Sorting nexin 17 accelerates internalisation yet retards degradation of P-selectin

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ABSTRACT

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. Following 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 since it only present at the plasma membrane of endothelial cells for a few minutes early in the inflammatory response (McEver et al., 1989; Hattori et al., 1989). Uncontrolled surface appearance of P-selectin would lead to chronic leukocyte recruitment, but its rapid internalisation (Setiadi et al., 1995; Blagoveshchenskaya et al., 1998a) 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 receptor 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 since its overexpression results in furin mislocalisation 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 internalisation 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 following secretagogue action (Stenberg et al. 1985; Hattori et al. 1989) while 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 internalisation 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 following internalisation from the plasma membrane.
MATERIALS AND METHODS

Antibodies

Mouse monoclonal antibodies used were from AMS Biotechnology (Oxon, UK) or (PE-labelled) from DAKO diagnostica (Hamburg, Germany). Mouse monoclonal antibody 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 monoclonal antibody against transferrin receptor (H68.4) was obtained from Zymed Laboratories Inc (San Francisco, CA, USA). EEA1 antibody was purchased from Transduction Laboratories (Lexington, KY, USA) and the monoclonal anti His from Roche (Basel, Switzerland). Monoclonal anti-lyso-bisphosphatidic acid (LBPA) antibody was a kind gift by Jean Gruenberg (Geneva, Switzerland). Mouse monoclonal antibody 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 Laboratories.

Constructs

ssHRP<sup>P-selectin</sup> and ssHRP<sup>P-selectin<sub>763</sub></sup> (chimera with a deletion of the cytoplasmic C1 and C2 domains) were described previously (Norcott<sup>et al</sup>., 1996; Blagoveshchenskaya<sup>et al</sup>., 1998b). The generation of full-length SNX17 cDNA and the SNX17-GFP expression plasmid pK64-GFP were described previously (Florian<sup>et al</sup>., 2001). To generate a His-tagged SNX17 construct (pSNX17-HH) a 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 tetracyclin controlled SNX17-GFP variant was made as follows: Into the vector pTRE (Clontech, Palo Alto, CA, USA), cut with PvuII/HindIII, was ligated a blasticidin resistance gene (Eco32I/HindIII) from the vector pEF-bsd (Invitrogen) to yield pTRE-bsd. Then SNX17-GFP was cloned via Nhel(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 HindI fragment from pEGFP-endo (Clontech) was ligated into pCR-Script (Stratagene, La Jolla, CA, USA), 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 Ltd, Paisley, UK) supplemented with 20 % fetal calf serum (Hyclone, Logan, UT, USA), 10 units/ml of heparin (Sigma, St. Louis, MO, USA), 50 µg/ml gentamicin (Invitrogen Ltd, Paisley, UK) and 30 mg/ml of 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 (0.5-2x10⁶ 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 permeabilised 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 labelled secondary antibody for 40 min.
Confocal images were obtained using a MRC 1024 laser scanner (Bio-Rad, Hercules, CA, USA) attached to an Optiphot 2 microscope (Nikon, Garden City, NY, USA). Images were collated using Adobe Photoshop (Adobe Systems, Mountain View, CA, USA).

**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). 24 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 supernants 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 6 separate nucleofections.

**Pre and post-lysis binding experiments**

Pre-lysis 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 hours after nucleofection cells were washed in ice cold PBS and lysed in 50 mM Tris, 500 mM NaCl, 1 % Triton X-100 with protease inhibitors. Lysates were passed twice through a 25G gauge needle and centrifuged at 12,000 rpm for 10 min at 4 °C and supernatants were incubated on a rotator for 1 hour 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, 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).

