Residues Throughout the Cytoplasmic Domain Affect the Internalization Efficiency of P-selectin*

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The cytoplasmic domains of many membrane proteins have short sequences, usually including a tyrosine or a di-leucine, that function as sorting signals. P-selectin is an adhesion receptor for leukocytes that is expressed on activated platelets and endothelial cells. Its 35-residue cytoplasmic domain contains signals for sorting into regulated secretory granules, for endocytosis, and for movement from endosomes to lysosomes. The domain has a membrane-distal sequence, YGVFTNAAF, that resembles some tyrosine-based signals. We studied the effects of deletions and mutations in the cytoplasmic tail of human P-selectin on its internalization in clathrin-coated pits of transfected Chinese hamster ovary cells. Mutations and deletions in the putative tyrosine-based motif did not clearly implicate these residues as critical components of a short internalization signal. Indeed, a construct containing a truncated 18-residue cytoplasmic domain with a single substitution (K761A/H773Stop) was internalized nearly three times as fast as wild-type P-selectin; this construct contained no di-leucine, tyrosine, or other known sorting motif. Substitution of residues throughout the cytoplasmic domain affected the internalization rate of P-selectin. Furthermore, the cytoplasmic domain of P-selectin mediated faster internalization when attached to the extracellular and transmembrane domains of the low density lipoprotein receptor than when attached to the corresponding domains of P-selectin. Thus, we were unable to identify a short internalization signal in the cytoplasmic tail of P-selectin. Residues throughout the cytoplasmic domain, and perhaps the transmembrane sequence to which the domain is attached, affect the efficiency of internalization.

The cellular trafficking of many membrane proteins appears to be directed by short sorting signals in the cytoplasmic domains (reviewed in Refs. 1 and 2). The most studied signal has an aromatic residue, usually a tyrosine, within a sequence of four to six amino acids (3–6). The tyrosine is often separated from a hydrophobic residue by two other amino acids (7, 8). Tyrosine-based signals mediate endocytosis of membrane proteins through clathrin-coated pits (1, 2) and direct sorting of membrane proteins to a variety of intracellular compartments (9–16). "Di-leucine" motifs are a second group of signals that mediate the internalization and sorting of membrane proteins (17–21). Some tyrosine-based signals form a β turn (5, 22–24), whereas the structures of di-leucine motifs are unknown. Tyrosine- or di-leucine-based signals may also function when inserted into the cytoplasmic domains of other membrane proteins (1, 2). Cytoplasmic domains with tyrosine-based signals bind to adaptin proteins of clathrin coats of the plasma membrane or trans-Golgi network (25–28). However, direct binding of tyrosine- or di-leucine-based motifs to adaptins has not been demonstrated.

Membrane proteins of the endoplasmic reticulum have retrieval signals with the consensus sequence KXX, located at the extreme C termini of their cytoplasmic domains (29). These signals interact with coatomers (30), which were originally described on transport vesicles of the Golgi complex (31). One C-terminal sequence, KKFF, has been shown to mediate internalization of membrane proteins through clathrin-coated pits (32, 33). These findings suggest that coatomer-mediated retrieval of membrane proteins is mechanistically related to clathrin-dependent sorting. The data also suggest that diverse sequences may create related sorting signals.

The selectins are a family of three type I membrane glycoproteins that initiate leukocyte adhesion to the vessel wall by interacting with cell-surface carbohydrates (reviewed in Ref. 34). The surface expression of the selectins is tightly regulated, a means to control the extent of leukocyte recruitment into sites of inflammation. P-selectin is located in the membrane of secretory granules of platelets and endothelial cells (35–38). Upon stimulation of the cells with thrombin or other agonists, P-selectin is redistributed to the plasma membrane, where it mediates adhesion of leukocytes (39–41). The protein is rapidly internalized from the surface of activated endothelial cells (42). Some of the internalized P-selectin molecules return to the trans-Golgi network, where they are sorted into secretory granules (43). When expressed in CHO cells, which lack regulated secretion, P-selectin is constitutively delivered to the cell surface and then internalized (44–46). P-selectin is also efficiently targeted from endosomes to lysosomes for degradation; this mechanism limits recycling of internalized P-selectin to the plasma membrane (46).

