Sorting Nexin 17 Accelerates Internalization Yet Retards Degradation of P-selectin

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Submitted February 23, 2004; Revised April 6, 2004; Accepted April 7, 2004
Monitoring Editor: Juan S. Bonifacino

The transient appearance of P-selectin on the surface of endothelial cells helps recruit leukocytes into sites of inflammation. The tight control of cell surface P-selectin on these cells depends on regulated exocytosis of Weibel-Palade bodies where the protein is stored and on its rapid endocytosis. After endocytosis, P-selectin is either sorted via endosomes and the Golgi apparatus for storage in Weibel-Palade bodies or targeted to lysosomes for degradation. A potential player in this complex endocytic itinerary is SNX17, a member of the sorting nexin family, which has been shown in a yeast two-hybrid assay to bind P-selectin. Here, we show that overexpression of SNX17 in mammalian cells can influence two key steps in the endocytic trafficking of P-selectin. First, it promotes the endocytosis of P-selectin from the plasma membrane. Second, it inhibits the movement of P-selectin into lysosomes, thereby reducing its degradation.

INTRODUCTION

To effectively interact with their environment, cells regulate the numbers of receptors at their surface. The leukocyte receptor P-selectin is an extreme example of this because it is only present at the plasma membrane of endothelial cells for a few minutes early in the inflammatory response (Hattori *et al.*, 1989; McEver *et al.*, 1989). Uncontrolled surface appearance of P-selectin would lead to chronic leukocyte recruitment, but its rapid internalization (Blagoveshchenskaya *et al.*, 1998a; Setiadi *et al.*, 1995) and subsequent endocytic trafficking (Subramaniam *et al.*, 1993; Arribas and Cutler, 2000; Straley and Green, 2000) is such as to preclude its uncontrolled return to the plasma membrane.

Recently, a new family of proteins likely to play a major role in regulating endocytic membrane traffic have emerged: the sorting nexins (SNXs; Worby and Dixon, 2002). A role for SNXs in endocytic trafficking in eukaryotic cells is being established by demonstrating that these proteins are located on endosomes, that their overexpression can modulate cell surface receptor trafficking, and that they can bind a number of receptors in a variety of assays. For example, SNX1 affects delivery of EGF recep-

tor and protease activated receptor-1 to lysosomes (Kurten *et al.*, 1996; Wang *et al.*, 2002); SNX3 overexpression inhibits EGF receptor transport to the lysosome, while inhibiting it prevents transferrin receptor (TfnR) recycling (Xu *et al.*, 2001b); SNX15 has been implicated in trafficking between endosomes and the TGN because its overexpression results in furin mislocalization and a delayed processing of several furin substrates (Barr *et al.*, 2000).

In a yeast two-hybrid screen Florian *et al.* (2001) found that SNX17 binds to P-selectin. SNX17 is unusual because as well as the family-defining PX (Phox-homology) domain, it also contains a truncated FERM (Four.1 protein, Ezrin, Radixin, Moesin) domain, which is found in proteins that act as linkers connecting cell surface transmembrane proteins to the actin cytoskeleton (Chishti *et al.*, 1998). SNX17 is located on an early endosomal compartment, and it binds the LDL receptor and related molecules (Stockinger *et al.*, 2002) as well as P-selectin. It has also been shown that the delivery of LDL to degradative compartments is increased by overexpressing SNX17, possibly arising from increased internalization and recycling rates, and paralleling the effect of SNX1 on the EGF receptor (Kurten *et al.*, 1996).

At steady state P-selectin is stored in secretory organelles within platelets and endothelial cells from where it transiently appears at the plasma membrane after secretagogue action (Stenberg *et al.*, 1985; Hattori *et al.*, 1989), whereas SNX17 is on endosomes. We have therefore determined the physiological significance of an interaction between P-selectin and SNX17. We find that overexpression of SNX17 can cause an acceleration of the internalization of P-selectin plus a diminution in degradation of HRP-P-selectin chimeras. We find P-selectin accumulating within an SNX17-positive endosomal compartment through which P-selectin travels after internalization from the plasma membrane.

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E04-02-0143. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E04-02-0143.

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Abbreviations used: AK6, anti-P-selectin antibody; EEA1, early endosome antigen; FERM, four.1 protein, ezrin, radixin, moesin; LAMP-1, lysosome-associated membrane protein; LBPA, lyso-bisphosphatidic acid; LDL, low-density lipoprotein; M6PR, mannose-6-phosphate receptor; PI(3)P, phosphatidylinositol 3-phosphate; PX, phox homology; SNX, sorting nexin; TfnR, transferrin receptor; WPB, Weibel Palade body

MATERIALS AND METHODS

Antibodies

Mouse monoclonal antibodies used were from AMS Biotechnology (Oxon, UK) or (PE-labeled) from DAKO diagnostica (Hamburg, Germany). Mouse mAb against CD63 (clone IB5) was a kind gift of Prof Mark Marsh (MRC: LMCB, UCL, London, UK). Rabbit polyclonal antibody against LAMP1 was a kind gift of Prof. Colin Hopkins (Imperial College, London, UK). Mouse mAb against transferrin receptor (H68.4) was obtained from Zymed Laboratories (San Francisco, CA). EEA1 antibody was purchased from Transduction Laboratories (Lexington, KY) and the monoclonal anti-His from Roche (Basel, Switzerland). Monoclonal antilyso-bisphosphatidic acid (LBPA) antibody was a kind gift by Jean Gruenberg (Geneva, Switzerland). Mouse mAb against TGN46 was a kind gift of Vas Ponnambalam (Leeds, UK). Rabbit polyclonal antibody against M6PR was a kind gift of Bernard Hoflack (Technical University of Dresden, Germany). Secondary antibodies conjugated with Texas Red were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Constructs

ssHRP^{P-selectin} and ssHRP^{P-selectin763} (chimera with a deletion of the cytoplasmic C1 and C2 domains) were described previously (Norcott *et al.*, 1996; Blagoveshchenskaya *et al.*, 1998b). The generation of full-length SNX17 cDNA and the SNX17-GFP expression plasmid pK64-GFP were described previously (Florian *et al.*, 2001). To generate an His-tagged SNX17 construct (pSNX17-HH) an SNX17 cDNA fragment obtained by *NdeI/BamHI* digestion of pK64-GFP was ligated into pcDNA3.1-HisA. pDHH-SNX17*15.03 was generated by inserting the SNX17 fragment 15.03 (missing the first 116 amino acids of full-length SNX17; Florian *et al.*, 2001) via *EcoRI/XhoI* into pcDNA3.1-HisA. The tetracycline controlled SNX17-GFP variant was made as follows: Into the vector pTRE (Clontech, Palo Alto, CA), cut with *PvuII/HindIII*, was ligated a blasticidin resistance gene (*Eco32I/HindIII*) from the vector pEF-bsd (Invitrogen, Paisley, UK) to yield pTRE-bsd. Then SNX17-GFP was cloned via *NheI*(blunt)/*EcoRI* from pK64-GFP into the pTRE-bsd vector (*Cfr42I*(blunt)/*EcoRI*) to achieve pTRE-bsd-SNX17-GFP. The vector pTet-On was bought from Clontech. SNX1 was gained by PCR and ligated via *EcoRI/XhoI* into pcDNA3.1-HisA to achieve pDHH-SNX1. A full-length P-selectin construct was generated as follows. The EGFP *Cfr42I* and *HinI* fragment from pEGFP-endo (Clontech) was ligated into pCR-Script (Stratagene, La Jolla, CA), containing a primed ER-signal, HA-tag, and factor Xa cleavage site (EHX). This EHX-GFP-fusion was cut out with *EcoRI* and *Bsp1407I* and ligated into pCR-Script containing primed transmembrane domain (TM) and the cytosolic tail of P-selectin. The resultant fusion was then inserted via *EcoRI* and *BamHI* sites into pTet-On, from which the tet-responsive transcription activator (rtTA) had been removed (as a *EcoRI* and *BamHI* fragment). The resulting construct (pPGEx) was digested with *EcoRI/Eco47III* to remove the EHX to TM fragment and primed P-selectin (ER signal to TM) was ligated in its place to yield a full-length P-selectin construct (pPEX).