Post-lysis binding: HEK-293 cells were subject to single plasmid nucleofections with ssHRP\textsuperscript{P-selectin}, ssHRP\textsuperscript{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\textsuperscript{P-selectin} or ssHRP\textsuperscript{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 visualised using a HRP conjugated anti-mouse secondary 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/10\textsuperscript{6} cells). 12 h post transfection SNX17-GFP expression was induced by the addition of doxycyclin (0.1 to 20 µg/ml) for another 12 h. Cells were then detached from culture dishes in Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-free HBSS and the resulting single cell suspension was adjusted to a final cell density of about 10,000 cells/µl. Cells were incubated for 30 min with 10 µg/ml of mouse anti-P-selectin antibody on ice. Cells were warmed to 37 °C to allow internalisation 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, USA). Flow cytometrical analysis of forward scatter light, sideward scatter light, green fluorescence (FL1) and deep red fluorescence (FL4) was performed on a
Becton & Dickinson (Mountain View, CA, USA) FacsCalibur cytometer. Data were recorded with CellQuest Pro software (Becton & 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 non-transfected control cells were gated as positives in either fluorescence detector channel.
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 co-localisation 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 following histamine stimulation of HUVECs nucleofected with SNX17-GFP were carried out. 30 min following internalisation, AK6 immunoreactivity co-localises 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 non-transfected HUVECs, AK6 internalised for 30 min is not seen in rings, but in small punctae, and by 1 h after internalisation it can be seen in ribbon-like adaptor protein 1 positive structures as previously described.

To further characterise these SNX17-positive structures, co-staining 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 co-staining organelles (Figure 1C inset) shows that the TfnR signal is concentrated in discrete patches on the perimeter membrane of SNX17-positive rings. Partial co-localisation 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 co-localisation was found with the late endosomal and lysosomal markers CD63 and LAMP1 (Figure 1E 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 localisation 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 co-localised with the TfnR (Figure 2A) and with early endosomal antigen 1 (EEA1) on ring-like structures (Figure 2C). EEA1 showed the greatest co-localisation with SNX17 that we have observed of any marker. As with HUVECs, no co-localisation 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).

While 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, since they were also seen with anti-EEA1 in non-transfected 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 while 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 localised to an endosomal compartment. P-selectin internalises into a transferrin positive endosome (Blagoveshchenskaya et al., 1998b; Blagoveshchenskaya et al., 1999; Straley and Green, 2000; Arribas and Cutler 2000) before further processing leads either to its recycling to the TGN (Straley and Green, 2000; Arribas and Cutler 2000) or to delivery to the lysosome (Green et al., 1994; Blagoveshchenskaya et al., 1998a, b; Blagoveshchenskaya et al., 2000a; Arribas and Cutler 2000). We found that internalised 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 synthesised 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 internalise 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\(^{\text{P-selectin}}\) (Blagoveshchenskaya \textit{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 analysed the effect of SNX17 on ssHRP\(^{\text{P-selectin}}\) 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 ± 0.5 % of ssHRP\(^{\text{P-selectin}}\) was clipped one day after nucleofection rising to 71.6 ± 0.6 % by day three (Figure 3A). Transient co-expression of SNX17-GFP with ssHRP\(^{\text{P-selectin}}\) reduced HRP clipping (Figure 3A) so that at one day post nucleofection only 27.8 ± 1.8 % of ssHRP\(^{\text{P-selectin}}\) was clipped, rising by day three to 52.2 ± 1.2 %. The maximal effect of SNX17-GFP was seen at two days post nucleofection, when co-expression of SNX17 caused a 42 % reduction in clipping. The effect of SNX17 on clipping of ssHRP\(^{\text{P-selectin}}\) increased with the ratio of SNX17-GFP to ssHRP\(^{\text{P-selectin}}\) 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\textsuperscript{P-selectin}. 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 (approx 5-10 %) than in HEK-293 cells (> 30 %). ssHRP\textsuperscript{P-selectin} expressed alone showed a similar HRP clipping profile to that seen in HEK-293 cells with 49.4 ± 0.8 % and 66.3 ± 0.1 % being clipped one and two days post nucleofection respectively (Figure 3C). Co-expression of SNX17-GFP reduced HRP clipping to 40.9 ± 0.1 % and 59.4 ± 0.9 % one and two days post nucleofection respectively. The maximal effect of SNX17 on ssHRP\textsuperscript{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. Firstly the lower percentage of HUVEC expressing SNX17-GFP, plus the lower level of protein expression of our constructs in HUVECs, secondly the sequestration of ssHRP\textsuperscript{P-selectin} within the WPB away from SNX17, and thirdly 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 down-regulation (Kurten et al., 1996) while it has the opposite effect on PAR (Wang et al., 2002) thus paralleling the positive effects of SNX17.
on LDL-R versus 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 a similar effect on P-selectin 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.