The 35-residue cytoplasmic tail of human P-selectin contains

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1 The abbreviations used are: CHO, Chinese hamster ovary; BSA, bovine serum albumin; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; LDL, low density lipoprotein; mAb, monoclonal antibody; NHS, N-hydroxysuccinimide; TBS, Tris-buffered saline; MESNA, 2-mercaptoethanesulfonate; ELISA, enzyme-linked immunosorbent assay.
P-selectin Cytoplasmic Domain

The conservation of amino acid sequences in the cytoplasmic domains of P-selectin from different species. The number for amino acid positions correspond to the human sequence. Residues in other species that are identical to the human counterpart are marked with a dash. The cytoplasmic domain from each species has 35 amino acids. The sequence data are from the following references: human (47), murine (48), rat (49), and bovine (50). The ovine sequence has been deposited in GenBank™ with the accession number L34270 (S. A. Burns, E. J. Neufeld, and J. J. Donady, unpublished data). The canine sequence has been deposited in GenBank™ with the accession number M88170 (A. M. Manning, W. E. Sanders, Jr., G. L. Kukielka, M. Dore, C. L. Rosenblum, H. L. Hawkins, L. H. Michael, M. L. Entman, C. W. Smith, A. L. Beaudet, and D. C. Anderson, unpublished data).

Fig. 1. Conservation of amino acid sequences in the cytoplasmic domains of P-selectin from different species. The numbers for amino acid positions correspond to the human sequence. Residues in other species that are identical to the human counterpart are marked with a dash. The cytoplasmic domain from each species has 35 amino acids. The sequence data are from the following references: human (47), murine (48), rat (49), and bovine (50). The ovine sequence has been deposited in GenBank™ with the accession number L34270 (S. A. Burns, E. J. Neufeld, and J. J. Donady, unpublished data). The canine sequence has been deposited in GenBank™ with the accession number M88170 (A. M. Manning, W. E. Sanders, Jr., G. L. Kukielka, M. Dore, C. L. Rosenblum, H. L. Hawkins, L. H. Michael, M. L. Entman, C. W. Smith, A. L. Beaudet, and D. C. Anderson, unpublished data).

The following monoclonal antibodies (mAbs) were used: S12, M88170 (A. M. Manning, W. E. Sanders, Jr., G. L. Kukielka, M. Dore, C. L. Rosenblum, H. L. Hawkins, L. H. Michael, M. L. Entman, C. W. Smith, A. L. Beaudet, and D. C. Anderson, unpublished data).

Internalization Assay by Removal of Surface-bound Antibody—The assay exploited the ability of avidic buffers to remove mAb G1 bound to P-selectin on the surface of transfected CHO cells. The assay was modified from that used to measure rapid internalization of the mannose-phosphate insulin-like growth factor II receptor (8). In preliminary experiments, saturating concentrations (1.5 μg/ml) of 125I-G1 or 125I-S12 were incubated for 30 min on ice with confluent CHO cells transfected with a tail-less form of P-selectin that is not internalized. After removing the unbound antibody, the cells were exposed to buffers of varying pH for 3–10 min at 37°C and then bound antibody was measured in a gamma scintillation counter (see below). It was found that incubation with 0.1 mM acetate buffer containing 0.15 M NaCl, pH 4, for as little as 3 min removed greater than 90% of the G1 specifically bound at pH 7.4. In contrast, the pH 4 buffer removed less than 20% of mAb S12 bound at pH 7.4 after 10 min; buffers at pH 4.5 or higher removed essentially none of the prebound S12.

In the assay, confluent CHO cells in 12-well plates were placed on ice for 10 min and washed four times with prewarmed medium (minimum Eagle’s medium, 1% FBS). The wells were filled with 500 μl of medium containing 10 μg/ml biotinylated mAb S12 and incubated at 37°C for 30 min. After washing the cells three times with 1 ml of prewarmed medium, the cells were cultured until confluent, transferred to a 100-mm Petri dish, and selected for transfectants with medium containing 400 μg/ml (active concentration) of G418 (Geneticin, Life Technologies, Inc.). After 2 weeks, colonies were isolated, expanded, and screened for P-selectin expression by binding of 125I-S12.

The following monoclonal antibodies—mAbs S12 and G1 were labeled with Na125I using the IODO-GEN reagent (Pierce) as described previously (44).