Tissue Culture

Human umbilical vein endothelial cells (HUVECs) were obtained from TCS-Cellworks (Bucks, UK). The cells were grown in M199 (Invitrogen) supplemented with 20% fetal calf serum (Hyclone, Logan, UT), 10 U/ml heparin (Sigma, St. Louis, MO), 50 µg/ml gentamicin (Invitrogen), and 30 mg/ml endothelial cell growth supplement (Sigma) under 5% CO₂ at 37°C. They were seeded on 1% gelatin coated plates or coverslips and used at passages 2–5. HEK-293 cells were obtained from Clontech and cultured in alpha-MEM, 10% FCS, and 50 µg/ml gentamicin. CHO cells were maintained as monolayers in DME supplemented with 10% fetal bovine serum (PAA Laboratories, Karlsruhe, Germany), 2 mM glutamine, and 4 µg/ml ciprofloxacin (Bayer AG, Leverkusen, Germany) under 6.5% CO₂ at 37°C.

Transfection of Cells

Constructs (0.5–5 µg) were expressed in HUVEC and HEK-293 cells (0.5–2 × 10⁶ cells) using nucleofector technology (Amaxa GmbH, Köln, Germany) following the manufacturers instructions for each of the cell types. CHO cells were transfected by electroporation as described previously (van den Hoff *et al.*, 1992).

Immunofluorescence Staining

Cells were fixed in 4% paraformaldehyde in PBS for 15 min, quenched, and permeabilized in a solution of 50 mM ammonium chloride and 0.2% saponin in PBS for 15 min, incubated with primary antibody in PBS supplemented with 1% gelatin and 0.02% saponin for 1 h, and then incubated with labeled secondary antibody for 40 min. Confocal images were obtained using a MRC 1024 laser scanner (Bio-Rad, Hercules, CA) attached to an Optiphot 2 microscope (Nikon, Garden City, NY). Images were collated using Adobe Photoshop (Adobe Systems, Mountain View, CA).

P-selectin Antibody Uptake

Cells seeded on coverslips were washed and incubated in M199 medium containing 2 mg/ml BSA and 10 mM HEPES, pH 7.4 (release medium, RM), for 30 min at 37°C. Cells were stimulated with histamine (10^{−4} M) in RM for 15 min at 37°C, in the presence of the anti-P-selectin antibody (10 µg/ml). The cells were washed three times with RM, incubated in RM for 30 min at 37°C, fixed, and incubated with the secondary antibody.

Wortmannin Treatment

CHO grown on glass cover slips were nucleofected with SNX17-GFP (10 µg DNA/10⁶ cells). Twenty-four hours later they were incubated with 50 nM wortmannin (Merck Biosciences GmbH, Schwalbach, Germany) for 15 min. Cells were then washed, fixed and examined by epifluorescence microscopy.

HRP Clipping Assay

Lysosomal targeting was evaluated using an HRP-clipping assay involving Triton X-114 partitioning. Cells on 60-mm dishes were placed on ice, washed twice with ice-cold PBS, and lysed in 1 ml of 1% Triton X-114 (from a precondensed stock; see Bordier, 1981) in PBS containing 20 mM EDTA and protease inhibitors (1:500 dilution, Sigma) for 30 min. Lysates were centrifuged for 5 min at 13,000 × g at 4°C to remove detergent-insoluble material. Phase separation was carried out by heating supernatants mixed at 900 rpm at 37°C for 3 min followed by centrifugation for 1 min at 13,000 × g at room temperature. The upper, aqueous phase was transferred to a tube containing 0.1 ml of 10% Triton X-114, and 0.9 ml of PBS was added to the lower detergent phase. Tubes were incubated on ice for 15 min. The partitioning was repeated and the HRP activity in the final detergent and aqueous phases was determined in triplicate by a kinetic OD assay as described previously (Norcott *et al.*, 1996). For analyses with HUVECs 100-mm dishes of cells were used. Data were expressed as the percentage of the total lysate HRP activity in the soluble phase (clipped chimera) and represents the mean of the results from six separate nucleofections.

Pre- and Postlysis Binding Experiments

Prelysis Binding. HEK-293 cells were nucleofected with ssHRP^{P-selectin} or ssHRP^{P-selectin763} in combination with a control plasmid, pSNX17-HH or pDHH-SNX1. 24 h after nucleofection cells were washed in ice-cold PBS and lysed in 50 mM Tris, 500 mM NaCl, and 1% Triton X-100 with protease inhibitors. Lysates were passed twice through a 25-gauge needle and centrifuged at 12,000 rpm for 10 min at 4°C, and supernatants were incubated on a rotator for 1 h at 4°C with 100 µl of probond slurry (Invitrogen; equilibrated in lysis buffer). Beads were collected by centrifugation for 2 min at 1000 rpm and washed five times with 50 mM Tris, 500 mM NaCl, 0.2% Triton X-100, and 30 mM imidazole. Bound material was released by incubating beads with 200 µl of 50 mM Tris, 500 mM NaCl, and 0.2% Triton X-100 containing 300 mM imidazole for 30 min at 4°C. The HRP in the elutant was determined using an OPD assay as previously described (Norcott *et al.*, 1996).

Postlysis Binding. HEK-293 cells were subject to single plasmid nucleofections with ssHRP^{P-selectin}, ssHRP^{P-selectin763}, pSNX17-HH, or pDHH-SNX1. His-tagged sorting nexins were isolated from HEK cells as previously stated. Beads prebound with either His-tagged SNX17 or His-tagged SNX1 were then dipped into HEK lysates containing ssHRP^{P-selectin} or ssHRP^{P-selectin763} for 1 h at 4°C. Elutants were subjected to SDS-PAGE separation on 8% gels and analyzed by Western blotting using a monoclonal anti-His antibody. Bands were visualized using a HRP-conjugated anti-mouse secondary antibody followed by ECL detection.

