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Cytoplasmic Domain of P-Selectin (CD62) Contains the Signal for Sorting into the Regulated Secretory Pathway

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P-selectin (CD62), formerly called GMP-140 or PADGEM, is a membrane protein located in secretory storage granules of platelets and endothelial cells. To study the mechanisms responsible for the targeting of P-selectin to storage granules, we transfected its cDNA into COS-7 and CHO-K1 cells, which lack a regulated exocytic pathway, or into AtT20 cells, which are capable of regulated secretion. P-selectin was expressed on the plasma membrane of COS-7 and CHO-K1 cells but was concentrated in storage granules of AtT20 cells. Immunogold electron microscopy indicated that the electron-dense granules containing P-selectin in AtT20 cells also stored the endogenous soluble hormone ACTH. Activation of AtT20 cells with 8-Br-cAMP increased the surface expression of P-selectin, consistent with agonist-induced fusion of granule membranes with the plasma membrane. Deletion of the last 23 amino acids of the 35-residue cytoplasmic domain resulted in delivery of P-selectin to the plasma membrane of AtT20 cells. Replacement of the cytoplasmic tail of tissue factor, a plasma membrane protein, with the cytoplasmic domain of P-selectin redirected the chimeric molecule to granules. We conclude that the cytoplasmic domain of P-selectin is both necessary and sufficient for sorting of membrane proteins into the regulated pathway of secretion.

INTRODUCTION

Many soluble and membrane proteins in eukaryotic cells are transported to various destinations within membrane-bound vesicles. During synthesis these proteins enter the endoplasmic reticulum (ER).[1] They may then travel through the Golgi complex by a constitutive, or default, pathway to the cell surface. However, proteins containing specific transport signals may be directed to other locations if the cell possesses the appropriate recognition machinery. Thus, signals cause retention or retrieval of proteins in the ER (Munro and Pelham, 1987; Jackson *et al.*, 1990) or in specific cisternae of the Golgi (Machamer and Rose, 1987; Colley *et al.*, 1989). Other signals direct sorting of proteins in the trans-Golgi network (Griffiths and Simons, 1986) for delivery to lysosomes (Kornfeld and Mellman, 1989; Williams and Fukuda, 1990; Peters *et al.*, 1990) or to secretory storage granules (Burgess and Kelly, 1987; Kelly, 1991; Tooze, 1991). The latter process is termed regulated secretion because the storage granules fuse with the plasma membrane and release their contents in response to specific cell agonists.

Soluble proteins targeted to the regulated secretory pathway are stored in granules that typically have an electron-opaque content or "dense core" when examined by electron microscopy. The sorting of these proteins has been studied extensively in AtT20 cells, a murine pituitary cell line that packages endogenous

[1] Abbreviations used: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; HBSS, Hanks' balanced salt solution; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PCR, polymerase chain reaction; ST, stop-transfer.