Internalization Assay by Removal of Surface-bound Antibody—The assay exploited the ability of avidic buffers to remove mAb G1 bound to P-selectin on the surface of transfected CHO cells. The assay was modified from that used to measure rapid internalization of the mannose-phosphate insulin-like growth factor II receptor (8). In preliminary experiments, saturating concentrations (1.5 μg/ml) of 125I-G1 or 125I-S12 were incubated for 30 min on ice with confluent CHO cells transfected with a tail-less form of P-selectin that is not internalized. After removing the unbound antibody, the cells were exposed to buffers of varying pH for 3–10 min at 37°C and then bound antibody was measured in a gamma scintillation counter (see below). It was found that incubation with 0.1 mM acetate buffer containing 0.15 M NaCl, pH 4, for as little as 3 min removed greater than 90% of the G1 specifically bound at pH 7.4. In contrast, the pH 4 buffer removed less than 20% of mAb S12 bound at pH 7.4 after 10 min; buffers at pH 4.5 or higher removed essentially none of the prebound S12.

In the assay, confluent CHO cells in 12-well plates were placed on ice for 10 min and washed four times with prewarmed medium (minimum Eagle’s medium, 1% FBS). The wells were filled with 500 μl of medium containing 10 μg/ml 125I-G1 were added to each well. After 30 min on ice, the cells were washed rapidly four times with ice-cold medium. To each of two wells, 500 μl of prewarmed 0.1 mM acetate, 0.15 M NaCl, pH 4, were added, whereas to each of the other wells 500 μl of prewarmed medium were added. The plates were then transferred immediately to a 37°C water bath where they were floated without their lids. At 1-min intervals, the medium containing the released 125I-G1 was collected from a well and replaced with 500 μl of 0.1 mM acetate buffer, 0.15 M NaCl, pH 4. After an additional 10-min incubation at 37°C, the acetate buffer was removed, and the cells were solubilized with 1 ml of 1% NH4OH.

In each experiment, the initial rate of internalization was determined as the sum of the radioactivity of cell lysate (acid-resistant bound 125I-G1), medium (spontaneous release of bound 125I-G1), and acid eluate (original surface-bound 125I-G1). At each subsequent time, the amount of radioactivity released or internalized was plotted as the percentage of the total radioactivity bound to the surface at time 0. The amount remaining on the cell surface was obtained by subtracting the sum of the released and internalized radioactivity from the initial surface-bound radioactivity. Spontaneous release of bound 125I-G1 was less than 10% of the total radioactivity. In each analysis, the initial rate of internalization was calculated by measuring the maximal slope of the uptake curve. This usually followed a short lag of 15–30 s. In some cases the lag period extended up to 2 min, and the lag period in the N782A mutant was 4–6 min. The wild-type construct was analyzed, the wild-type form was also included in the experiment. The internalization rate of each mutant was examined in at least two different clonal lines. At least two separate experiments were performed with each clone, except for the C766A/H773Stop mutant where one clone was examined four times.

Internalization Assay by Removal of Surface-bound Biotin—Cells in 12-well plates were placed on ice and washed four times with 1 ml of chilled HBSS. Aliquots of 200 mg/ml sulfosuccinimidyl (Pierce), stored in MeSO at 20°C, were thawed immediately prior to use and diluted in chilled HBSS to a final concentration of 0.5 mg/ml (53, 54). The cells were incubated with 0.5 ml of the biotin suspension on ice for 20 min, washed three times with a fresh ice-cold suspension, and then washed twice more with a fresh ice-cold suspension, and then washed twice more with a fresh ice-cold suspension. After washing, the cells were incubated with 0.5 ml of the biotin suspension on ice for 20 min. One set of cells was kept on ice. Warmed HBSS containing 1% globulin-free BSA was added to the other cells, which were then transferred to a 37°C water bath. At various times, the latter cells were returned to ice and washed once with 1 ml of chilled HBSS.

For each time point, half of the cells were treated with 10 mM sodium 2-morpholinosydnonimine (SNSNA) (Sigma) in 1 ml of 0.1 mM NaCl, pH 8.5. The other half was treated with the Tris buffer without MESNA. The solutions were incubated with the cells for 30 min on ice. The MESNA solutions were replaced with fresh solution at 10-min intervals. After three washes with 1 ml of 5 mg/ml iodoacetamide in HBSS, the cells were lysed with 250 μl/plex of 1% Triton X-100 in 100 mM sodium phosphate, pH 8, containing 0.3 unit/ml aprotinin, 1 μM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, and 5 mM iodoaceticamide. Following centrifugation of each lysate at 10,000 × g, the supernatant was transferred to a new microcentrifuge tube and stored.
at 4 °C until analysis.