Flow Cytometry

HEK-293 cells were simultaneously nucleofected with the P-selectin-expressing vector pPEX, pTRE-bsd-SNX17-GFP, and pTet-On (1 µg of each DNA/10⁶ cells). Twelve hours posttransfection SNX17-GFP expression was induced by the addition of doxycycline (0.1–20 µg/ml) for another 12 h. Cells were then detached from culture dishes in Ca²⁺- and Mg²⁺-free HBSS, and the resulting single cell suspension was adjusted to a final cell density of ~10,000 cells/µl. Cells were incubated for 30 min with 10 µg/ml mouse anti-P-selectin antibody on ice. Cells were warmed to 37°C to allow internalization to proceed for differing periods of time and the remaining plasma membrane P-selectin was stained with 2 µg/ml Alexa 647-coupled secondary antibodies (Molecular Probes, Eugene, OR). Flow cytometrical analysis of forward scatter light, sideward scatter light, green fluorescence (FL1), and deep red fluorescence (FL4) was performed on a Becton and Dickinson (Mountain View, CA) FacsCalibur cytometer. Data were recorded with CellQuest Pro software (Becton and Dickinson) and evaluated off-line with WinMDI 2.8 software (Joseph Trotter, <http://facs.scripps.edu>). Cells with a fluorescence higher than those of 97% of nontransfected control cells were gated as positives in either fluorescence detector channel.

Figure 1. Overexpressed SNX17 and endogenous P-selectin colocalize in endosomes of HUVECs in secretagogue stimulated cells only. (A) P-selectin and SNX17-GFP do not colocalize in resting HUVECs. HUVECs were nucleofected with SNX17-GFP (5 μ g). One day later, cells were stained with anti-P-selectin antibody (AK6). (B) Endocytosed anti-P-selectin antibody colocalizes with SNX17. One day postnucleofection with SNX17-GFP (5 μ g) HUVECs were stimulated with histamine (10^{-4} M) for 15 min in the presence of anti-P-selectin antibody AK6 (10 μ g/ml). After a 30-min incubation in RM (MATERIALS AND METHODS) cells were fixed and processed for microscopy. (C–F) Localization of SNX17 overexpressed in HUVECs. HUVECs were nucleofected with SNX17-GFP (5 μ g) and 1 day postnucleofection cells were processed for microscopy and stained with the indicated marker: (C) TfR, (D) M6PR, (E) CD63, and (F) LAMP1. For all images primary antibodies were visualized with relevant donkey secondary antibodies conjugated with Texas Red as described in MATERIALS AND METHODS. Bars, 10 μ m.

RESULTS

Intracellular Location of SNX17

We have examined the location of SNX17 within cells expressing P-selectin. In HUVECs (human umbilical vein endothelial cells) P-selectin immunoreactivity (Bonfanti *et al.*, 1989) is found in the cigar-like Weibel-Palade bodies (WPBs;

Weibel and Palade, 1964), whereas transiently expressed SNX17-GFP was seen both in the cytosol and concentrated in punctae throughout the cell (Figure 1A). Some of these GFP-positive punctae were large enough that a lack of internal staining can be observed (“rings”), indicating that SNX17-GFP is associated with the perimeter membrane of these organelles (Figure 1C). Occasionally the GFP signal

highlights what appear to be cytoskeletal structures (Figure 1E). There is no significant colocalization with P-selectin immunoreactivity in WPBs.

To determine whether P-selectin might interact with SNX17 during its endocytic trafficking, anti-P-selectin antibody (AK6) uptake studies after histamine stimulation of HUVECs nucleofected with SNX17-GFP were carried out. Thirty minutes after internalization, AK6 immunoreactivity colocalizes with SNX17-GFP in the rings (Figure 1B). P-selectin expression in HUVECs is heterogeneous and thus not all cells will take up the AK6 antibody. In nontransfected HUVECs, AK6 internalized for 30 min is not seen in rings, but in small punctae, and by 1 h after internalization it can be seen in ribbon-like adaptor protein 1-positive structures as previously described (unpublished data and Arribas and Cutler, 2000). Overexpressed SNX17 thus causes an accumulation of internalized anti-P-selectin antibodies in ring-like endosomes.

To further characterize these SNX17-positive structures, costaining with antibodies to marker proteins was carried out. A significant but incomplete overlap in immunoreactivity was seen with the TfnR (Figure 1C). A closer look at costaining organelles (Figure 1C, inset) shows that the TfnR signal is concentrated in discrete patches on the perimeter membrane of SNX17-positive rings. Partial colocalization was also seen with the cation-independent mannose-6-phosphate receptor (M6PR; Figure 1D). However, high magnification of M6PR/SNX17-positive structures shows that in contrast to the distribution of TfnR, the M6PR immunoreactivity is concentrated in the lumen of the SNX17-positive structures. This indicates that there must be internal membranes present within the "rings," i.e., that these must be multivesicular endosomes. M6PR is usually thought of as a late endosomal marker (Griffiths *et al.*, 1988), although it has been shown to traffic through the early endosome (Hirst *et al.*, 1998). The presence of M6PR immunoreactivity in SNX17-positive structures could be explained by trapping (upon overexpression of SNX17) of M6PR. However, no significant colocalization was found with the late endosomal and lysosomal markers CD63 and LAMP1 (Figure 1, E and F). SNX17 overexpression is therefore not causing the significant perturbation of the endocytic pathway that would be indicated by "compartment mixing" in HUVECs under the conditions used in these experiments.

SNX17 is ubiquitously expressed (Nomura *et al.*, 1994), so we have also determined the localization of SNX17-GFP in CHO and HEK-293 cells. SNX17-GFP showed a similar distribution to that found in HUVECs in these cells, despite being expressed at a higher level (both more cells and a brighter fluorescence). SNX17-GFP thus partially colocalized with the TfnR (Figure 2A) and with early endosomal antigen 1 (EEA1) on ring-like structures (Figure 2C). EEA1 showed the greatest colocalization with SNX17 that we have observed of any marker. As with HUVECs, no colocalization was seen with a marker of the late endosome (LBPA, Figure 2B), of lysosomes (LAMP1, unpublished data), the trans-Golgi network (TGN46, Figure 2D), or of recycling endosomes (Rab11, unpublished data).

Although the above data indicate that there is no major remodelling of the endocytic pathway resulting from SNX17 expression in HEK-293 cells, SNX17-GFP sometimes accumulated in larger ring structures (Figure 2) or even in rare aggregates of fused rings. The rings were not simply a consequence of SNX17 expression, because they were also seen with anti-EEA1 in nontransfected cells. However, the frequency of the larger rings did increase

and the appearance of the aggregates did correlate with the level of SNX17 expression (as assessed by the brightness of GFP signal). Thus at moderate levels of expression SNX17-GFP was seen in punctae and in rings, whereas at higher levels of expression SNX17-GFP was seen in larger rings and occasionally in aggregate ring structures. Overexpression of SNX17 may at least partially drive the formation of these structures, as was the case with SNX15 (Barr *et al.*, 2000). However, unlike SNX15 overexpression, these enlarged SNX17-positive structures do not contain lysosomal markers.

SNX17 Retards the Degradation of P-selectin

Our data indicate that SNX17 is localized to an endosomal compartment. P-selectin internalizes into a transferrin-positive endosome (Blagoveshchenskaya *et al.*, 1998b, 1999; Arribas and Cutler 2000; Straley and Green, 2000) before further processing leads either to its recycling to the TGN (Arribas and Cutler 2000; Straley and Green, 2000) or to delivery to the lysosome (Green *et al.*, 1994; Blagoveshchenskaya *et al.*, 1998a, 1998b, 2000a; Arribas and Cutler 2000). We found that internalized anti-P-selectin accumulates in an SNX17-positive endosome that is TfnR-positive and that may also accumulate M6PR. This suggests that overexpressed SNX17 is retarding delivery of P-selectin to the lysosome and/or to the TGN. We have determined whether SNX17 overexpression modulates P-selectin degradation in HEK-293 cells. Because HEK-293 cells lack a storage compartment, newly synthesized P-selectin will be delivered to the plasma membrane (Disdier *et al.*, 1992; Green *et al.*, 1994; Blagoveshchenskaya *et al.*, 1998a; Straley *et al.*, 1998). It will then internalize and be delivered to lysosomes or recycle via the TGN, to enter a new round of exo- and endocytosis. The final destination of all this traffic is delivery to the lysosome, a process that can be easily monitored.

