Determinants in the cytoplasmic domain of P-selectin required for sorting to secretory granules

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P-selectin is a granule membrane protein of platelets and endothelial cells that is expressed at the plasma membrane after cell activation. To determine which residues in its cytoplasmic tail are important for sorting to storage granules during biosynthesis, we expressed P-selectin mutants in AtT-20, a murine cell line with secretory granules that contain the hormone corticotropin ('ACTH'). Immunofluorescence microscopy of permeabilized cells revealed that wild-type P-selectin and mutants with alanine substitutions at 14 different positions in the cytoplasmic tail were concentrated in the tips of the cellular processes, which contain the majority of corticotropin granules. However, targeting to the cell tips was greatly decreased for Tyr⁷⁷⁷ → Ala, Tyr⁷⁷⁷ → Phe, Gly⁷⁷⁸ → Ala, Phe⁷⁸⁰ → Ala and Leu⁷⁶⁸/Asn⁷⁶⁹ → Ala/Ala mutants. The reduced presence of these mutants in

corticotropin granules was confirmed by immunoelectron microscopy. Stimulation of AtT-20 transfectants with 8-Br-cAMP resulted in a significant increase in membrane expression of wild-type P-selectin, but in only a marginal increase in the surface expression of the five mutants. Antibody binding studies with intact and permeabilized cells demonstrated that the percentage of P-selectin that is expressed on the surface of the cells was considerably higher for these mutants than for wild-type P-selectin (6%), ranging from ~20% for the Gly⁷⁷⁸ and Phe⁷⁸⁰ mutants to 63% for the Leu⁷⁶⁸/Asn⁷⁶⁹ mutant. Taken together, these results indicate that Tyr⁷⁷⁷, Gly⁷⁷⁸ and Phe⁷⁸⁰ form part of an atypical tyrosine-based motif, which also requires the presence Leu⁷⁶⁸ and/or Asn⁷⁶⁹ to mediate sorting of P-selectin to secretory granules.

INTRODUCTION

In cells with regulated secretion, protein storage granules fuse with the plasma membrane in response to a stimulus, resulting in the release of soluble proteins and in the cell-surface expression of membrane proteins. P-selectin is a 140 kDa membrane glycoprotein that is stored in platelet α granules and in Weibel–Palade bodies of endothelial cells. In response to activation of these cells with thrombin or other strong agonists, P-selectin is translocated to the cell surface, where it functions as a receptor for PSGL-1 (P-selectin glycoprotein ligand-1), a ligand that is present on most leucocytes [1–3].

Various studies have indicated that information required for intracellular trafficking of P-selectin is located in its 35-amino-acid cytoplasmic tail, which is composed of a 'stop transfer' region (residues 755–761) followed by the C1 domain (residues 762–772) and the C2 domain (residues 773–789). Disdier et al. [4] showed that deletion of the C-terminal 23 amino acids of P-selectin abrogated its sorting to corticotropin ('ACTH') storage granules in the mouse pituitary cell line AtT-20. More recent experiments with cytoplasmic tail deletion mutants indicated that information required for the sorting of P-selectin to storage granules in PC12 cells is present within the sequences comprising residues 763–772 and 777–782 [5]. The signal that determines the rapid internalization of P-selectin at the plasma membrane may consist of amino acids scattered throughout the cytoplasmic domain [6]. Finally, information required for rapid transport of P-selectin to lysosomes after internalization has been shown to

be present in the C1 domain [7], in which a putative Lys-Cys-Pro-Leu motif containing the critical Pro⁷⁶⁷ residue has recently been identified [8].

Over the past few years, much has been learned about the nature of trafficking signals of membrane proteins and about the machinery involved in their recognition. These signals are often short linear sequences in the cytoplasmic tail of the molecules, such as the dileucine motifs and the tyrosine-based signals, e.g. the Asn-Pro-Xaa-Tyr sequences and the Tyr-Xaa-Xaa- Φ (YXX Φ)-like signals (where Xaa or X denotes any amino acid and Φ is a large hydrophobic residue). While all three types of signal mediate internalization of proteins from the plasma membrane, both YXX Φ and dileucine-type motifs also function in other forms of membrane trafficking, such as the sorting of proteins from the *trans*-Golgi network (TGN) to endosomes, lysosomes, basolateral surfaces of polarized epithelial cells and specialized organelles [9–11]. *In vitro*, both YXX Φ and dileucine signals have been found to bind to the medium (μ 2) chain of the adaptor protein-2 (AP2) complex [12–14], the heterotetrameric protein complex that is associated with the clathrin coat around endocytic vesicles at the plasma membrane. Similarly, some YXX Φ and dileucine motifs interact *in vitro* with the μ 1 chain of the AP1 complex, which is present on clathrin-coated vesicles that transport proteins out of the TGN [12–14].

Transport of constitutively expressed membrane proteins from the TGN to the plasma membrane is generally believed to be independent of sorting signals, and is mediated by the ill-defined constitutive secretory vesicles. Sorting of membrane proteins

Abbreviations used: AP, adaptor protein; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hanks' balanced salt solution; TGN, *trans*-Golgi network.

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from the TGN to storage granules, on the other hand, may involve recognition of specific signals by unknown adaptins in the nascent secretory granules at the surface of the TGN. Alternatively, the major sorting step may be the retrieval of proteins destined for other locations from maturing granules (the 'sorting by retention' hypothesis [15]) via API containing, clathrin-coated vesicles, as has been shown for the TGN-associated membrane protein furin [16].

As yet, signals that sort membrane proteins to storage granules have not been defined. To characterize further sorting information for P-selectin, we have performed site-directed mutagenesis, replacing amino acids in the cytoplasmic domain with alanine. The effect of these mutations on the targeting to storage granules was studied by expressing the mutants in AtT-20 cells. Our results show the presence of at least two regions in the cytoplasmic domain of P-selectin that contribute to the sorting signal. One of these comprises the C2 domain sequence Tyr⁷⁷⁷-Gly⁷⁷⁸-Phe⁷⁸⁰, while a second region involves Leu⁷⁶⁸ and/or Asn⁷⁶⁹ in the C1 domain of the cytoplasmic tail.

