after cell surface biotin labeling as described in the legend of Figure 4. All of the indicated substitutions at L768 supported rapid degradation. (B) The indicated P-selectin mutants were expressed in CHO cells. Half-lives were measured after cell surface biotin labeling. Although the L768A mutant exhibited the same long half-life seen in NRK cells, the polar substitution L768N was degraded rapidly.

the behavior of P-selectin and the chimeric protein LLP containing the same mutations, which we attribute to a subtle change in the conformation or orientation of the cytoplasmic domain in the chimeric protein compared with the native protein. Also, because Blagoveshchenskaya *et al.* (1998) did not measure the rate of internalization or the rate of delivery to lysosomes directly, it is unclear whether the altered distribution of the P767A mutant in their assays is due in whole or in part to a defect in internalization activity, which we observed for the equivalent mutation in the chimeric LLP construct. For both the internalization activity (Setiadi *et al.*, 1995) and the endosomal sorting activity (this study; see below), the results suggest that the sorting determinants in P-selectin are critically dependent on the tertiary structure of the cytoplasmic domain. It is perhaps not surprising that small changes in the structure or orientation of the cytoplasmic domain may lead to significant changes in the results of the mutagenesis experiments. At a minimum, we believe the results obtained using the native protein are likely to represent a more accurate view of the sorting activity.

One mechanism for endosomal sorting of membrane proteins is aggregation of proteins in the plane of the membrane (Anderson *et al.*, 1982; Hopkins and Trowbridge, 1983; Mellman and Plutner, 1984; von Figura *et al.*, 1984; Wolins *et al.*, 1997). Although these examples of aggregation sufficient to drive recycling receptors to lysosomes are mediated by interactions among lumenal domains and large polyvalent ligands, and formation of small aggregates does not cause a significant change in transport to lysosomes (Marsh *et al.*, 1995), it remains possible that self-aggregation mediated by cytoplasmic domains could drive endosomal sorting events. We have attempted to detect aggregation of native P-selectin by velocity sedimentation of octylglucoside-, CHAPS-, or Brij 96-solubilized cell lysates and by chemical cross-linking in intact cells (Straley, Daugherty, and Green, unpublished observations). We have been unable to detect any large oligomers (see also Ushiyama *et al.*, 1993). We cannot exclude that self-aggregation may occur within endosomes. However, in the absence of any evidence that P-selectin undergoes any significant self-aggregation, we assume that, as for many other selective sorting events, endosomal sorting is mediated by specific interactions with other proteins.

The majority of post-Golgi sorting determinants defined to date contain tyrosine-dependent motifs or dileucine motifs, and both types of motifs have been shown to act either as internalization signals, lysosomal targeting signals, or both (Sandoval and Bakke, 1994; Marks *et al.*, 1997). Several signals have been identified that either require structural information in addition to tyrosine motifs or dileucine motifs (Johnson and Kornfeld, 1992; Le Borgne *et al.*, 1993; Dietrich *et al.*, 1994; Motta *et al.*, 1995; Pond *et al.*, 1995; Vijayasaradhi *et al.*, 1995; Chen *et al.*, 1997; Lin *et al.*, 1997) or, less frequently, appear to be unrelated to these signals (Reich *et al.*, 1996; Odorizzi and Trowbridge, 1997; Schweizer *et al.*, 1997; Subtil *et al.*, 1997). We considered the possibility that the endosomal sorting determinant represents a degenerate dileucine motif, because dileucine motifs are known to participate in lysosomal targeting of some proteins (Letourneur and Klausner, 1992; Sandoval and Bakke, 1994; Vijayasaradhi *et al.*, 1995; Dietrich *et al.*, 1997). However, in cases analyzed to date, the N-terminal leucine in dileucine motifs is intolerant of replacement with methionine or valine (Letourneur and Klausner, 1992; Dietrich *et al.*, 1997), while L768 could be replaced with either of these residues and with asparagine. It therefore appears unlikely that L768 functions as part of a dileucine-type sorting determinant. The C1 region does contain a sequence, DGKC, that is similar to the ubiquitination signal required for internalization of the yeast α factor receptor (Rohrer *et al.*, 1993; Hicke and Riezman, 1996). None of these residues, however, was necessary for rapid turnover of P-selectin. Thus, our data, as well as a study analyzing the internalization activity in P-selectin (Setiadi *et al.*, 1995), suggest that both the internalization and endosomal sorting determinants in the cytoplasmic domain of P-selectin do not conform to any of the reported sorting signals, at least at the level of primary structure.