Delivery to lysosomes can be quantified by an HRP-clipping assay on cells nucleofected with ssHRP^{P-selectin} (Blagoveshchenskaya *et al.*, 1998b). This assay exploits the protease resistance of the HRP domain and uses Triton X-114 partitioning between detergent binding (hydrophobic) and aqueous phases of the HRP activity to determine the amount of hydrophilic HRP released (or clipped) from its membrane anchor by proteolysis, thereby providing an indirect but accurate (Blagoveshchenskaya and Cutler, 2000b) measure of lysosomal targeting.

We analyzed the effect of SNX17 on ssHRP^{P-selectin} on different days after transfection (Figure 3). This was important because we use a double-transient transfection for these experiments. We thus wished to determine that any effect observed was relatively stable over time and was not only seen at some particular ratio of expression or time after nucleofection but was a robust phenomenon. In HEK-293 cells $48.7 \pm 0.5\%$ of ssHRP^{P-selectin} was clipped 1 day after nucleofection rising to $71.6 \pm 0.6\%$ by day 3 (Figure 3A). Transient coexpression of SNX17-GFP with ssHRP^{P-selectin} reduced HRP clipping (Figure 3A) so that at one day post-nucleofection only $27.8 \pm 1.8\%$ of ssHRP^{P-selectin} was clipped, rising by day 3 to $52.2 \pm 1.2\%$. The maximal effect of SNX17-GFP was seen at 2 days postnucleofection, when coexpression of SNX17 caused a 42% reduction in clipping. The effect of SNX17 on clipping of ssHRP^{P-selectin} increased with the ratio of SNX17-GFP to ssHRP^{P-selectin} DNA used (Figure 3B). We used a ratio of 10:1 for all further HEK-293 studies.

We have established in HEK-293 cells that overexpression of SNX17 reduces degradation of ssHRP^{P-selectin}.

Figure 2. Overexpressed SNX17 is localized to early endosomes in HEK-293 cells. One day postnucleofection with SNX17-GFP (5 μ g) HEK-293 cells were fixed, processed for microscopy and stained with the indicated markers: (A) TfnR, (B) LBPA, (C) EEA1, and (D) TGN46. Primary antibodies were visualized with relevant donkey secondary antibodies conjugated with Texas Red as described in MATERIALS AND METHODS. Bars, 10 μ m.

Does this also occur in cells normally expressing P-selectin? The HRP-clipping assays were therefore repeated using nucleofected HUVECs. The efficiency of nucleofection with SNX17 was much lower in HUVECs ($\sim 5\text{--}10\%$) than in HEK-293 cells ($> 30\%$). ssHRP^{P-selectin} expressed alone showed a similar HRP-clipping profile to that seen in HEK-293 cells with $49.4 \pm 0.8\%$ and $66.3 \pm 0.1\%$ being clipped one and two days postnucleofection, respectively (Figure 3C). Coexpression of SNX17-GFP reduced HRP clipping to $40.9 \pm 0.1\%$ and $59.4 \pm 0.9\%$ 1 and 2 days postnucleofection, respectively. The maximal effect of SNX17 on ssHRP^{P-selectin} in HUVECs was thus 40% of the effect seen in HEK-293 cells. This smaller effect is probably due to three differences between the two cell types. First, the lower percentage of HUVEC expressing SNX17-GFP, plus the lower level of protein expression of our constructs in HUVECs; second, the sequestration of ssHRP^{P-selectin} within the WPB away from SNX17; and third, the presence of a population of endogenous P-selectin potentially competing for binding to SNX17.

SNX1 Binds to P-selectin But Does Not Affect Its Lysosomal Delivery

The data described above raises the questions of whether SNX17 and P-selectin directly interact and whether the effect on P-selectin is specific to SNX17. To address these issues together, we have used SNX1 overexpression experiments in parallel with SNX17. We have chosen SNX1 for these experiments because the effect of overexpression of SNX1 on EGF-R is to increase its downregulation (Kurten *et al.*, 1996), whereas it has the opposite effect on PAR (Wang *et al.*, 2002), thus paralleling the positive effects of SNX17 on LDL-R vs. its negative effect on P-selectin. Importantly, SNX1 can also enlarge the EEA1-positive compartment when overexpressed. If that enlargement in and of itself were to cause the retardation of P-selectin, then SNX1 ought to have an effect on P-selectin similar to that seen for SNX17, even if it did not bind P-selectin directly. We therefore decided to test both the binding and any effect on P-selectin degradation caused by SNX1 in parallel to SNX17.

Figure 3. SNX17 overexpression reduces the clipping of HRP-P-selectin. (A) HEK-293 cells nucleofected with either ssHRP^{P-selectin} (■) or SNX17-GFP and ssHRP^{P-selectin} (□) were seeded onto 60-mm plates. At 1 to 3 days after nucleofection cells were lysed and Triton X-114 partitioned, and HRP activity in aqueous and detergent phases was determined as described in MATERIALS AND METHODS. The data are presented as percentage of total activity present in the aqueous phase (i.e., clipped HRP). (B) HEK-293 cells were nucleofected with either ssHRP^{P-selectin} or SNX17-GFP and ssHRP^{P-selectin} at a 2:1 (■), 5:1 (□) and 10:1 (■) ratio of DNA. Cells were seeded onto 60-mm dishes and lysed and Triton X-114 partitioned, and HRP activity in aqueous and detergent phases was determined at 1 and 2 days postnucleofection. Data are shown as difference between the percentage of HRP activity in aqueous phase for cells nucleofected with ssHRP^{P-selectin} and that for cells nucleofected with SNX17-GFP and ssHRP^{P-selectin}. (C) HUVEC were nucleofected with either ssHRP^{P-selectin} (■) or SNX17-GFP and ssHRP^{P-selectin} (□) and seeded onto 100-mm plates. Cells were then treated as in A. Each bar represents the mean $\pm$ SD of six independent determinations.

HEK-293 cells were transfected with ssHRP^{P-selectin} or with ssHRP^{P-selectin763}. The latter variant lacks the cytoplasmic tail of P-selectin, which would be responsible for any relevant and specific binding activity. Either together with the selectins, or separately, cells were transfected with pDHH-SNX1 or pSNX17-HH. Binding was then determined in lysates from cells coexpressing the two potential partners or in extracts from cells expressing the two components separately that were mixed after lysis. Isolation of the SNX partner followed by HRP assay to determine how much P-selectin is bound (Figure 4A), reveals several important findings. First, we confirm the interaction between both SNX17 and P-selectin seen in a yeast two-hybrid screen (Florian *et al.*, 2001). Second, we report for the first time an interaction between SNX1 and P-selectin. This is found using either pre- or postlysis interaction of the two proteins. Third, in both cases the interaction is dependent on the presence of the cytoplasmic domain of the selectin.