MATERIALS AND METHODS

Antibodies

Mouse monoclonal anti-P-selectin antibodies RUU 1.18 (kindly provided by Dr. H. K. Nieuwenhuis, University Hospital, Utrecht, The Netherlands) and CLB-thromb/6 have been described previously [17]. A rabbit antiserum named 'Kathy', containing antibodies specific for corticotropin [18], was kindly donated by Dr. R. E. Mains (Johns Hopkins University, Baltimore, MD, U.S.A.).

Cells

AtT-20/D16v-F2 mouse pituitary tumour cells were obtained from the American Type Culture Collection (A.T.C.C. no. CRL 1795). AtT-20 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Gibco-BRL, Grand Island, NY, U.S.A.) supplemented with 10 % (v/v) foetal bovine serum (Gibco), 4 mM glutamine, non-essential amino acids (Gibco), 100 units/ml penicillin and 100 µg/ml streptomycin, in a humidified atmosphere of 15 % CO₂ at 37 °C.

Generation of P-selectin C2 domain alanine substitution mutants

P-selectin cDNA, amplified by PCR from an endothelial cDNA library and inserted into *XbaI*/*Bam*HI-digested expression vector pSVL (Pharmacia LKB, Uppsala, Sweden), was a gift from Dr. J. Voorberg and Dr. R. D. Fontein (CLB, Amsterdam, The Netherlands). The cDNA differed from the published sequence [19] at three positions, leading to two amino acid substitutions, both in the extracellular domain of the molecule: T⁵³⁵ → A (Met¹²⁵ → Lys), A²⁰⁸⁵ → T (Thr⁶⁴² → Ser) and A²³⁸⁴ → G. After subcloning of the cDNA into expression vector pcDNA3 (see below) and expression in CHO cells, P-selectin containing these mutations mediates the binding of human neutrophils and human myeloid cell line cells U937 and HL-60 (P. W. Modderman and E. A. Beuling, unpublished work). Site-directed mutagenesis was performed by the PCR overlap extension method [20], using two outer primers, 5' CTCAGGAAACAGGGTTGGTCC 3' (nt 2610–2630; antisense) and 5' GATGGTCAGCTACTCCACCAACC 3' (nt 1900–1922; sense), and twelve sets of complementary mutagenic primers for the production of alanine substitution mutants (see Figure 1). PCR reactions were performed with *Pfu* polymerase (Stratagene, La Jolla, CA, U.S.A.). For the first three mutants (Tyr⁷⁷⁷ → Phe, Tyr⁷⁷⁷ → Ala and

Figure 1 Mutants used to study P-selectin sorting

The P-selectin cytoplasmic domain is depicted, showing the stop transfer (ST) and the C1 and C2 domains; the latter two are encoded by exons 15 and 16 respectively. In the upper part of the figure, alanine substitutions of a number of residues in the C2 domain are indicated, as is the Tyr⁷⁷⁷ → Phe substitution. The lower part of the figure displays the C1 domain deletion mutant (ΔC1) and the PS(HL) construct; the latter is identical to wild-type P-selectin except for the presence of a His-Leu dipeptide (HL) between the ST and C1 domains. In addition, multiple and single alanine substitutions of PS(HL) are shown.

Ser⁷⁸⁸ → Ala), the PCR-amplified mutagenic fragments were digested with *XbaI* and *Afl*II, gel-purified and ligated to *XbaI*/*Afl*II-digested pSVL from which the corresponding wild-type cDNA fragment had been excised. The cDNA for wild-type and mutant P-selectins was subsequently subcloned into the *Hind*III and *Bam*HI sites in expression vector pcDNA3 (Invitrogen, San Diego, CA, U.S.A.). Additional C2 domain mutants were produced using pcDNA3.P-selectin and the methods described above, except that *Xcm*I and *Afl*II digestion was used for insertion of the mutant cDNA fragments. In all cases, sequencing was performed to verify that the inserted DNA fragments were correct.

Generation of P-selectin C1 domain mutants

A C1 domain (residues 762–772) deletion mutant (ΔC1) was prepared by splice overlap extension-PCR [21], using the two outer primers described above and two mutagenic primers: 5' agacaaaaCACCTAGGAACATATGGAGTT 3' (corresponding to codons 759–761 followed by codons 773–779) and 5'

tcctaggtgTTTTGTCTGAAACGCTTCT 3' (corresponding to codons 775–773 followed by codons 761–755). *XcmI* and *AflII* digestion was used to exchange the mutant cDNA fragment with the corresponding wild-type fragment of P-selectin cDNA. Additional mutants were prepared by ligating synthetic oligonucleotides coding for mutant C1 domains into Δ C1 cDNA digested with *AflII* at P-selectin position 2479. For example, to restore the wild-type C1 domain, the following oligonucleotides were used: 5' CTAGATGATGGGAAATGCCCTTGAATC-CTCACAGCCAT 3' and 5' CTAGATGGCTGTGAGGATT-CAAGGGGATTTCCCATCAT 3'. The resulting cDNA encodes a cytoplasmic domain that is identical to that of wild-type P-selectin, except for the presence of two amino acids (His and Leu) that are inserted between the stop transfer region and the C1 domain (see Figure 1). All C1 domain alanine substitution mutants were prepared similarly, and all contained the inserted His-Leu dipeptide. Sequencing confirmed that the inserted DNA fragments were correct for all mutants.

Transfections

AtT-20 cells were detached by trypsinization, washed once with complete DMEM and three times with serum-free RPMI medium (Gibco), and resuspended at 10^7 cells/ml in RPMI (0 °C). Wild-type and mutant P-selectin cDNAs subcloned in pcDNA3 (10 μ g) were transfected into AtT-20 cells (2.5×10^6 cells) by electroporation using the Bio-Rad gene pulser set at 230 V and 960 μ F. Electroporated cells were plated in T75 flasks in DMEM and allowed to recover for 2 days before selection with 0.4 mg/ml Geneticin (G418 sulphate; Gibco). Geneticin-resistant colonies were picked after 10–14 days of selection and screened for P-selectin expression by immunofluorescence staining of permeabilized cells.