HEK-293 cells were transfected with ssHRP<sup>P-selectin</sup> or with ssHRP<sup>P-selectin<sub>763</sub></sup>. 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 co-expressing 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. Firstly, we confirm the interaction between both SNX17 and P-selectin seen in a yeast two-hybrid screen (Florian et al., 2001). Secondly, we report for the first time an interaction between SNX1 and P-selectin. This is found using either pre- or post-lysis interaction of the two proteins. Thirdly, 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, since 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 localisation 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 following co-expression, SNX17*15.03 can still bind P-selectin, albeit at a reduced level (about 32 %) compared to 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) since this has a direct bearing on possible mechanisms of action of SNX17. By HRP clipping analysis following co-expression, 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 internalisation of P selectin from the plasma membrane.**

By FACS analysis we discovered (data not shown) that overexpression of SNX17 reduces levels of P-selectin at the plasma membrane. This might correspond to the increased rate of LDL-R internalisation reported by Stockinger for SNX17 overexpression (Stockinger *et al* 2002). However unlike Stockinger and co-workers, 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 lead to increased internalisation of P-selectin over that of control levels. (Figure 6). In addition Fig. 6 shows that levels of internalised P selectin fall at 30 mins of warming before rising again; mostly likely due to recycling. This data indicates that SNX17 does not enhance intracellular accumulation of P-selectin by blockade of recycling.
DISCUSSION

Modulation of the endocytic behaviour of cell surface receptors by the sorting nexin family is emerging as an important aspect of their control. In this paper we examine the functional relationship between SNX17 and the leukocyte receptor P-selectin. We show that the two proteins co-localise following internalisation of P-selectin in HUVECs, and that P-selectin can bind SNX17 in HEK293 cells. We show that heterologous expression of SNX17 increases internalisation 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 co-localises with internalised P-selectin whose accumulation it causes within the endosome. In addition, membrane-associated SNX17 best co-localises with EEA1 but also overlaps with the TfnR. SNX17-GFP did not co-localise 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 co-localises 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 since there is overlap with TfnR but not with CD63 and LBPA. Interestingly, endogenous SNX15 in COS-7 cells also shows some, albeit infrequent, co-localisation in puncta with M6PR (Barr et al., 2000). However, SNX15 overexpression also leads to the presence of markers from early endosomes, late endosomes and lysosomes within the same abnormal structures (Barr et al., 2000). Our data show that while 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.

While 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 co-localising 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 co-localisation 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 since while SNX15 and SNX17 partially co-localise with EEA1, they also show association with Rab5 (Barr et al., 2000) and Rab4 (Stockinger et al., 2002) respectively. Since PI(3)P is found throughout early endosomes (Stenmark and Gillooly, 2001), specific localisation to a particular subcompartment is likely to be achieved through additional protein/protein interactions. Interestingly it has been recently shown that SNX1 localisation 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
localisation. Clearly, binding to P-selectin alone is not sufficient to drive SNX17 localisation since 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 behaviour 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. Since this alteration might itself have led to a non-specific 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. While this simplest model is attractive as explaining the retardation in lysosomal delivery, it does not easily explain the increase in internalisation that we observe, which requires a second mode of action. Further, without evoking additional binding partners it cannot explain why a 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 since 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 internalisation, recycling and degradation, the steady-state levels of receptors on the plasma membrane and the kinetics of attenuation of their signalling 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-synthesised 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, b; Blagoveshchenskaya et al., 1999; Strasser et al., 1999; Blagoveshchenskaya and Cutler, 2000a; Straley and Green, 2000; Daugherty et al., 2001; Blagoveshchenskaya et al., 2002; 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 internalisation of P-selectin. However, both Stockinger and co-workers (Stockinger et al., 2002) and ourselves see SNX17 mainly concentrated on endosomal membranes making it difficult to see how it might influence internalisation, 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 internalisation 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 co-workers[Straley and Green, 2000]) delivery to which is blocked by overexpressing SNX17, then the differential effect of SNX17 on LDL receptor versus P-selectin can thereby be explained.