These data strongly suggest that the effects of SNX17 on P-selectin may arise from a direct interaction between the two proteins. If so, then does SNX1 have a similar effect on P-selectin trafficking? We carried out a standard HRP-clipping assay on cells transfected with P-selectin plus either pSNX17-HH or pDHH-SNX1 and compared the effect of the two on clipping (Figure 4C). We conclude that SNX1, despite binding to P-selectin and despite being expressed at similar levels in these experiments (Figure 4B) has only one fifth the effect of SNX17 and thus does not significantly retard delivery of the leukocyte receptor to the lysosome. We therefore conclude that the effects of SNX17 on P-selectin are specific

and that the effects of SNX overexpression on EEA1-positive endosomes are not responsible for this effect.

The endosomal association of SNX17 depends on both the activity of PI-3-kinase and the presence of a PX domain, unlike its binding to P-selectin and its functioning, which are partially independent

The binding of SNX17 to P-selectin is not solely controlled by the cytoplasmic tail of the leukocyte receptor, because in HUVECs the two proteins are not found together (Figure 1). It is likely that SNX17, like e.g., EEA1 and SNX3 (Xu *et al.*, 2001a), is located at the early endosome through binding of its PX domain to the phosphatidylinositol-3-phosphate (PI(3)P), in which membranes of early endosomes are enriched (Gillooly *et al.*, 2000). This association can be disrupted by inhibition of the PI-3-kinase with wortmannin (Wurmser *et al.*, 1999; Xu *et al.*, 2001b). We tested whether the endosomal localization of SNX17 would be similarly altered by PI-3-kinase inhibition. As with EEA1 (unpublished data), the SNX17-GFP signal became exclusively cytosolic with no evident vesicle-associated signal shortly after the addition of 50 nM wortmannin (Figure 5B). This implies that the binding of SNX17 to endosomes is dependent on 3-phosphoinositides. An SNX17 variant lacking the PX domain (SNX17*15.03; schematic diagram shown in Figure 5A) also showed only a diffuse cytoplasmic distribution in HEK-293 cells (Figure 5B, panel D). Altogether, this indicates that the PX domain, and therefore PI-binding is indeed necessary for the endosomal distribution of SNX17.

We have tested whether loss of the endosomal location also ablates binding of SNX17 to P-selectin. We find that

Figure 4. SNX17 and SNX1 both bind to P-selectin but only SNX17 has a major effect on HRP clipping. (A) HEK-293 cells were transfected with ssHRP^{P-selectin} or with ssHRP^{P-selectin763} either in combination with or separately with pDHH-SNX1 or pSNX17-HH, which encode the respective His-tagged SNX's. Binding was then determined in lysates from cells coexpressing the two potential partners (prelysis) or in extracts from cells expressing the two components separately that were mixed after lysis (postlysis). His-tagged complexes were isolated using Probond, eluted from the beads, and the HRP activity were determined as in MATERIALS AND METHODS. Data are presented as a percentage of the binding of SNX17 and to ssHRP^{P-selectin} for either method, ($\pm$ SE) and is representative of two independent experiments analyzed in triplicate. (B) Elutants from A were subjected to SDS-PAGE followed by Western blotting with an anti-His antibody. Bands were visualized using an HRP-conjugated

after coexpression, SNX17*15.03 can still bind P-selectin, albeit at a reduced level ($\sim$ 32%) compared with the wild-type SNX (Figure 5C). We have also examined whether anchoring of SNX17 to the endosome is required for its functional activity (i.e., its ability to retard trafficking of P-selectin), because this has a direct bearing on possible mechanisms of action of SNX17. By HRP-clipping analysis after coexpression, we find that while reduced to 56% of the effect of wild-type, a SNX17 variant lacking the PX domain can still retard lysosomal delivery of P-selectin (Figure 5D).

SNX17 Enhances the Internalization of P-selectin from the Plasma Membrane

By FACS analysis we discovered (unpublished data) that overexpression of SNX17 reduces levels of P-selectin at the plasma membrane. This might correspond to the increased rate of LDL-R internalization reported by Stockinger for SNX17 overexpression (Stockinger *et al.*, 2002). However unlike Stockinger and coworkers, who find increased lysosomal delivery of LDL, we find a reduced lysosomal targeting of P-selectin. One explanation of our data might be that SNX17 generally reduces exit of P-selectin from the endosome, not only to the lysosome but also to the plasma membrane, thereby reducing steady state levels at the cell surface by blocking recycling. To resolve this we established a time course of accumulation of P-selectin within cells by FACS analysis under control conditions and in the presence of SNX17-GFP expressed either at high or low levels from an inducible system. We find that increasing levels of SNX17 leads to increased internalization of P-selectin over that of control levels. (Figure 6). In addition Figure 6 shows that levels of internalized P-selectin fall at 30 min of warming before rising again, mostly likely because of recycling. These data indicate that SNX17 does not enhance intracellular accumulation of P-selectin by blockade of recycling.

DISCUSSION

Modulation of the endocytic behavior of cell surface receptors by the sorting nexin family is emerging as an important aspect of their control. In this article we examine the functional relationship between SNX17 and the leukocyte receptor P-selectin. We show that the two proteins colocalize after internalization of P-selectin in HUVECs and that P-selectin can bind SNX17 in HEK293 cells. We show that heterologous expression of SNX17 increases internalization of P-selectin from the plasma membrane, yet also reduces its delivery to the lysosome, leading to an internal accumulation in endosomes. SNX17 therefore has a major influence on the trafficking of P-selectin.

Cellular Distribution of SNX17 and P-selectin

Overexpressed SNX17 colocalizes with internalized P-selectin whose accumulation it causes within the endosome. In addition, membrane-associated SNX17 best colocalizes with EEA1 but also overlaps with the TfR. SNX17-GFP did not

anti-mouse secondary followed by ECL detection. (C) HEK-293 cells were nucleofected with ssHRP^{P-selectin} or ssHRP^{P-selectin763} in combination with a control plasmid, pSNX17-GFP, or pDHH-SNX1 and were subjected to TX114 partitioning. The HRP activity in the aqueous and detergent phases was determined as in MATERIALS AND METHODS. The data from six independent determinations are expressed as a percentage of the HRP-clipping activity of SNX17.