Immunofluorescence staining

AtT-20 transfectants grown on coverslips were washed once with Hanks' balanced salt solution (HBSS; Gibco) and fixed by incubation for 10 min at room temperature in HBSS/1% (v/v) paraformaldehyde. Cells were then washed three times with HBSS/1% (w/v) BSA and either incubated in HBSS/BSA (intact cells) or permeabilized by incubation in HBSS/BSA containing 0.02% (w/v) saponin for 30 min at room temperature. Subsequent incubation and washing steps were performed with either HBSS/BSA or HBSS/BSA/saponin. Cells were incubated for 30 min with anti-P-selectin antibody CLB-thromb/6 (1:250 dilution of ascites), washed three times and incubated for 30 min with FITC-labelled goat anti-(mouse Ig) antibodies (CLB, Amsterdam, The Netherlands). The coverslips were then washed three times and mounted for fluorescence microscopy. The cells were viewed under a Zeiss microscope (Carl Zeiss Inc., Thornwood, NY, U.S.A.) equipped with a confocal scanning laser microscope (Bio-Rad, Richmond, CA, U.S.A.).

Immunoelectron microscopy

Transfected AtT-20 cells were fixed with a mixture of 0.5% (v/v) glutaraldehyde and 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 h and pelleted in 10% (w/v) gelatin in PBS. Ultrathin frozen sections were incubated at room temperature with a mixture of rabbit anti-corticotropin antiserum Kathy (1:1000 dilution) and anti-P-selectin monoclonal antibody RUU 1.18 (ascites; 1:100), followed by incubation with a mixture of goat anti-(rabbit IgG) coupled to 5 nm gold and goat anti-(mouse IgG) coupled to 10 nm gold, both diluted 1:40 (Amersham, 's-Hertogenbosch, The Netherlands). In control experi-

ments, primary antibodies were replaced by non-relevant rabbit or mouse antibodies respectively. After immunolabelling, the cryosections were embedded in a mixture of methylcellulose and uranyl acetate and examined with a CM10 electron microscope (Philips, Eindhoven, The Netherlands). Targeting of P-selectin was quantified by determining the percentage of corticotropin-positive granules also containing P-selectin. For these assays, several cells, together containing at least 100 corticotropin granules, were examined.

Binding of 125 I-labelled monoclonal antibodies to intact and permeabilized cells

AtT-20 transfectants were seeded at 10^5 cells/well in 24-well plates, grown for 2 days, fixed with paraformaldehyde and either left intact or permeabilized with 0.02% (w/v) saponin as described above. For the determination of cell-surface and total cellular P-selectin, the cells were incubated, in triplicate, with a saturating concentration (3 μ g/ml) of 125 I-labelled antibody CLB-thromb/6 diluted in HBSS/BSA or HBSS/BSA/saponin for 30 min at room temperature. Cells were then washed three times with the corresponding buffer and lysed by addition of 0.5 ml of 2% (w/v) SDS. Binding was determined by measuring radioactivity in the cell lysates with a γ -radiation counter. Non-specific binding was assessed by performing the assay in the presence of a 50-fold excess of unlabelled CLB-thromb/6.

To determine the effect of activation of AtT-20 cells on P-selectin surface expression, AtT-20 transfectants were incubated, in triplicate, for 2 h at 37 °C and at 5% CO₂ in medium containing 3 μ g/ml 125 I-labelled CLB-thromb/6 with or without the agonist 8-Br-cAMP (5 mM). The cells were washed three times with HBSS/1% BSA before they were lysed; antibody binding was determined as described above for fixed cells. Non-specific binding was assessed as above. The 8-Br-cAMP-induced increase in specific binding was calculated as: (binding to stimulated cells minus binding to control cells)/binding to control cells $\times$ 100%.

RESULTS

Cellular localization of Tyr⁷⁷⁷ mutants detected by immunofluorescence staining

Tyrosine-based motifs are known to be important for trafficking of membrane proteins [9–11]. The cytoplasmic domain of P-selectin contains a single tyrosine, which is part of a potential YXX Φ -like motif (Tyr⁷⁷⁷-Gly⁷⁷⁸-Val⁷⁷⁹-Phe⁷⁸⁰; Figure 1). To study the role of Tyr⁷⁷⁷ in membrane trafficking, we replaced Tyr⁷⁷⁷ with either alanine (Tyr⁷⁷⁷ $\rightarrow$ Ala) or phenylalanine (Tyr⁷⁷⁷ $\rightarrow$ Phe), as phenylalanine can replace the tyrosine residue in some, but not all, tyrosine-based signals [9]. Wild-type and mutant P-selectins were stably expressed in mouse pituitary AtT-20 cells and their subcellular distribution was studied by immunofluorescence microscopy. Consistent with previous observations [4,22], wild-type P-selectin appeared to be concentrated in the tips of the cell processes when saponin-permeabilized cells were incubated with the P-selectin monoclonal antibodies CLB-thromb/6 (Figure 2) or RUU 1.18 (results not shown). Less intense granular staining was observed throughout the rest of the cytoplasm, while staining of the cell surface was very weak. A similar staining pattern was seen for Ser⁷⁸⁸ $\rightarrow$ Ala P-selectin, a mutant in which Ser⁷⁸⁸, the major phosphorylated serine residue in platelet P-selectin [23], was replaced with alanine (Figure 2). In contrast, the two Tyr⁷⁷⁷ mutants, Tyr⁷⁷⁷ $\rightarrow$ Ala and Tyr⁷⁷⁷ $\rightarrow$ Phe, were not concentrated in the tips of the cell processes; however, as for wild-type P-selectin, granular staining was seen throughout

Figure 2 Distribution of wild-type and mutant P-selectins in permeabilized AtT-20 cells

AtT-20 transfectant cells were fixed, permeabilized with saponin and stained by incubation with a monoclonal antibody against P-selectin, followed by incubation with FITC-labelled goat anti-(mouse Ig). Wild-type (WT) and Ser⁷⁸⁸ → Ala (S788A) P-selectins are strongly expressed in the tips of the cell processes. Weaker granular staining is observed throughout the cytoplasm. Staining of the Tyr⁷⁷⁷ → Phe (Y777F) and Tyr⁷⁷⁷ → Ala (Y777A) mutants is seen in cytoplasmic granules and at the cell surface, as determined by focusing on different levels of the cells. Bar = 50 µm.