In many cases, amino acid substitutions that produce mutant sorting phenotypes have been interpreted as identifying residues and/or sorting motifs that interact with the sorting machinery directly. Indeed, recent reports directly demonstrate binding of adaptor subunits to the same short peptide sequences that have been defined as sorting determinants by mutagenesis (Ohno *et al.*, 1995, 1996; Boll *et al.*, 1996). However, we believe that it is highly unlikely that this interpretation applies to our study of P-selectin. In the C1 region defined as essential for sorting by deletion mutagenesis, only one amino acid residue, L768, is critical for the sorting activity, and it is tolerant of multiple substitutions. It seems quite implausible that the endosomal sorting determinant (or any sorting determinant) consists of a single amino acid side chain, particularly because conservative substitutions, and even a nonconservative substitution (L768N), of that residue maintain the sorting activity. Rather, the results suggest either that L768 is part of a larger structure that is remarkably tolerant of nonconservative substitutions or that L768 does not interact directly with sorting machinery at all but is required for correct folding or orientation of a different part of the cytoplasmic domain that does interact with the sorting machinery.

At least two different indirect roles for L768 in the function of the endosomal sorting determinant could

be envisioned. L768 may be required for proper folding of the cytoplasmic domain to create a sorting determinant that does not include residue 768 directly. Perhaps the side chain of residue 768 is completely or partially buried in the interior of the normally folded cytoplasmic domain, where substitution with other large residues may not grossly disrupt normal folding, whereas substitution with a small side chain could cause significant disruption of the tertiary structure. Alternatively, the structure of the sorting determinant per se may not depend on L768, but the orientation of the sorting determinant with respect to the plane of the membrane, and thus its availability to interact with sorting machinery, may depend on a conformation requiring the presence of a large side chain at position 768. This model would be consistent with a sorting determinant that does not include the C1 region at all but is inaccessible when C1 is deleted or when the determinant is repositioned by changing L768 to alanine.

Another possibility is that the surface that interacts with the sorting machinery is formed at least in part by the peptide backbone. This hypothesis offers one explanation of the ability of the sorting activity to tolerate so many different alanine substitutions in the C1 region. Direct or indirect participation of L768 in formation of the sorting determinant would be plausible in this scenario, although our results preclude the possibility that the side chain itself can confer specificity to the determinant. A few other mutagenesis studies in which no obvious small motif could be identified have also pointed to the possible participation of the peptide backbone in forming some sorting determinants (Naim and Roth, 1994; Motta *et al.*, 1995).

A final consideration is that the short cytoplasmic domains typical of many Type I and Type II membrane proteins may not have a single stable tertiary structure in living cells. Rather, multiple conformations may occur and may serve to interact with different types of sorting machinery in different locations (Harrison, 1996; Lin *et al.*, 1997). In this situation it is conceivable that a number of substitutions could be tolerated outside of the interacting surface without preventing the correct conformation from occurring. Also, mutations that prevent conformations necessary for one sorting event such as endosomal sorting may not prevent conformations required for other sorting events such as rapid internalization, consistent with the observed differences in sequence requirements for these two activities in P-selectin (Green *et al.*, 1994; Setiadi *et al.*, 1995). Further progress in understanding the structure of cytoplasmic domains under physiological conditions and characterization of endosomal sorting machinery will be required to address these possibilities.

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