Figure 5. Both PI3 kinase activity and the PX domain of SNX17 are needed for endosomal localization, but function is only partially dependent on its PX domain. (A) Schematic diagram of domain structure of SNX17*15.03 encoding His-tagged sorting nexin 17 lacking a PX domain. Known domains (PX, FERM) are boxed and amino acid length are indicated underneath. The site at which pDHH-SNX17*15.03 begins is indicated with an arrow above. (B) Wortmannin treatment or loss of the PX domain relocate SNX17 to the cytosol. CHO or HEK293 (unpublished data) cells nucleofected with SNX17-GFP 1 day postnucleofection were incubated with medium (A) or medium containing 50 nM wortmannin (B) for 15 min and examined by epifluorescence microscopy. HEK-293 cells nucleofected with pSNX17-HH (C) and pDHH-SNX17*15.03 (D) were fixed 1 day postnucleofection and examined by confocal microscopy. Bars, 10 μ m. (E) HEK-293 cells were nucleofected with ssHRP^{P-selectin} and pSNX17-HH or pDHH-SNX17*15.03. After 24 h His-tagged complexes were isolated using Probond and eluted from

Figure 6. SNX17 expression enhances the internalization of P-selectin from the cell surface. HEK-293 cells were transfected with pPEX, encoding for P-selectin, and an inducible SNX17-GFP variant (pTRE-bsd-SNX17-GFP). SNX17-GFP expression not induced (control cells) or induced with 2 μ g/ml doxycycline for 12 h. Cells were labeled with anti-P-selectin antibody on ice, warmed for indicated times at 37°C to allow internalization then fixed, stained with Alexa 647-labeled secondary antibody and analyzed by flow cytometry as described in materials and methods. Data points shown are means ($\pm$ SE) from three independent experiments are shown from control cells ($\square$), cells gated as expressing low SNX17-GFP ($\blacksquare$), and cells gated as expressing high levels of SNX17-GFP ($\blacktriangle$).

colocalize with CD63/LBPA, LAMP1, TGN46, and Rab11. This intracellular distribution is thus in broad agreement with that found by Stockinger *et al.* (2002), who showed that heterologously expressed SNX17 colocalizes with EEA1 and Rab4 but not LAMP1.

In addition to the accumulation of P-selectin, we also find M6PR overlapping with SNX17. It is unusual to see significant levels of M6PR at steady state in structures that also contain TfnR and EEA1; rather, it is normally seen at the TGN and late endosomes plus a small amount on the plasma membrane with the exact distribution being cell-type dependent (Griffiths *et al.*, 1988; Kornfeld and Mellman, 1989; Hirst *et al.*, 1998). The SNX17/M6PR-positive structures are early rather than late endosomes because there is overlap with TfnR but not with CD63 and LBPA. Interestingly, endogenous SNX15 in COS-7 cells also shows some, albeit infrequent, colocalization in puncta with M6PR (Barr *et al.*, 2000). However, SNX15 overexpression also leads to the presence of markers for early endosomes, late endosomes, and lysosomes within the same abnormal structures (Barr *et al.*, 2000). Our data

the beads, and the HRP activity was determined as in MATERIALS AND METHODS. Data are expressed as a percentage of the binding ssHRP^{P-selectin} and pSNX17-HH. Each bar represents the mean $\pm$ SE of at least five independent determinations. (D) HEK-293 cells were nucleofected with ssHRP^{P-selectin} and pSNX17-HH or pDHH-SNX17*15.03 and after 24 h were subjected to TX114 partitioning. HRP activity in aqueous and the detergent phases were measured, and the clipping activity was determined. Data are expressed as a percentage of clipping that occurs with ssHRP^{P-selectin} and pSNX17-HH obtained from five independent experiments.

show that although we cause some enlargement of the endosomes, we are not seeing any real mixing of compartments caused by SNX17 in this system. We therefore suspect that the M6PR is entering the early endosome from the plasma membrane or the TGN in the normal way but as with P-selectin is then failing to exit.

Although we have no electron microscopic data on the structure of the SNX17 compartment, we can obtain some clear suggestions from our immunofluorescence of the enlarged endosomes. We see EEA1 and SNX17 colocalizing on the perimeter, with TfnR associated only in discrete patches. In contrast, the M6PR staining was restricted to the lumen. SNX17 is thus most likely on the perimeter of an endosome with significant internal vesicles and from which tubules containing recycling TfnR extend (Geuze *et al.*, 1987; Stoorvogel *et al.*, 1996).

Targeting of SNX17

The endosomal location of SNX17 is disrupted by either inhibiting PI-3-kinase or deleting its PX domain. Thus it is likely located on the endosome by its PX domain binding the 3-phosphoinositides that are enriched in endosomal membranes, as is the case for other SNXs (Sato *et al.*, 2001). The SNX17 colocalization with EEA1 and findings from other groups on the lipid preference of SNXs indicate that the most likely candidate for SNX17 association is PI(3)P. PI(3)P has been demonstrated to be present on limiting membranes of early endosomes and on internal vesicles of multivesicular endosomes (Gillooly *et al.*, 2000). However, we only detected SNX17 by immunofluorescence on the limiting membrane.

Our data and that from Stockinger *et al.* (2002) show that SNX17 occupies a subset of EEA1-positive vesicles, which is also true for other SNXs (SNX1, SNX15). SNXs probably occupy overlapping locations because although SNX15 and SNX17 partially colocalize with EEA1, they also show association with Rab5 (Barr *et al.*, 2000) and Rab4 (Stockinger *et al.*, 2002), respectively. Because PI(3)P is found throughout early endosomes (Stenmark and Gillooly, 2001), specific localization to a particular subcompartment is likely to be achieved through additional protein/protein interactions. Interestingly it has been recently shown that SNX1 localization to its endosomal compartment is dependent both on the presence of the PX domain but also the predicted C-terminal coiled-coil domain (Zhong *et al.*, 2002). In addition to its N-terminal PX domain SNX17 also contains part of a predicted FERM domain and a 200 aa carboxy-terminal stretch with no established function. It is possible that these regions could be involved in SNX17 localization. Clearly, binding to P-selectin alone is not sufficient to drive SNX17 localization because we do not find the SNX on WPBs.

Specificity and Significance of Action of SNX17 on P-selectin

One of our findings is that in addition to SNX17, P-selectin binds to SNX1. However, overexpression of this SNX does not have a significant effect on the lysosomal targeting of P-selectin. This shows that either the binding may be of no functional significance or that another assay of P-selectin behavior is required. However, we can conclude that despite being found in the early endosome, and presumably encountering P-selectin there, binding to SNX1 has very different functional consequences from binding to SNX17.

One implication of the failure of SNX1 to retard delivery of P-selectin to lysosomes is that this occurs despite

the parallel abilities of the two SNXs (and indeed possibly any PIP-binding protein) to distort the early endosomal architecture. Because this alteration might itself have led to a nonspecific and indirect effect on P-selectin trafficking, the fact that SNX1 does not affect the HRP-clipping assay is important. Our conclusion that the effect of SNX17 is of physiological significance is also bolstered by the observation that a SNX17 variant that lacks a PX domain and therefore cannot be affecting the endosomal architecture can still affect lysosomal targeting (Figure 5). Finally, it should be noted that even grossly altering the endosomal morphology by treatment with wortmannin, leading to enlarged endosomes with few inner vesicles, does not necessarily block lysosomal delivery (Futter *et al.*, 2001).

Mechanism of Action of SNX17

The simplest model for SNX17 action is that it is retained in the early endosome by its binding to PI(3)P and that when it binds passing P-selectin, the receptor becomes trapped by the retention of its binding partner. Although this simplest model is attractive because explaining the retardation in lysosomal delivery, it does not easily explain the increase in internalization that we observe, which requires a second mode of action. Further, without evoking additional binding partners it cannot explain why an SNX17 lacking a PX domain is able to retard lysosomal delivery of P-selectin. One potential explanation is that the fall in efficiency of its effect (the mutant can only block 50% as well as the wild type) reflects some contribution of simple trapping but that other factors must also be involved. However, this interpretation may itself be too simple because the binding of SNX17*15.03 is also reduced relative to wild type. Clearly, although we have a model system with which to explore the mechanism of SNX17 action, considerable work is needed to go beyond ruling out the simplest model as we have done so far.