Figure 3 Expression of wild-type and mutant P-selectins on the surface of intact AtT-20 cells

Paraformaldehyde-fixed cells were stained by incubation with anti-P-selectin monoclonal antibody, followed by incubation with FITC-labelled goat anti-(mouse Ig). In contrast with wild-type (WT) and Ser⁷⁸⁸ → Ala (S788A) P-selectins, both Tyr⁷⁷⁷ mutants (Y777F and Y777A) are strongly expressed at the cell surface. Bar = 50 µm.

the cytoplasm. In addition, the Tyr⁷⁷⁷ mutants displayed appreciable cell-surface staining (Figure 2). The stronger cell-surface expression of the Tyr⁷⁷⁷ mutants compared with that of wild-type and Ser⁷⁸⁸ → Ala P-selectins was even more evident when intact, paraformaldehyde-fixed cells were used for immunofluorescence staining (Figure 3). Much of the granular cytoplasmic staining observed for wild-type and mutant P-selectins is

Figure 4 Localization of wild-type P-selectin and the Ser⁷⁸⁸ → Ala mutant compared with localization of corticotropin

Immunogold double-labelling of frozen thin sections of AtT-20 cells transfected with wild-type (a) or Ser⁷⁸⁸ → Ala (b) P-selectin was performed with rabbit antiserum Kathy against corticotropin and monoclonal antibody RUU 1.18 against P-selectin. Labelling of both proteins was restricted to granules (g). Corticotropin (5 nm gold) was dispersed in the granular matrix, whereas P-selectin was predominantly observed near the granule membranes (10 nm gold; arrows). P-selectin staining was also present in structures (v) resembling 'condensing vacuoles' (see the text). Bar = 100 nm.

probably due to their presence in the endosomal compartment as a result of internalization at the plasma membrane. Indeed, incubation of AtT-20 transfectants with antibody CLB-thromb/6 for 30 min at 37 °C, followed by fixation, permeabilization and staining with FITC-labelled goat anti-(mouse Ig), revealed granular cytoplasmic fluorescence similar to that seen with directly fixed permeabilized cells (results not shown).

Ultrastructural localization of Tyr⁷⁷⁷ mutants

The tips of the long cellular projections of AtT-20 cells are known to contain storage granules for corticotropin, a hormone that is released upon stimulation of the cells with agonists such as 8-Br-cAMP [24]. Previous ultrastructural studies [4,22] have demonstrated that P-selectin is co-localized with corticotropin in these granules. Figure 4 shows that both wild-type and Ser⁷⁸⁸ → Ala P-selectins are present in organelles that also stain for corticotropin in immunoelectron microscopy. For wild-type P-selectin, 23 % of corticotropin-containing granules stained for P-selectin, compared with 27 % for Ser⁷⁸⁸ → Ala P-selectin. P-selectin was also detected in the Golgi apparatus and in structures resembling 'condensing vacuoles' (cf Figure 4a), large dilations of the TGN of AtT-20 cells previously described by Tooze and Tooze [25]. Furthermore, P-selectin staining was occasionally observed in granules presumed to correspond to endocytic vesicles, sometimes displaying the morphology of multivesicular bodies. Very little surface staining was observed. Both Tyr⁷⁷⁷ mutants, Tyr⁷⁷⁷ → Ala and Tyr⁷⁷⁷ → Phe, were also detected in the Golgi compartment and in the endocytic-like vesicles. However, P-selectin staining of corticotropin-positive granules was

Figure 5 Localization of the Tyr⁷⁷⁷ → Ala and Tyr⁷⁷⁷ → Phe P-selectin mutants compared with localization of corticotropin

The cellular localization of corticotropin and P-selectin in AtT-20 cells transfected with the Tyr⁷⁷⁷ → Ala (a) or Tyr⁷⁷⁷ → Phe (b) mutants was examined by double-labelling performed as described for experiments depicted in Figure 4. For both mutants, the granules (g), numerous at the tips of the cell processes, were labelled for corticotropin only (5 nm gold). Label for P-selectin (10 nm gold; arrows) was predominantly present at the cell surface. Bar = 100 nm.

Table 1 Ratio of surface-expressed to total P-selectin for wild-type and mutant molecules

P-selectin expression on the surface of AtT-20 transfectant cells was determined by measuring the specific binding of ¹²⁵I-labelled anti-P-selectin monoclonal antibody CLB-thromb/6 to paraformaldehyde-fixed cells. Total P-selectin expression was measured with paraformaldehyde-fixed cells permeabilized with saponin. The data represent means ± S.D. of the ratio of surface to total P-selectin expression, with the number of assays performed indicated in parentheses.

P-selectin	(Surface-expressed P-selectin/total P-selectin) × 100 %
Wild type	5.8 ± 1.3 (6)
Ser ⁷⁸⁸ → Ala	7.0 ± 1.6 (4)
Tyr ⁷⁷⁷ → Ala	40.3 ± 10.4 (4)
Tyr ⁷⁷⁷ → Phe	28.0 ± 8.8 (5)
Gly ⁷⁷⁸ → Ala	21.8 ± 1.1 (3)
Phe ⁷⁸⁰ → Ala	21.4 ± 5.7 (3)
Leu ⁷⁶⁸ /Asn ⁷⁶⁹ → Ala/Ala	63.3 ± 4.5 (3)
ΔC1	49.1 ± 4.1 (4)

reduced when compared with that of wild-type P-selectin: for both the Tyr⁷⁷⁷ → Ala and the Tyr⁷⁷⁷ → Phe mutants, only 2 % of the corticotropin-containing granules were positive for P-selectin. Staining for these mutants at the plasma membrane was more extensive than that for wild-type or Ser⁷⁸⁸ → Ala P-selectins (Figure 5).

Determination of surface and total Tyr⁷⁷⁷ mutant expression

To quantify the proportion of total cellular P-selectin expressed at the cell surface, ¹²⁵I-labelled monoclonal antibody binding studies were performed with fixed intact and fixed saponin-permeabilized cells. The surface expression of P-selectin as a

percentage of total expression is indicated in Table 1. For wild-type and Ser⁷⁸⁸ → Ala P-selectins, only 6 and 7 % respectively of the total amount of the molecule appeared to be present at the cell surface. In contrast, larger proportions of the Tyr⁷⁷⁷ → Ala and Tyr⁷⁷⁷ → Phe mutants were expressed at the plasma membrane (40 and 28 % respectively; Table 1), consistent with the notion that the bulk of these two mutant proteins may be directly transported from the TGN to the cell surface during biosynthesis. The relatively large proportion of both Tyr⁷⁷⁷ mutants that is nevertheless detected intracellularly (60–70 %) most likely represents newly synthesized P-selectin present in the Golgi apparatus and internalized P-selectin present in the endosomal compartment. Differences between wild-type and mutant P-selectins were not due to different levels of expression, as the binding of ¹²⁵I-labelled antibody CLB-thromb/6 to equal numbers of permeabilized wild-type and mutant P-selectin transfectants differed by a factor less than 1.5.