The amount of internalized biotinylated P-selectin protected from MESNA cleavage was quantitated by an ELISA consisting of a preincubation step and a detection step. In the preincubation step, cell lysates were added to microtiter wells coated with T10, a mAAb directed to the platelet αIIbβ3. In the detection step, the preincubated lysates were transferred to microtiter wells coated with αIIbβ3 which was then biotinylated with biotin-avidin alkaline phosphatase and a 50 μM solution of NADPH were added to each well for 15 min at room temperature. The absorbence of the product was read at 495 nm on a fluorometric microplate reader interfaced to Softmax software (Molecular Devices, Palo Alto, CA).

To establish the linear range of detection for the ELISA, serial dilutions of cell lysate were assayed by biotinylated cells that were not incubated at 37 °C or treated with MESNA. Comparable dilutions were used for the experimental wells; absorbance values within the linear range of the assay were then corrected for the dilution factor. The amounts of cell lysate were subtracted to obtain the specific P-selectin-dependent signals. The specificity of the assay was further determined by demonstrating that preincubation of the detection wells with 2 μM NADPH completely eliminated the specific signal. At each time point and all antibody concentrations, the absorbance values of the control T10-detection wells were lower than 10% of the corresponding samples in the S12, G1, and W40-coated wells and were subtracted to obtain the specific P-selectin-dependent signals. The absorbence values of the control T10-detection wells were lower than 10% of the corresponding samples in the S12, G1, and W40-coated wells and were subtracted to obtain the specific P-selectin-dependent signals.

Effect of Hypertonic Medium on Endocytosis of P-selectin—Hypertonic medium containing sucrose was used to block clathrin-mediated endocytosis as described previously (58, 59). The addition of 0.45 M sucrose to a minimum Eagle's medium, 1% FBS increased its osmolality from 290 to 770 mOsm/kg as determined by an osmometer (OSMETTE A, Precision System, Inc., Natick, MA). Confluent cells in 24-well plates were pretreated with α-minimal Eagle's medium, 1% FBS in the presence or absence of 0.45 M sucrose at 37 °C for 15 min; they were then washed with 0.3% (w/v) Pronase in Ringer's solution; the other half was treated with Ringer's solution without Pronase. After 1 h on ice, the solutions were removed, and their radioactivity was counted in a gamma scintillation counter.

RESULTS

Measurement of Internalization of P-selectin in CHO Cells—The cytoplasmic domain of human P-selectin contains 35 residues encoded by three exons (60) (Fig. 2). The first seven amino acids, encoded by the distal part of exon 14, include a putative stop-transfer (ST) signal. The next 11 residues (C1) are encoded by exon 15 and the final 17 residues (C2) are encoded by exon 16. In previous studies, Asp-762 was incorrectly listed as part of the ST region rather than the C1 segment, although constructs were prepared according to the correct intron-exon boundaries (60). Thus, a previous "tail-less" construct with a stop codon introduced after the ST region ended at Lys-761 (60) (Fig. 2). The first seven amino acids, encoded by three exons (60) (Fig. 2). The first seven amino acids, encoded by three exons (60) (Fig. 2). The first seven amino acids, encoded by three exons (60) (Fig. 2). The first seven amino acids, encoded by three exons (60) (Fig. 2). The first seven amino acids, encoded by three exons (60) (Fig. 2). The first seven amino acids, encoded by three exons (60) (Fig. 2). The first seven amino acids, encoded by three exons (60) (Fig. 2). The first seven amino acids, encoded by three exons (60) (Fig. 2). The first seven amino acids, encoded by three exons (60) (Fig. 2).

Effect of Cytoplasmic Acidification on Internalization of P-selectin—The effect of cytoplasmic acidification on internalization of P-selectin and selected constructs was studied by modification of previously described methods (56–58). Confluent cells in 24-well plates were washed three times with 500 μl of Ringer's solution (103 mM NaCl, 5.6 mM Na2HPO4, 5.36 mM KCl, 1.5 mM CaCl2, pH 7.4) containing 1% FBS, alone or in the presence of 10 mM acetic acid which lowered the pH to 5.2. The plates were incubated at 37 °C for 5 min and then put on ice. The ability of acetic acid to induce cytoplasmic acidification was confirmed by addition to the cells of 50 μM membrane-permeant pH probe, 50 μM NADPH were added to each well for 15 min at room temperature. Then, 50 μM of reconstituted amplifier solution containing alcohol dehydrogenase and diaphorase were added. After 15 min at room temperature, the reaction was stopped by the addition of 50 μl of 0.3 M H2SO4.

In comparing the internalization rate of wild-type P-selectin with the LDL receptor and the chimeric LLP protein, the biotinylation assay was performed as described previously (46).