Effect of SNX17 on Physiological Functioning of P-selectin

The physiological importance of the SNX family lies in their ability to influence the endocytic trafficking of plasma membrane receptors. In principal, by controlling rates of internalization, recycling, and degradation, the steady-state levels of receptors on the plasma membrane and the kinetics of attenuation of their signaling can be modulated. P-selectin operates at the plasma membrane to bind leukocytes, but is stored within WPBs. How can endosomal SNX17 affect the functioning of this receptor?

One possibility arises from the fact that the amounts of P-selectin at the plasma membrane will reflect levels in the WPBs. Can SNX17 affect delivery to the WPB of newly synthesized and/or of recycling P-selectin? It has been reported that accumulation of P-selectin within WPBs is adaptor protein 3-dependent and therefore most likely occurs via an endosomal intermediate (Daugherty *et al.*, 2001). In addition, SNX17 may influence P-selectin levels in the WPB by controlling recycling to this organelle. In either case, SNX17 must affect the endocytic traffic of this leukocyte receptor. We have shown in HEK-293 cells that this does indeed occur. It is important to point out that a great deal is known about the endocytic trafficking of P-selectin in heterologous systems (Green *et al.*, 1994; Setiadi *et al.*, 1995; Norcott *et al.*, 1996; Blagoveshchenskaya *et al.*, 1998a, 1998b, 1999, 2002; Strasser *et al.*, 1999; Blagoveshchenskaya and Cutler, 2000a; Straley and Green, 2000; Daugherty *et al.*, 2001; Kaur and Cutler, 2002), and we are confident that an analysis of P-

selectin trafficking through the endocytic pathway in HEK-293 cells will not differ significantly from, e.g., that in PC12, H.Ep.2, CHO or most importantly HUVECs. We therefore believe that SNX17 will be involved in physiological control of the endocytic sorting of P-selectin, as indicated by controlling the amount of HRP-P-selectin reaching the lysosome where degradation occurs. Whether there is a similar fall in delivery to WPBs or the TGN will be the subject of further work.

The second way in which SNX17 can modulate P-selectin function would be if it affects the time spent on the plasma membrane by this transiently appearing receptor. We do indeed find a decreased level of P-selectin on the plasma membrane of cells overexpressing SNX17, which is, like its effect on LDL receptor (and indeed the effect of SNX1 on EGF receptor), due to an increased rate of internalization of P-selectin. However, both Stockinger and coworkers (Stockinger *et al.*, 2002) and ourselves see SNX17 mainly concentrated on endosomal membranes making it difficult to see how it might influence internalization, although we do find some cells where we cannot rule out a plasma membrane location (e.g., Figure 1D). The fact that a soluble form of SNX17 that is not restricted to the endosomal membrane by its PX domain can still influence the lysosomal targeting of P-selectin may also be relevant.

However, even if SNX17 is increasing the internalization of both LDL receptor and P-selectin, only one of these two proteins shows reduced surface levels as a result. This difference presumably reflects their different itineraries. If recycling to the plasma membrane from early endosomes is unaffected by SNX17 as we find for P-selectin, then levels of LDL receptor at the cell surface may well be unaffected. However, if P-selectin were to recycle to the plasma membrane primarily from the late endosome (then via the TGN, as suggested by Green and coworkers; Straley and Green, 2000) delivery to, which is blocked by overexpressing SNX17, then the differential effect of SNX17 on LDL receptor vs. P-selectin can thereby be explained.

In conclusion, we report that SNX17 can reduce P-selectin levels on the plasma membrane by enhancing its internalization as well as retarding its delivery to lysosomes. Both of these effects will either directly or indirectly (the latter) control the ability of this receptor to function in leukocyte recruitment.

ACKNOWLEDGMENTS

T.S. thanks Dr A. Gerber for giving the opportunity to use the flow cytometer of the Institute of Immunology, Otto-von-Guericke University Magdeburg. D.C. and R.W. thank M. Hannah and other members of the Cutler lab, members of the LMCB, and C. Futter for helpful discussions and reading the manuscript. R.W., M.R., and D.C. are supported by the Medical Research Council. Parts of this work were supported by a "Start-up" project (No 5, NBL3) of the Magdeburger Forschungsverbund to P.K.

REFERENCES

Arribas, M., and Cutler, D.F. (2000). Weibel-Palade body membrane proteins exhibit differential trafficking after exocytosis in endothelial cells. *Traffic* 1, 783–793.

Barr, V.A., Philips, S.A., Taylor, S.I., and Renfrew Haft, C. (2000). Overexpression of a novel sorting nexin, SNX15, affects endosome morphology and protein trafficking. *Traffic* 1, 904–916.

Blagoveshchenskaya, A.D., Hewitt, E.W., and Cutler, D.F. (1998a). A balance of opposing signals within the cytoplasmic tail controls the lysosomal targeting of P-selectin. *J. Biol. Chem.* 273, 27896–27903.

Blagoveshchenskaya, A.D., Norcott, J.P., and Cutler, D.F. (1998b). Lysosomal targeting of P-selectin is mediated by a novel sequence within its cytoplasmic tail. *J. Biol. Chem.* 273, 2729–2737.

Blagoveshchenskaya, A.D., Hewitt, E.W., and Cutler, D.F. (1999). A complex web of signal-dependent trafficking underlies the triorganellar distribution of P-selectin in neuroendocrine PC12 cells. *J. Cell Biol.* 145, 1419–1433.

Blagoveshchenskaya, A.D., and Cutler, D.F. (2000a). Biochemical analyses of trafficking with horseradish peroxidase-tagged chimeras. *Methods Enzymol.* 327, 45–60.

Blagoveshchenskaya, A.D., and Cutler, D.F. (2000b). Sorting to synaptic-like microvesicles from early and late endosomes requires overlapping but not identical targeting signals. *Mol. Biol. Cell* 11, 1801–1814.

Blagoveshchenskaya, A.D., Hannah, M.J., Allen, S., and Cutler, D.F. (2002). Selective and signal-dependent recruitment of membrane proteins to secretory granules formed by heterologously expressed von Willebrand factor. *Mol. Biol. Cell* 13, 1582–1593.

Bonfanti, R., Furie, B.C., Furie, B., and Wagner, D.D. (1989). PADGEM (GMP140) is a component of Weibel-Palade bodies of human endothelial cells. *Blood* 73, 1109–1112.

Bordier, C. (1981). Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* 256, 1604–1607.

Chishti, A. H. *et al.* (1998). The FERM domain: a unique module involved in the linkage of cytoplasmic proteins to the membrane. *Trends Biochem. Sci.* 23, 281–282.

Daugherty, B.L., Straley, K.S., Sanders, J.M., Phillips, J.W., Disdier, M., McEver, R.P., and Green, S.A. (2001). AP-3 adaptor functions in targeting P-selectin to secretory granules in endothelial cells. *Traffic* 2, 406–413.

Disdier, M., Morrissey, J.H., Fugate, R.D., Bainton, D.F., and McEver, R.P. (1992). Cytoplasmic domain of P-selectin (CD62) contains the signal for sorting into the regulated secretory pathway. *Mol. Biol. Cell* 3, 309–321.

Florian, V., Schlüter, T., and Bohnensack, R. (2001). A new member of the sorting nexin family interacts with the C-terminus of P-selectin. *Biochem. Biophys. Res. Commun.* 281, 1045–1050.