Activation-induced surface expression of Tyr⁷⁷⁷ mutants

Previous studies have shown that, concomitant with corticotropin release, increased surface expression of P-selectin occurs after stimulation of AtT-20 transfectants with the cAMP analogue 8-Br-cAMP [4,22]. However, whereas redistribution of P-selectin to the plasma membranes of activated platelets and endothelial cells is very rapid [1], complete release of corticotropin from stimulated AtT-20 cells takes several hours. Release of P-selectin was therefore measured by incubating cells for 2 h with ¹²⁵I-labelled antibody (CLB-thromb/6), in the presence or absence of 8-Br-cAMP. Since plasma membrane P-selectin is rapidly internalized by endocytosis [6,26,27], 'cell-associated' radioactivity in these experiments represents the sum of surface-bound and internalized antibody. Initial experiments indicated that, even in the absence of 8-Br-cAMP, the amount of antibody that became associated with the wild-type P-selectin cells during the 2 h incubation period was approx. 20-fold greater than the amount bound to paraformaldehyde-fixed cells (results not shown). Thus significant amounts of P-selectin are expressed at the plasma membrane (and subsequently internalized) during this period, presumably as a result of the 'basal' [28] or 'constitutive-like' [29] secretion of storage granules that is known to occur in AtT-20 cells. 8-Br-cAMP increased the levels of ¹²⁵I-labelled CLB-thromb/6 associated with wild-type and Ser⁷⁸⁸ → Ala P-selectin-transfected cells by 44 and 56 % respectively (Table 2). Similar results have been reported for wild-type P-selectin by Disdier et al. [4]. In contrast, the levels of ¹²⁵I-labelled CLB-thromb/6 associated with the Tyr⁷⁷⁷ → Phe and Tyr⁷⁷⁷ → Ala mutant cells were not affected by the presence of 8-Br-cAMP (Table 2). Thus these data are consistent with the notion that significant amounts of wild-type and Ser⁷⁸⁸ → Ala P-selectins, but not of the Tyr⁷⁷⁷ mutant P-selectins, are present in the corticotropin-containing storage granules and become expressed at the cell surface during activation.

Alanine substitution of additional residues in the C2 domain of P-selectin

The data presented so far indicate that Tyr⁷⁷⁷ is critical for the sorting of P-selectin to storage granules. As mentioned above, Tyr⁷⁷⁷ is part of a potential YXXΦ-type sorting signal (Tyr⁷⁷⁷-Gly⁷⁷⁸-Val⁷⁷⁹-Phe⁷⁸⁰). In addition to such typical four-amino-acid-type YXXΦ motifs, however, signals have been described in which amino acids N-terminal to the tyrosine are also critical for sorting [9–11]. Therefore, to study the role of residues around Tyr⁷⁷⁷, we prepared alanine substitution mutants for Leu⁷⁷⁴, Gly⁷⁷⁵ and Thr⁷⁷⁶, and for Gly⁷⁷⁸, Val⁷⁷⁹ and Phe⁷⁸⁰. Immuno-

Table 2 Stimulation of cell-surface expression of wild-type and mutant P-selectins by 8-Br-cAMP

AtT-20 transfectants were incubated for 2 h at 37 °C in complete culture medium in the presence of ^{125}I -labelled antibody CLB-thromb/6, in either the absence or the presence of 5 mM 8-Br-cAMP. The percentage increase in specific ^{125}I -CLB-thromb/6 binding to stimulated cells relative to the binding to untreated cells is indicated; results are means $\pm$ S.D., with the numbers of experiments indicated in parentheses.

P-selectin	$\frac{(\text{Bound to 8-Br-cAMP cells}) - (\text{bound to untreated cells})}{(\text{Bound to untreated cells})} \times 100\%$
Wild type	44 $\pm$ 9 (4)
Ser ⁷⁸⁸ $\rightarrow$ Ala	56 $\pm$ 14 (4)
Tyr ⁷⁷⁷ $\rightarrow$ Ala	0 $\pm$ 3 (4)
Tyr ⁷⁷⁷ $\rightarrow$ Phe	0 $\pm$ 6 (5)
Gly ⁷⁷⁸ $\rightarrow$ Ala	4 $\pm$ 9 (6)
Phe ⁷⁸⁰ $\rightarrow$ Ala	7 $\pm$ 8 (6)
Leu ⁷⁶⁸ /Asn ⁷⁶⁹ $\rightarrow$ Ala/Ala	3 $\pm$ 3 (3)

Figure 6 Distribution of P-selectin C2 domain mutants in permeabilized AtT-20 cells

The distribution of C2 domain alanine substitution mutants in AtT-20 cells was studied as described in the legend to Figure 2. Mutants are designated using one-letter symbols for amino acids, e.g. L774A is the notation for the Leu⁷⁷⁴ $\rightarrow$ Ala mutant. While most mutants are predominantly present in the tips of the cell processes, stronger granular staining of other parts of the cytoplasm is seen for the Gly⁷⁷⁸ $\rightarrow$ Ala and Phe⁷⁸⁰ $\rightarrow$ Ala mutants. Bar = 25 μm .

fluorescence staining of permeabilized AtT-20 transfectants expressing these mutants is shown in Figure 6. With the exception of the mutants of Gly⁷⁷⁸ and Phe⁷⁸⁰ (see below), staining of all of the transfected mutant molecules was clearly concentrated in the tips of the cell processes, with less intense granular staining in other regions of the cytoplasm, similar to the distribution of the wild-type molecule (cf. Figure 2). Cell-surface expression, assessed by immunofluorescence staining of intact cells, was not detectable for any of these mutants.

A second potential targeting signal in the C2 domain is suggested by comparison of the sequence of P-selectin [19] with those of two other human granule-associated membrane proteins, vacuolar H⁺-ATPase membrane subunit Ac45 [30] and peptidyl-glycine α -amidating mono-oxygenase [31]. A common motif in the cytoplasmic domains of these proteins is S/TXXXFD, corresponding to P-selectin Thr⁷⁸¹-Asn-Ala-Ala-Phe⁷⁸⁵-Asp⁷⁸⁶. Immunofluorescence staining of permeabilized AtT-20 cells expressing mutants with alanine substitution of each of the consensus residues in the putative S/TXXXFD motif showed that all three mutants were concentrated in the tips of the cell processes (Figure 6), while no staining of fixed intact cells was detectable (results not shown). Thus the data show that the consensus residues of the proposed S/TXXXFD motif are not critical for targeting of P-selectin to secretory granules.