To study the endocytosis of P-selectin independently of its sorting into secretory granules, we used CHO cells, which lack the regulated secretory pathway. In transfected CHO cells, wild-type P-selectin is primarily distributed on the plasma membrane and in endosomes (44, 46). We expressed in these cells a series of constructs with various mutations and deletions in the cytoplasmic domain of P-selectin (Fig. 2). All constructs contained the extracellular and transmembrane domains of P-selectin. To quantitate the rate of internalization of P-selectin in CHO cells, we modified a method used to study rapid internalization of the mannos-6-phosphate/insulin-like growth factor-II receptor (8). Cells were incubated at 4 °C with G1, a mAAb to P-selectin that is rapidly dissociated from its ligand when exposed to acidic buffers. Following a wash, the cells were quickly warmed to 37 °C to initiate internalization of P-selectin with its bound antibody. At 1-min intervals, the medium was collected to measure spontaneously released antibody, and the cells were incubated with acetate buffer at pH 4 to remove surface-bound antibody. The cells were then lysed to measure the radioactivity of internalized G1 that was protected from the acid-dissociation procedure.

As measured by this assay, wild-type P-selectin was rapidly internalized, reaching a plateau 10 min after warming the cells...
less construct remained primarily on the cell surface. Thus, after warming the cells to 37°C (Fig. 4). In contrast, the tail-biotinylated P-selectin. As measured by this assay, wild-type that was resistant to MESNA, relative to the initial surface-calculation the percentage of internalized biotinylated P-selectin incubated with or without MESNA. An ELISA was used to following warming to 37°C, the cells were returned to ice and permeant reducing agent, MESNA (53, 54, 63). At intervals in which cells surface proteins were modified at 4°C with sulfo-NHS-ss-biotin, which can be cleaved with the membrane-impermeant reducing agent, MESNA (53, 54, 63). At intervals following warming to 37°C, the cells were returned to ice and incubated with or without MESNA. An ELISA was used to calculate the percentage of internalized biotinylated P-selectin that was resistant to MESNA, relative to the initial surface-biotinylated P-selectin. As measured by this assay, wild-type P-selectin was also rapidly internalized during the first 10 min after warming the cells to 37°C (Fig. 4). In contrast, the tail-less construct remained primarily on the cell surface. Thus, binding of antibody did not induce the rapid internalization of P-selectin. We routinely used the antibody-binding assay because it was simpler to perform. The internalization rate was determined by measuring the slope of the initial linear portion of the curve, as described under “Experimental Procedures.”

Contributions of the C1 and C2 Regions to Internalization—We initially focused on potential internalization signals in the C2 region, because it contains three aromatic residues within the sequence, YGVFNTAASF. The sequence NAAF has weak similarity to the NPXY internalization signal that was originally described in the receptor for low density lipoprotein (3, 64). The sequence YGVF has an aromatic residue (Tyr) separated by two amino acids from a hydrophobic residue (Phe), a characteristic of many tyrosine-based internalization signals (1, 2, 8).

We first tested a construct with an internal deletion of C1, ΔD762-S772, which fused the C2 region directly to the ST segment. This construct was internalized at ~90% of the rate of wild-type P-selectin (Fig. 5A). However, a construct in which a stop codon was substituted for Asn-782 in ΔD762-S772 was endocytosed at only 30% the rate of wild-type. These data indicate that, in the absence of C1, the C2 domain mediates internalization, and both halves of C2 are required for its optimal function.

To examine the role of the C2 region in the context of the entire cytoplasmic domain, we measured the endocytosis of a series of constructs with C-terminal deletions of increasing size. Unexpectedly, deletions of C2, including all three aromatic residues, had little or no effect on the internalization rate (Fig. 5B). A construct lacking the entire C2 region, H773Stop, was still internalized at ~50% the rate of wild-type P-selectin. Deletion of an additional six residues (P767Stop) eliminated internalization. Thus, in the absence of C2, the C1 domain also mediates internalization, and both halves of C1 are required for its optimal function. The collective results indicate that either C1 or C2 is capable of mediating endocytosis when fused to the ST segment. However, the data do not establish whether C1 and C2 contribute independent internalization signals within the intact cytoplasmic domain. It should be noted that the sum of the internalization rates of the C1 (H773Stop) and C2 (ΔD762-S772) constructs is higher than that of wild-type P-selectin.