In conclusion, we report that SNX17 can reduce P-selectin levels on the plasma membrane by enhancing its internalisation, 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.
Acknowledgements

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### Abbreviations list

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AK6</td>
<td>anti-P-selectin antibody</td>
</tr>
<tr>
<td>EEA1</td>
<td>early endosome antigene</td>
</tr>
<tr>
<td>FERM</td>
<td>four.1 protein, ezrin, radixin, moesin</td>
</tr>
<tr>
<td>LAMP-1</td>
<td>lysosome associated membrane protein</td>
</tr>
<tr>
<td>LBPA</td>
<td>lyso-bisphosphatidic acid</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>M6PR</td>
<td>mannose 6-phosphate receptor</td>
</tr>
<tr>
<td>PI(3)P</td>
<td>phosphatidylinositol 3-phosphate</td>
</tr>
<tr>
<td>PX</td>
<td>phox homology</td>
</tr>
<tr>
<td>SNX</td>
<td>sorting nexin</td>
</tr>
<tr>
<td>TfnR</td>
<td>transferrin receptor</td>
</tr>
<tr>
<td>WPB</td>
<td>Weibel Palade body</td>
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REFERENCES


Figure Legends

**Fig. 1.** Overexpressed SNX17 and endogenous P-selectin co-localise in endosomes of HUVECs in secretagogue stimulated cells only.  (A) P-selectin and SNX17-GFP do not co-localise in resting HUVECs. HUVECs were nucleofected with SNX17-GFP (5 µg). One day later, cells were stained with anti-P-selectin antibody (AK6).  (B) Endocytosed anti-P-selectin antibody co-localises with SNX17. One day post nucleofection with SNX17-GFP (5 µg) HUVECs were stimulated with histamine (10\(^{-4}\) M) for 15 min in the presence of anti-P-selectin antibody AK6 (10 µg/ml). After a 30 min incubation in RM (Methods) cells were fixed and processed for microscopy.  (C-F) Localisation of SNX17 overexpressed in HUVECs.  HUVECs were nucleofected with SNX17-GFP (5 µg) and one day post nucleofection cells were processed for microscopy and stained with the indicated marker:  (C) TfnR,  (D) M6PR,  (E) CD63 and (F) LAMP1. For all images primary antibodies were visualised with relevant donkey secondary antibodies conjugated with Texas-Red as described in materials and methods. Bars, 10 µm.

**Fig. 2.** Overexpressed SNX17 is localised to early endosomes in HEK-293 cells. One day post nucleofection with SNX17-GFP (5 µg) 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 visualised with relevant donkey secondary antibodies conjugated with Texas-Red as described in methods and materials. Bars, 10 µm.
Fig. 3. SNX17 overexpression reduces the clipping of HRP-P-selectin. (A) HEK-293 cells nucleofected with either ssHRP$^\text{P-selectin}$ (■) or SNX17-GFP and ssHRP$^\text{P-selectin}$ (□) were seeded onto 60 mm plates. At one to three days after nucleofection cells were lysed, Triton X-114 partitioned and HRP activity in aqueous and detergent phases determined as described in materials and methods. The data is presented as percentage of total activity present in the aqueous phase (i.e. clipped HRP). (B) HEK-293 cells were nucleofected with either ssHRP$^\text{P-selectin}$ or SNX17-GFP and ssHRP$^\text{P-selectin}$ at a 2:1 (■); 5:1 (□) and 10:1 (■) ratio of DNA. Cells were seeded onto 60 mm dishes and lysed, Triton X-114 partitioned and HRP activity in aqueous and detergent phases determined at one and two days post nucleofection. Data is shown as difference between the percentage of HRP activity in aqueous phase for cells nucleofected with ssHRP$^\text{P-selectin}$ and that for cells nucleofected with SNX17-GFP and ssHRP$^\text{P-selectin}$. (C) HUVEC were nucleofected with either ssHRP$^\text{P-selectin}$ (■) or SNX17-GFP and ssHRP$^\text{P-selectin}$ (□) and seeded onto 100 mm plates. Cells were then treated as in A. Each bar represents the mean ± standard deviation of six independent determinations.