Characterization of Gly⁷⁷⁸ $\rightarrow$ Ala and Phe⁷⁸⁰ $\rightarrow$ Ala mutants

For both the Gly⁷⁷⁸ $\rightarrow$ Ala and Phe⁷⁸⁰ $\rightarrow$ Ala mutants, concentration of fluorescence staining at the tips of the cell processes was observed in only a minority of the cells (Figure 6). In the great majority of the cells, however, granular staining of other regions of the cytoplasm appeared to be much more intense. For both mutants, this pattern of immunofluorescence staining was observed with a number of independent clones, obtained in separate transfections. Cell-surface staining was weak for both mutants. Immunoelectron microscopy showed that only a few corticotropin-containing granules in the Gly⁷⁷⁸ $\rightarrow$ Ala and Phe⁷⁸⁰ $\rightarrow$ Ala transfectants (3 and 4% respectively) also stained for P-selectin, compared with 23% for wild-type P-selectin (see above).

We further measured the recruitment of granule-contained P-selectin to the plasma membrane induced by 8-Br-cAMP. The secretagogue increased the amount of ^{125}I -labelled antibody CLB-thromb/6 associated with the Gly⁷⁷⁸ $\rightarrow$ Ala and Phe⁷⁸⁰ $\rightarrow$ Ala transfectants by approx. 4 and 7% respectively (Table 2). These values are significantly lower than those observed for wild-type P-selectin (44%), but slightly higher than those found for the two Tyr⁷⁷⁷ mutants (0%; Table 2). Although the increase in antibody binding induced by 8-Br-cAMP cannot be taken as a direct measure of sorting to the secretory granules, these results nevertheless suggest that targeting of the Gly⁷⁷⁸ and Phe⁷⁸⁰ mutants to these organelles is greatly decreased.

The relative cell-surface expression of the mutants was determined by performing ^{125}I -labelled monoclonal antibody binding assays with intact and saponin-permeabilized cells. These assays demonstrated that the fraction of total cellular P-selectin expressed at the cell surface was $\sim$ 20% for both the Gly⁷⁷⁸ $\rightarrow$ Ala and Phe⁷⁸⁰ $\rightarrow$ Ala mutants (Table 1). Thus relative cell-surface expression was lower than that of the Tyr⁷⁷⁷ $\rightarrow$ Phe and Tyr⁷⁷⁷ $\rightarrow$ Ala mutants (28 and 40% respectively), but considerably higher than that of wild-type P-selectin (6%). The higher cell-surface expression of the Gly⁷⁷⁸ $\rightarrow$ Ala and Phe⁷⁸⁰ $\rightarrow$ Ala mutants relative to that of wild-type P-selectin was not due to higher levels of total P-selectin expression, as indicated by the comparable binding of ^{125}I -labelled CLB-thromb/6 to equal numbers of permeabilized cells.

Together, the results presented here indicate that Gly⁷⁷⁸ and Phe⁷⁸⁰ are part of the P-selectin sorting signal, consistent with the findings of Norcott et al. [5] that information for sorting of P-selectin to storage granules is contained within residues 777–782 of the cytoplasmic tail. Furthermore, these data indicate that Tyr⁷⁷⁷-Phe⁷⁸⁰ is not a typical YXX Φ signal, as, in addition to Tyr⁷⁷⁷ and Phe⁷⁸⁰, the Gly⁷⁷⁸ residue appears to contribute to the signal.

Figure 7 Distribution of P-selectin C1 domain mutants in permeabilized and intact AtT-20 cells

The distribution of mutants in permeabilized and intact AtT-20 cells was studied as described in the legends to Figures 2 and 3. The PS(HL) mutant, identical to wild-type P-selectin except for the insertion of a His-Leu dipeptide between the stop transfer and C1 domains, is predominantly present in the tips of the cell processes and is undetectable at the cell surface. In contrast, PS(HL) mutants in which either all even-numbered (C1-EVEN-ALA) or all odd-numbered (C1-ODD-ALA) residues of the original C1 domain were substituted with alanines were not concentrated at the cell tips, but displayed strong cell-surface staining. Bar = 25 μ m.

Alanine substitution of residues in the C1 domain

Recent studies have shown that deletion of C1 domain residues 763–772 abrogated the sorting of chimaeric molecules containing the P-selectin cytoplasmic domain to storage granules in PC12 cells [5]. Similarly, we found that a mutant in which the entire C1 domain (residues 762–772) was deleted (Δ C1) was not concentrated in the tips of the cell elongations of AtT-20 cells (results not shown), whereas its relative cell-surface expression was high (49 %; Table 1). To determine which residues in the C1 domain are responsible for the sorting of P-selectin, we prepared a number of mutants with alanine substitutions in this domain. These mutants all contained two additional residues, His and Leu, inserted between the stop transfer domain and the first residue (Asp⁷⁶²; original numbering) of the restored C1 domain (Figure 1). Expression of a such a construct containing the 'wild-type' C1 domain, PS(HL), in AtT-20 cells showed that P-selectin was localized in the tips of the processes, with undetectable staining of intact cells (Figure 7). Thus the inserted His and Leu residues do not affect sorting to the granules, which is also evident from results with most of the additional mutants (see below).

To first exclude the possibility that the defective sorting of the

Figure 8 Distribution of C1 domain single alanine substitution mutants in permeabilized AtT-20 cells and of the Leu⁷⁶⁸/Asn⁷⁶⁹ → Ala/Ala mutant in permeabilized and intact AtT-20 cells

The distribution of mutants in permeabilized and intact AtT-20 cells was studied as described in the legends to Figures 2 and 3. Designation of the mutants using one-letter symbols for amino acid residues is as in previous figures. With the exception of the Leu⁷⁶⁸/Asn⁷⁶⁹ mutant, the C1 domain mutants are concentrated at the tips of cell processes. The Leu⁷⁶⁸/Asn⁷⁶⁹ mutant displays granular fluorescence throughout the cytoplasm and is present at the cell surface of permeabilized and intact cells. Bar = 25 μ m.