Contributions of Individual Residues to Internalization of Constructs Lacking the C2 Region—The H773Stop construct, which has only the ST and C1 domains, lacks the tyrosine-, di-leucine-, and KKFF-based motifs that have been described as internalization signals. The only aromatic residue is Phe-758, which is not in a sequence that resembles known short aromatic residue-based signals (1, 2). To examine which residues might contribute to internalization of the H773Stop construct, we prepared a series of constructs in which each residue from Phe-758 to Ser-772 was replaced by alanine (Fig. 6). Mutations of Asp-762, Asp-763, Pro-767, Leu-768, and Pro-770 significantly decreased internalization. The other mutations had no effect on internalization or even increased the internalization rate. Remarkably, the K761A/H773Stop mutant was internalized nearly 30% faster than wild-type P-selectin containing the entire cytoplasmic domain. These data do not provide clear rules as to the sequence requirements for internalization of H773Stop, but do indicate that very rapid internalization is mediated by a sequence that has none of the previously described short motifs.

Contributions of Individual Residues to Internalization of Constructs Containing the Entire Cytoplasmic Domain—Because mutation of Asp-762, Asp-763, Pro-767, Leu-768, and Pro-770 to alanine each significantly reduced the internaliza-

**Fig. 2. Amino acid sequences of the cytoplasmic domains of human P-selectin constructs.** The 35 residues of the wild-type cytoplasmic domain have been assigned to the ST, C1, and C2 domains according to exon-intron boundaries. The name of each construct is listed at the left of its schematic diagram. Internal deletions of the indicated residues are preceded by a Δ. The nomenclature for point mutations is as follows: S788Stop means that the codon for Ser-788 is replaced by a stop codon; Y777A means that the codon for Tyr-777 is replaced by alanine.

| Construct | ST | L-selectin | RKLKKG |
|-----------|---|------------|--------|
| Wild-type | | | |
| Tail-less | | | |
| ΔD762-S772 | | | |
| S788Stop | | | |
| D767A | | | |
| A783Stop | | | |
| N782Stop | | | |
| T781Stop | | | |
| Y779Stop | | | |
| Y777Stop | | | |
| G775Stop | | | |
| H773Stop | | | |
| P767Stop | | | |
| P758A/H773Stop | A | | |
| K759A/H773Stop | A | | |
| Q760A/H773Stop | A | | |
| Y761A/H773Stop | A | | |
| D762A/H773Stop | A | | |
| D763A/H773Stop | A | | |
| D764A/H773Stop | A | | |
| K765A/H773Stop | A | | |
| C766A/H773Stop | A | | |
| P767A/H773Stop | A | | |
| L768A/H773Stop | A | | |
| N769A/H773Stop | A | | |
| P770A/H773Stop | A | | |
| K771A/H773Stop | A | | |
| Δ772A/H773Stop | A | | |
| D762A | A | | |
| D763A | A | | |
| P767A | A | | |
| L768A | A | | |
| P770A | A | | |
| Y777A | A | | |
| G775A | A | | |
| Y779A | A | | |
| P780A | A | | |
| T781A | A | | |
| N782A | A | | |
| A783G | A | | |
| A784G | A | | |
| P785G | A | | |
tion rate of the H773Stop construct, we next examined the effect of these mutations in the context of the entire cytoplasmic domain (Fig. 7A). Four of the five mutations partially reduced the internalization rate compared to that of wild-type P-selectin. A simple interpretation of this result is that each of these mutations eliminated an internalization signal in C1 but did not affect an independent internalization signal in C2. However, mutation of Asp-762 to alanine in the context of the entire cytoplasmic tail increased, rather than decreased, internalization. The disparity in the effects of this mutation on the wild-type protein relative to the H773Stop construct suggests that interpretations about how specific residues affect internalization of P-selectin must be made cautiously.

We also mutated each residue in C2 from Tyr-777 to Phe-785 within the context of the entire cytoplasmic domain (Fig. 7B). Mutation of Gly-778 to alanine within the putative YGVF motif markedly increased internalization. This result is consistent with previous observations that glycine is poorly tolerated at the second position of four-residue tyrosine-based internalization signals.
tion signals (8). However, mutation of Phe-780 to alanine also increased internalization; this result is not consistent with the requirement for a hydrophobic residue at the fourth position of tyrosine-based signals (8). Furthermore, mutation of Asn-782 or Phe-785 significantly inhibited internalization (Fig. 7B), even though deletion of sequences containing these residues had little or no effect on internalization (Fig. 5B).