Fig. 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$^\text{P-selectin}$ or with ssHRP$^\text{P-selectin763}$ 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 co-expressing the two potential partners (pre-lysis) or in extracts from cells expressing the
two components separately that were mixed after lysis (post lysis). His-tagged complexes were isolated using Probond, eluted from the beads, and the HRP activity determined as in materials and methods. Data is presented as a percentage of the binding of SNX17 and to ssHRP$^{p-selectin}$ for either method, (+/- Std error) 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 visualised using an HRP conjugated 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 is expressed as a percentage of the HRP clipping activity of SNX17.

**Fig. 5.** Both PI3 kinase activity and the PX domain of SNX17 are needed for endosomal localisation, 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 domainKnown 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 (data not shown) cells nucleofected with SNX17-GFP one day post nucleofection 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 one day post nucleofection and examined by confocal microscopy. Bars, 10 µm (C) HEK-293 cells were co-nucleofected with ssHRP\textsuperscript{P-selectin} and pSNX17-HH or pDHH-SNX17*15.03. After 24 hours His-tagged complexes were isolated using ProBond, eluted from the beads and the HRP activity determined as in materials and methods. Data is expressed as a percentage of the binding ssHRP\textsuperscript{P-selectin} and pSNX17-HH. Each bar represents the mean ± standard error of at least 5 independent determinations. (D) HEK-293 cells were co-nucleofected with ssHRP\textsuperscript{P-selectin} and pSNX17-HH or pDHH-SNX17*15.03 and after 24 hours were subjected to TX114 partitioning. HRP activity in aqueous and the detergent phases were measured and the clipping activity determined. Data is expressed as a percentage of clipping that occurs with ssHRP\textsuperscript{P-selectin} and pSNX17-HH obtained from five independent experiments.

**Fig. 6.** SNX17 expression enhances the internalisation 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 µg/ml doxycyclin for 12 h.. Cells were labelled with anti-P-selectin antibody on ice, warmed for indicated times at 37 °C to allow internalisation then fixed, stained with Alexa 647-labelled secondary antibody and analysed by flow cytometry as described in materials and methods. Data points shown are means (+/-) Standard error from three independent experiments are shown from control
cells ( ), cells gated as expressing low SNX17-GFP ( ) and cells gated as expressing high levels of SNX-GFP ( ).
A

B

C

ssHRP-selectin + SNX17

ssHRP-selectin + SNX1

ssHRP-selectin + control

ssHRP-selectin763 + SNX17

Relative % binding

Relative % clipping

SNX: 1 1 17 17

Post lysis

Pre lysis

pSNX17-HH + ssHRP-selectin

pSNX17-HH + ssHRP-selectin 763

pDHH-SNX1 + ssHRP-selectin

DHPSNX1 + ssHRP-selectin 763

Relative % clipping
A

B

C

D

pSNX17-HH + ssHRPP-selectin
pDHH-SNX17*15.03 + ssHRPP-selectin

% Binding
Relative % clipping

C

D

pSNX17-HH + ssHRPP-selectin
pDHH-SNX17*15.03 + ssHRPP-selectin

A

B

C

D