C1 domain deletion mutant was due to abnormal spacing of the Tyr⁷⁷⁷-Gly⁷⁷⁸-Xaa-Phe⁷⁸⁰ motif relative to the membrane, we prepared mutants in which either all even-numbered (Asp⁷⁶², Gly⁷⁶⁴...Ser⁷⁷²) or all odd-numbered (Asp⁷⁶³, Lys⁷⁶⁵...His⁷⁷¹) residues of the C1 domain were replaced by alanines (C1-EVEN-ALA and C1-ODD-ALA respectively). Neither of these mutants was localized in the cell tips, and both were detected in relatively large amounts on the surface of intact cells (Figure 7). These results suggest that it is not the altered distance of the Tyr⁷⁷⁷-Gly⁷⁷⁸-Xaa-Phe⁷⁸⁰ motif from the membrane that is responsible for the abnormal sorting of the Δ C1 mutant, but rather indicate that residues in the C1 domain are critical for sorting to the granules. We then prepared a series of mutants with alanine substitutions in the C1 domain, choosing residues that are conserved between human [19] and murine, rat, ovine, bovine and canine P-selectin ([6] and references therein). Figure 8 shows that most of these PS(HL) mutants (with alanine substitutions at residues Asp⁷⁶², Asp⁷⁶³, Gly⁷⁶⁴, Pro⁷⁶⁷, Pro⁷⁷⁰ and Ser⁷⁷²) were sorted to the tips of the cell processes and that none of them was present in significant amounts at the surface of the AtT-20 cells.

Only a PS(HL) mutant with alanine substitutions of the conserved residues Leu⁷⁶⁸ and Asn⁷⁶⁹ did not localize in the cell tips and was present at the cell surface in appreciable amounts

(Figure 8). In immunoelectron microscopy, 5 % of corticotropin-positive granules also stained for P-selectin in Leu⁷⁶⁸/Asn⁷⁶⁹ → Ala/Ala transfectants, compared with 23 % for wild-type P-selectin. ¹²⁵I-labelled CLB-thromb/6 binding studies with intact and permeabilized cells indicated that 63 % of the Leu⁷⁶⁸/Asn⁷⁶⁹ mutant was expressed at the cell surface (Table 1). Furthermore, incubation of the transfectant cells in the presence and absence of 8-Br-cAMP demonstrated that the agonist increased the amount of antibody associated with the cells by only 3 % (Table 2). Thus these results indicate that Leu⁷⁶⁸ and/or Asn⁷⁶⁹, together with Tyr⁷⁷⁷, Gly⁷⁷⁸ and Phe⁷⁸⁰, are all critical for the sorting of P-selectin to storage granules.

DISCUSSION

Four lines of evidence show that alanine or phenylalanine substitution of Tyr⁷⁷⁷, and alanine substitution of Gly⁷⁷⁸, Phe⁷⁸⁰ or the dipeptide sequence Leu⁷⁶⁸-Asn⁷⁶⁹, in the cytoplasmic domain of P-selectin results in reduced sorting of P-selectin to corticotropin granules in AtT-20 cells. (1) Immunofluorescence staining of permeabilized cells demonstrated that, whereas wild-type P-selectin and alanine substitution mutants for 14 other cytoplasmic-tail residues were concentrated in the tips of the cell processes, which contain the bulk of mature corticotropin storage granules, the five mutants mentioned above were not. (2) In immunoelectron microscopy, the percentage of P-selectin-positive granules in cells transfected with the five mutants was considerably lower than in the wild-type transfectant. (3) 8-Br-cAMP-induced recruitment of the five mutant molecules from granules to the plasma membrane was significantly lower than that observed for the wild-type molecule. (4) Increased relative cell-surface expression was noted for all five mutants in comparison with wild-type P-selectin.

Whereas sorting of all five mutants to corticotropin granules was clearly reduced, the phenotypes observed were not identical. For both the Gly⁷⁷⁸ and Phe⁷⁸⁰ mutants, concentration of fluorescence staining in the cell processes was observed in a minority of the cells. Evidently, recognition of the P-selectin sorting signal in these mutants is not as dramatically impaired as it is for the Tyr⁷⁷⁷ and Leu⁷⁶⁸/Asn⁷⁶⁹ P-selectins. The Gly⁷⁷⁸ and Phe⁷⁸⁰ mutants also displayed a weaker relative cell-surface expression than the other mutants with reduced sorting to granules (Table 1). Although possibly related to the less severe nature of the sorting defect, the weaker cell-surface expression might also be due to differences in the efficiency with which these molecules are endocytosed. In CHO cells, the wild-type molecule is internalized at a rate of ~7 % of surface-expressed P-selectin/min, while approximate values for the Tyr⁷⁷⁷ → Ala, ΔC1, Phe⁷⁸⁰ → Ala and Gly⁷⁷⁸ → Ala mutants are 4 %, 6 %, 10 % and 25 % respectively [6]. Clearly, if these mutants are internalized at similar rates in AtT-20 cells, the weaker relative surface expression of the Phe⁷⁸⁰ and Gly⁷⁷⁸ mutants may, at least partly, be due to their more rapid removal from the cell surface by endocytosis.

Sorting of membrane proteins to the plasma membrane via the constitutive secretory pathway is generally thought not to require specific sorting signals. Hence one would expect disruption of the P-selectin sorting signal to result in constitutive transport to the cell surface. This is indeed suggested by the increased relative membrane expression observed for the mutants with reduced sorting to the corticotropin granules. Also, the extent of internalization of ¹²⁵I-labelled antibody CLB-thromb/6 observed in experiments in which the antibody was incubated with mutant P-selectin transfectants at 37 °C in the absence of 8-Br-cAMP (results not shown) indicates that considerable amounts of P-

selectin are expressed at the plasma membrane during the 2-h incubation period. However, at present we cannot exclude the possibility that a fraction of the mutant molecules may be sorted directly from the TGN to the endosomal compartment.