The data in Fig. 7 indicate that the effects of mutations of individual residues in the context of the entire cytoplasmic domain are not always consistent with the effects produced by C-terminal deletions or by point mutations in the context of a cytoplasmic domain lacking the C2 region. Overall, the data do not clearly implicate a specific short sequence as the sole internalization signal. However, the results do suggest that many residues in the cytoplasmic domain contribute to optimal internalization of P-selectin. To further address this possibility, we substituted the seven-residue ST sequence of P-selectin with the six membrane-proximal residues of human L-selectin (65). This construct was internalized at less than half the rate of wild-type P-selectin, even though the sequences in the C1 and C2 regions remained intact (Fig. 8).

A Chimera Containing the Cytoplasmic Domain of P-selectin Attached to the Extracytoplasmic and Transmembrane Domains of the LDL Receptor Is Internalized Faster than Wild-type P-selectin—The preceding data suggest that many residues in the cytoplasmic domain contribute to the internalization efficiency of P-selectin. To determine whether the internalization function of the cytoplasmic domain is affected by the sequence to which it is attached, we directly compared the internalization rate of wild-type P-selectin with that of LLP, a chimeric protein in which the cytoplasmic domain of the LDL receptor was replaced with the cytoplasmic domain of P-selectin. Internalization was measured with a biotinylation procedure (46). As previously demonstrated (46), LLP was internalized extremely rapidly, at the same rate as the native LDL receptor (Fig. 9). In contrast, wild-type P-selectin was internalized at less than half the rate of LLP. The internalization rate of P-selectin measured by this biotinylation assay was similar to that obtained by the biotinylation procedure shown in Fig. 4 and by the antibody uptake assay. Thus, the site of membrane attachment affects the ability of the cytoplasmic domain of P-selectin to mediate internalization.

P-selectin Is Internalized in Clathrin-coated Pits—Membrane proteins are usually rapidly internalized through interactions of their cytoplasmic domains with clathrin-coated pits (66). To determine whether P-selectin was also internalized in clathrin-coated pits, we examined the effects of cytoplasmic acidification or addition of hypertonic medium (56–59). These perturbations prevent endocytosis of membrane proteins in clathrin-coated pits through different mechanisms. Acidification of the cytoplasm blocks budding of clathrin-coated vesicles, whereas incubation of cells in hypertonic medium prevents interaction of clathrin with membrane-bound adaptors (58).

The cytoplasm of CHO cells transfected with various P-
selectin constructs was acidified by incubation in medium containing acetic acid, which lowered the pH to 5.2. Because this pH affected the binding of mAb G1, we developed an alternative assay in which mAb S12, which binds efficiently to P-selectin at acidic pH, was incubated with cells and then removed with Pronase. The cumulative percentage of internalized P-selectin was measured over a 10-min period. The results of this assay indicated that cytoplasmic acidification markedly inhibited internalization of wild-type P-selectin, of the constructs containing either the C1 or the C2 domains, and of the constructs with point mutations that significantly accelerated the rate of internalization (Fig. 10A). Using the conventional G1-binding assay, we found that incubation of cells in hypertonic medium also prevented the internalization of all forms of P-selectin (Fig. 10B). These data suggest that P-selectin and the examined P-selectin constructs are internalized in clathrin-coated pits.

**DISCUSSION**

The cytoplasmic domain of P-selectin contains a signal(s) that mediates rapid endocytosis in clathrin-coated pits. Our results indicate that many residues in the cytoplasmic domain affect, directly or indirectly, the internalization of P-selectin. Deletions or substitutions throughout the cytoplasmic domain either increased or decreased the internalization rate. Constructs with either the C1 domain or the C2 domain fused directly to the ST region mediated internalization in clathrin-coated pits. Although the simplest interpretation of this result is that C1 and C2 each contains an independent internalization signal, the conformations of C1 and C2 in the deletion constructs may be very different from those in the intact cytoplasmic tail. Indeed, the sum of the internalization rates of the deletion constructs exceeded that of wild-type P-selectin. Thus, the data do not establish the presence of independent signals in C1 and C2 in the context of the entire cytoplasmic domain.

The construct containing only the ST and C1 domains (H773Stop) has no di-leucine-based motifs (17) or KKXX-type signals (29, 30, 32, 33). It has only one aromatic residue, Phe-758, and substitution of this residue with alanine did not affect the internalization rate. Replacement of Lys-761 with alanine in the H773Stop construct resulted in an internalization rate nearly three times that of wild-type P-selectin, similar to the rapid internalization rates of the transferrin and LDL receptors (1, 2). Therefore, a cytoplasmic sequence lacking any of the described short cytoplasmic signals can mediate very rapid internalization in clathrin-coated pits.