Futter, C.E., Collinson, L.M., Backer, J.M., and Hopkins, C.R. (2001). Human VPS34 is required for internal vesicle formation within multivesicular endosomes. *J. Cell Biol.* 155, 1251–64.

Geuze, H.J., Slot, J.W., and Schwartz, A.L. (1987). Membranes of sorting organelles display lateral heterogeneity in receptor distribution. *J. Cell Biol.* 104, 1715–1723.

Gillooly, D.J., Morrow, I.C., Lindsay, M., Gould, R., Bryant, N.J., Gaullier, J.-M., Parton, R.G., and Stenmark, H. (2000). Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells. *EMBO J.* 19, 4577–4588.

Green, S.A., Setiadi, H., McEver, R.P., and Kelly, R.B. (1994). The cytoplasmic domain of P-selectin contains a sorting determinant that mediates rapid degradation in lysosomes. *J. Cell Biol.* 124, 435–448.

Griffiths, G., Hoflack, B., Simons, K., Mellman, I., and S. Kornfeld. (1988). The mannose 6-phosphate receptor and the biogenesis of lysosomes. *Cell* 52, 329–341.

Hattori, R., Hamilton, K.K., Fugate, R.D., McEver, R.P., and Sims, P.J. (1989). Stimulated secretion of endothelial von Willebrand factor is accompanied by rapid redistribution to the cell surface of the intracellular granule membrane protein GMP-140. *J. Biol. Chem.* 264, 7768–7771.

Hirst, J., Futter, C.E., and Hopkins, C.R. (1998). The kinetics of mannose 6-phosphate receptor trafficking in the endocytic pathway in HEP-2 cells: the receptor enters and rapidly leaves multivesicular endosomes without accumulating in a prelysosomal compartment. *Mol. Biol. Cell* 9, 809–816.

Kaur, J., and Cutler, D.F. (2002). P-selectin targeting to secretory lysosomes of Rbl-2H2 cells. *J. Biol. Chem.* 277, 10498–10505.

Kornfeld, S., and Mellman, J. (1989). The biogenesis of lysosomes. *Annu. Rev. Cell Biol.* 5, 483–525.

Kurten, R.C., Cadena, D.L., and Gill, G.N. (1996). Enhanced degradation of EGF receptors by a sorting nexin, SNX1. *Science*, 272, 1001–1010.

McEver, R.P., Beckstead, J.H., Moore, K.L., Marshall-Carlson, L., and Bainton, D.F. (1989). GMP-140, a platelet alpha-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies. *J. Clin. Invest.* 84, 92–99.

Nomura, N. *et al.* (1994). Prediction of the coding sequences of unidentified human genes. II. The coding sequences of 40 new genes (K1AA0041-K1AA0080) deduced by analysis of cDNA clones from human cell line KG-1. *DNA Res.* 1, 251–262.

Norcott, J.P., Solari, R., and Cutler, D.F. (1996). Targeting of P-selectin to two regulated secretory organelles in PC12 cells. *J. Cell Biol.* 134, 1229–1240.

Sato, T.K., Overduin, M., and Emr, S.D. (2001). Location, location, location: Membrane targeting directed by PX domains. *Science* 294, 1881–1885.

- Setiadi, H., Disdier, M., Green, S.A., Canfield, W.M., and McEver, R.P. (1995). Residues throughout the cytoplasmic domain affect the internalization efficiency of P-selectin. *J. Biol. Chem.* 270, 26818–26826.
- Stenberg, P.E., McEver, R.P., Shuman, M.A., Jacques, Y.V., and Bainton, D.F. (1985). A platelet alpha-granule membrane protein (GMP-140) is expressed on the plasma membrane after activation. *J. Cell Biol.* 101, 880–886.
- Stenmark, H., and Gillooly, D.J. (2001). Intracellular trafficking and turnover of phosphatidylinositol 3-phosphate. *Semin. Cell Dev. Biol.* 12, 193–199.
- Stockinger, W., Sailler, B., Strasser, V., Recheis, B., Fasching, D., Kahr, L., Schneider, W.J., and Nimpf, J. (2002). The PX-domain protein SNX17 interacts with members of the LDL receptor family and modulates endocytosis of the LDL receptor. *EMBO J.* 21, 4259–4267.
- Stoorvogel, W., Oorschot, V., and Geuze, H.J. (1996). A novel class of clathrin-coated vesicles budding from endosomes. *J. Cell Biol.* 132, 21–33.
- Straley, K.S., Daugherty, B.L., Aeder, S.E., Hockenson, A.L., Kim, K., and Green, S.A. (1998). An atypical sorting determinant in the cytoplasmic domain of P-selectin mediates endosomal sorting. *Mol. Biol. Cell* 9, 1683–94.
- Straley, K.S., and Green, S.A. (2000). Rapid transport of internalized P-selectin to late endosomes and the TGN: roles in regulating cell surface expression and recycling to secretory granules. *J. Cell Biol.* 151, 107–116.
- Strasser, J.E., Arribas, M., Blagoveshchenskaya, A.D., and Cutler, D.F. (1999). Secretagogue-triggered transfer of membrane proteins from neuroendocrine secretory granules to synaptic-like microvesicles. *Mol. Biol. Cell* 10, 2619–2630.
- Subramaniam, M., Koedam, J.A., and Wagner, D.D. (1993). Divergent fates of P- and E-selectins after their expression on the plasma membrane. *Mol. Biol. Cell* 4, 791–801.
- van den Hoff, M.J., Moorman, A.F., and Lamers, W.H. (1992). Electroporation in 'intracellular' buffer increases cell survival. *Nucleic Acids Res.* 20, 2902.
- Wang, Y., Zhou, Y., Szabo, K., Renfrew Haft, C., and Trejo, J. (2002). Down-regulation of protease-activated receptor-1 is regulated by sorting nexin 1. *Mol. Biol. Cell* 13, 1965–1976.
- Weibel, E.R., and Palade, G.E. (1964). New cytoplasmic components in arterial endothelia. *J. Cell Biol.* 23, 101–112.
- Worby, C.A., and Dixon, J.E. (2002). Sorting out the cellular functions of sorting nexins. *Nat. Rev. Mol. Cell Biol.* 3, 919–931.
- Wurmser, A.E., Gary, J.D., and Emr, S.D. (1999). Phosphoinositide 3-kinases and their FYVE domain-containing effectors as regulators of vacuolar/lysosomal membrane trafficking pathways. *J. Biol. Chem.* 274, 9129–9132.
- Xu, Y., Seet, L., Hanson, B., and Hong, W. (2001a). The phox homology (PX) domain, a new player in phosphoinositide signalling. *Biochem. J.* 360, 513–530.
- Xu, Y., Hortsman, H., Seet, L., Wong, S.H., and Hong, W. (2001b). SNX3 regulates endosomal function through its PX-domain-mediated interaction with PtdIns(3)P. *Nat. Cell Biol.* 3, 658–666.
- Zhong, Q., Lazar, C.S., Tronchère, H., Sato, T.K., Meerloo, T., Yeo, M., Songyang, Z., Emr, S.D., and Gill, G.N. (2002). Endosomal localization and function of sorting nexin 1. *Proc. Natl. Acad. Sci. USA* 99, 6767–6772.