As yet, identification of signals that direct membrane proteins to storage granules has not been reported. Thus the P-selectin determinants critical for sorting identified in this study represent the first example of such a signal. Although belonging to the category of tyrosine-based signals, the P-selectin signal is clearly distinct from tyrosine-based signals that mediate internalization or targeting to other cellular locations. First, although both Tyr⁷⁷⁷ and Phe⁷⁸⁰ contribute to the P-selectin sorting signal, the Y⁷⁷⁷GVF⁷⁸⁰ sequence is not a typical YXXΦ motif. This was already suggested by the fact that, in contrast with most, but not all, YXXΦ-type signals [9], the YGVF sequence is not part of a β-turn, as predicted by structural analysis according to the methods of Chou and Fasman [32]. Furthermore, the P-selectin sorting signal differs from the tyrosine-based signals of a number of other plasma-membrane-expressed proteins in that the signal is not identical to, or co-linear with, the internalization signal, as internalization was shown to depend on residues scattered throughout the cytoplasmic domain, with no apparent critical role for the Tyr⁷⁷⁷ residue [6]. However, the feature that most clearly distinguishes the P-selectin signal from YXXΦ-type signals is the presence of a critical Y + 1 residue (Gly⁷⁷⁸). To our knowledge, tyrosine-based signals with both a critical Y + 1 residue and a critical large hydrophobic residue at the Y + 3 position have not been described.

A further distinguishing characteristic is the contribution of Leu⁷⁶⁸ and/or Asn⁷⁶⁹ to the sorting of P-selectin. The observation that sorting of P-selectin to the AtT-20 storage granules is greatly decreased in mutants in which the C1 domain is deleted (ΔC1) or mutated in different ways (C1-EVEN-ALA, C1-ODD-ALA and Leu⁷⁶⁸/Asn⁷⁶⁹ mutants) strongly suggests that the Tyr-Gly-Xaa-Phe sequence requires additional determinants to constitute the complete sorting signal. The individual contribution of Leu⁷⁶⁸ and Asn⁷⁶⁹ remains to be established. The fact that sorting is strongly reduced for Leu⁷⁶⁸/Asn⁷⁶⁹ → Ala/Ala as well as for C1-EVEN-ALA (containing Leu⁷⁶⁸ → Ala) and C1-ODD-ALA (containing Asn⁷⁶⁹ → Ala) suggests that Leu⁷⁶⁸ and Asn⁷⁶⁹ may both be critical for sorting. However, as C1-EVEN-ALA and C1-ODD-ALA both contain alanine substitutions of residues that have not been tested individually (Cys⁷⁶⁶ for C1-EVEN-ALA, and Lys⁷⁶⁵ and His⁷⁷¹ for C1-ODD-ALA), we cannot exclude the possibility that substitution of these residues with alanine would also interfere with recognition of the sorting signal. Although the presence of critical residues outside YXXΦ-type signals has not been reported, more complex tyrosine-based signals have been described in which critical residues are encountered at some distance from the critical tyrosine in the primary sequence. An example is the basolateral targeting signal of the low-density lipoprotein receptor, which comprises critical tyrosine-glutamine and glycine-tyrosine dipeptides as well as two acidic three-residue motifs that are not contiguous with the tyrosine motifs [33].

The finding that phenylalanine cannot replace Tyr⁷⁷⁷ in the sorting signal indicates that the hydroxy group of Tyr⁷⁷⁷ is important for recognition by the sorting machinery. A more direct demonstration of such a function in other signals is provided by recent work showing that either mutation of a tyrosine to phenylalanine or its phosphorylation inhibits the *in vitro* interaction of tyrosine-based signals with the μ2 chain of the AP2 complex [34–37]. For example, in the case of the T-cell molecule cytotoxic T-lymphocyte antigen-4, phosphorylation of Tyr²⁰¹ in the cytoplasmic tail abrogates its binding to μ2 *in vitro*

and, in intact cells, not only inhibits internalization of CTLA-4 but also creates a novel binding site for Src homology 2 (SH2) domain-containing signal transduction molecules [37]. By analogy, phosphorylation of P-selectin Tyr⁷⁷⁷ in the TGN might result in a switch from regulated to constitutive secretion, leading to increased expression on the surface of endothelial cells, such as was observed for the Tyr⁷⁷⁷ mutants in our AtT-20 transfectants. Permanent endothelial P-selectin surface expression has been observed in some types of chronic inflammation [38–41] and can be induced in cultured endothelial cells by cytokines such as interleukin-3 [42], interleukin-4 and oncostatin M [43]. It remains to be determined, however, whether increased membrane expression in these endothelial cells results from altered sorting of P-selectin or from saturation of the regulated secretion pathway as a result of increased P-selectin synthesis [42,43].

The function of P-selectin phosphorylation is at present unknown. Phosphorylation of Tyr⁷⁷⁷ and of cytoplasmic-tail serine, threonine and histamine residues occurs rapidly, but transiently, after stimulation of platelets with thrombin [23, 44,45]. In addition, P-selectin tyrosine phosphorylation can be induced by treating platelets with the tyrosine phosphatase inhibitor pervanadate, and also occurs *in vitro* in disulphide-linked complexes between P-selectin and the tyrosine kinase pp60^{c-src} [46]. To our knowledge, detailed studies of endothelial P-selectin phosphorylation have not been reported. For AtT-20 transfectants, P-selectin tyrosine phosphorylation was not detectable in immunoblotting and metabolic labelling studies of resting or pervanadate-treated cells, although tyrosine phosphorylation of a large number of AtT-20 proteins was increased by pervanadate (results not shown). Obviously, mechanisms that regulate P-selectin phosphorylation in platelets may be lacking in AtT-20 cells, implying that the AtT-20 model may not be suitable for the study of this aspect of P-selectin sorting.

In conclusion, our data show that residues Leu⁷⁶⁸ and/or Asn⁷⁶⁹, Tyr⁷⁷⁷, Gly⁷⁷⁸ and Phe⁷⁸⁰ in the cytoplasmic domain of P-selectin are all essential for recognition by the machinery that sorts P-selectin to storage granules. Naturally occurring mutations in these residues may increase the amount of P-selectin expressed at the surface of platelets and endothelial cells, thus potentially leading to excessive leucocyte adhesion, which might contribute to thrombosis and adverse reactions in inflammation.

We thank Dr. H. K. Nieuwenhuis and Dr. R. E. Mains for their gifts of antibodies, and Dr. J. Voorberg and Dr. R. D. Fontein for providing P-selectin cDNA. We gratefully acknowledge Dr. L. C. J. M. Oomen for assistance with scanning confocal microscopy. This work was supported by The Netherlands Heart Foundation, grant no. 93.054.

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