The C2 domain has three aromatic residues within the sequence YGVFTNAAF. The YGVF sequence resembles canonical four-residue tyrosine-based internalization signals where an aromatic residue is separated from a hydrophobic residue by two other amino acids (1, 2, 8). The YGFT sequence mediates rapid internalization when attached to a truncated cytoplasmic domain of the mannose 6-phosphate/insulin-like growth factor-11 receptor (8). In contrast, the function of this motif is less...
clear in the intact cytoplasmic domain of P-selectin, because mutation of Phe-780 to alanine actually increased the internalization rate. The NAAF sequence has slight similarity to the NPXY motif described in the LDL receptor (3, 64). Consistent with the possible importance of this sequence, mutation of either Asn-782 or Phe-785 significantly decreased the internalization rate. In marked contrast, deletion of the NAAF sequence had little effect on internalization. Thus, the mutagenesis studies do not clearly define a short internalization signal based on the aromatic amino acids.

Despite analyzing many constructs, we were unable to assign internalization signals to specific short amino acid sequences in the cytoplasmic domain of P-selectin. Our data contrast with many reports of short tyrosine, di-leucine, or C-terminal KKFF-like sequences that function as signals for internalization and other sorting functions (1, 2, 29, 30, 32, 33). These studies also relied on measurements of internalization or sorting of membrane proteins that were subjected to site-directed mutagenesis. It has been suggested that diverse short linear sequences form a family of related structures that interact with sorting proteins (1, 2, 67). Perhaps the cytoplasmic domain of P-selectin has a short linear signal that is not readily identified because many other residues contribute to its appropriate orientation. Alternatively, an internalization motif might be created by juxtaposition of residues from discontinuous portions of the amino acid sequence. Even putative linear sorting motifs in other proteins require further structural definition. Analysis by nuclear magnetic resonance indicates that some small peptides with tyrosine-based motifs form β turns in aqueous solutions (23, 24). However, one peptide containing a tyrosine-based internalization motif has been shown to form a nascent helix instead of a β turn in solution (68). The structure of an amino acid sequence may differ depending on whether it is studied as an isolated small peptide or as part of a larger peptide corresponding to the entire cytoplasmic domain (68). Furthermore, some linear peptides adopt stable structures only when bound to a membrane or to a macromolecular ligand (69, 70). It should be emphasized that the actual structures of cytoplasmic domains that contact the membrane surface, adaptins, or other sorting molecules have not been identified. Determination of these sites will require analysis of interacting proteins by co-crystallization and x-ray diffraction or by nuclear magnetic resonance.

The structural features in the cytoplasmic domain of P-selectin that mediate sorting to secretory granules and movement from endosomes to lysosomes are also unknown. Although deletion of the C1 region eliminates endosomal sorting with little affect on the internalization rate (46), it is unclear whether C1 presents an endosomal sorting signal in the intact cytoplasmic tail. Mutations in the cytoplasmic domain that differentially affect internalization, sorting to secretory granules, and delivery to lysosomes will indicate that the sorting signals have different structures, but will not clarify the details of the structures.

We found that the cytoplasmic domain of P-selectin mediated more rapid internalization when attached to the extracellular and transmembrane domains of the LDL receptor (the LLP construct) than when attached to the corresponding domains of P-selectin. Since the LDL receptor is not internalized in the absence of its own cytoplasmic domain (64), the faster internalization of LLP relative to wild-type P-selectin may be due to a different presentation of the P-selectin cytoplasmic tail. The LDL receptor forms oligomers in the membrane, but only in the presence of its own cytoplasmic domain; there is no clear correlation of oligomerization with the rate of internalization (71). The transmembrane domain of P-selectin promotes its oligomerization in solution, even in nonionic detergents above the critical micellar concentration (72). It is not known whether P-selectin also oligomerizes in the cell membrane and, if so, whether the oligomers affects the orientation and sorting functions of the cytoplasmic domain. LLP and P-selectin have similar half-lives in CHO cells (46), but the assay used for these measurements might not distinguish minor differences in endosomal sorting.

We conclude that site-directed mutagenesis does not always identify a short sorting signal in the cytoplasmic domain of a membrane protein. Our data indicate that many residues in the cytoplasmic domain of P-selectin contribute to internalization. The highly conserved sequence of the cytoplasmic domain of P-selectin may be required to create a structure that mediates not only internalization, but also sorting into regulated secretory granules and rapid movement from endosomes to lysosomes.

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