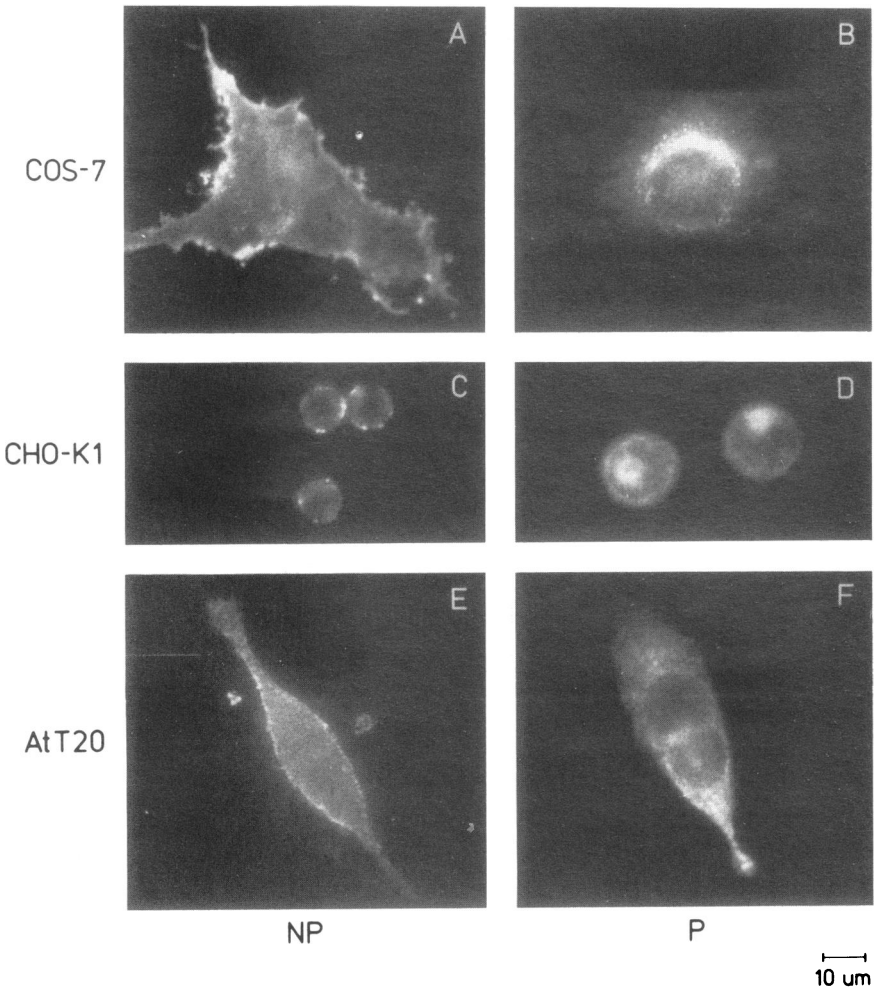

Figure 1. Immunofluorescence of transfected COS-7, CHO-K1, and AtT20 cells expressing P-selectin. Cells were stained with monoclonal antibodies to P-selectin followed by FITC- or Texas Red-conjugated anti-mouse IgGs. Cells in A, C, and E were not permeabilized (NP), whereas those in B, D, and F were permeabilized with saponin (P). Nonpermeabilized transfected AtT20 cells exhibited faint surface staining (E) when visualized in the absence of screening filters to increase sensitivity, but permeabilized cells had strong punctate intracellular staining (F) that was detectable even in the presence of filters.

hormones such as ACTH into storage granules (reviewed in Burgess and Kelly, 1987). Soluble proteins of other cells that are sorted for regulated secretion are also delivered to storage granules of AtT20 cells after transfection of their cDNAs. This finding suggests that the sorting machinery is conserved among different cell types and species. However, the mechanisms responsible for delivery of soluble proteins to storage granules remain unclear (Kelly, 1991).

Even less is known about the signals for sorting of membrane proteins to storage granules. Recently, the primary structures of some of these proteins have been determined by cDNA cloning (Johnston *et al.*, 1989a; Perin *et al.*, 1988, 1991). One of these molecules is P-selectin (CD62, formerly known as GMP-140 or PADGEM), a 140-kDa membrane glycoprotein identified in storage granules of two cell types: the α granules of platelets (Stenberg *et al.*, 1985; Berman *et al.*, 1986) and the Weibel-Palade bodies of endothelial cells (Bonfanti *et al.*, 1989; Hattori *et al.*, 1989; McEver *et al.*, 1989). P-selectin is a member of the selectin family of lectin-like adhesion molecules (reviewed in McEver, 1991). After stimulation of platelets and endothelial cells with agonists such as thrombin, P-selectin is rapidly redistributed from granule membranes to the cell surface where it mediates adhesion of leukocytes. Like other selectins, P-selectin has an N-terminal domain homologous to Ca^{2+}-dependent lectins, followed by an epidermal growth factor-like motif, a series of consensus repeats related to those in complement-binding proteins, a transmembrane domain, and a short cytoplasmic tail. P-selectin is the only selectin known to be located in secretory storage granules.

Targeting signals have been identified in the cytoplasmic domains of membrane proteins that are retained in the ER (Jackson *et al.*, 1990) or delivered to lysosomes (Peters *et al.*, 1990; Williams and Fukuda, 1990). Furthermore, cytoplasmic domains contain essential information for endocytosis of membrane proteins in coated pits (Chen *et al.*, 1990; Johnson *et al.*, 1990; Canfield *et al.*, 1991) and for shuttling of mannose 6-phosphate receptors between the trans-Golgi network and prely-

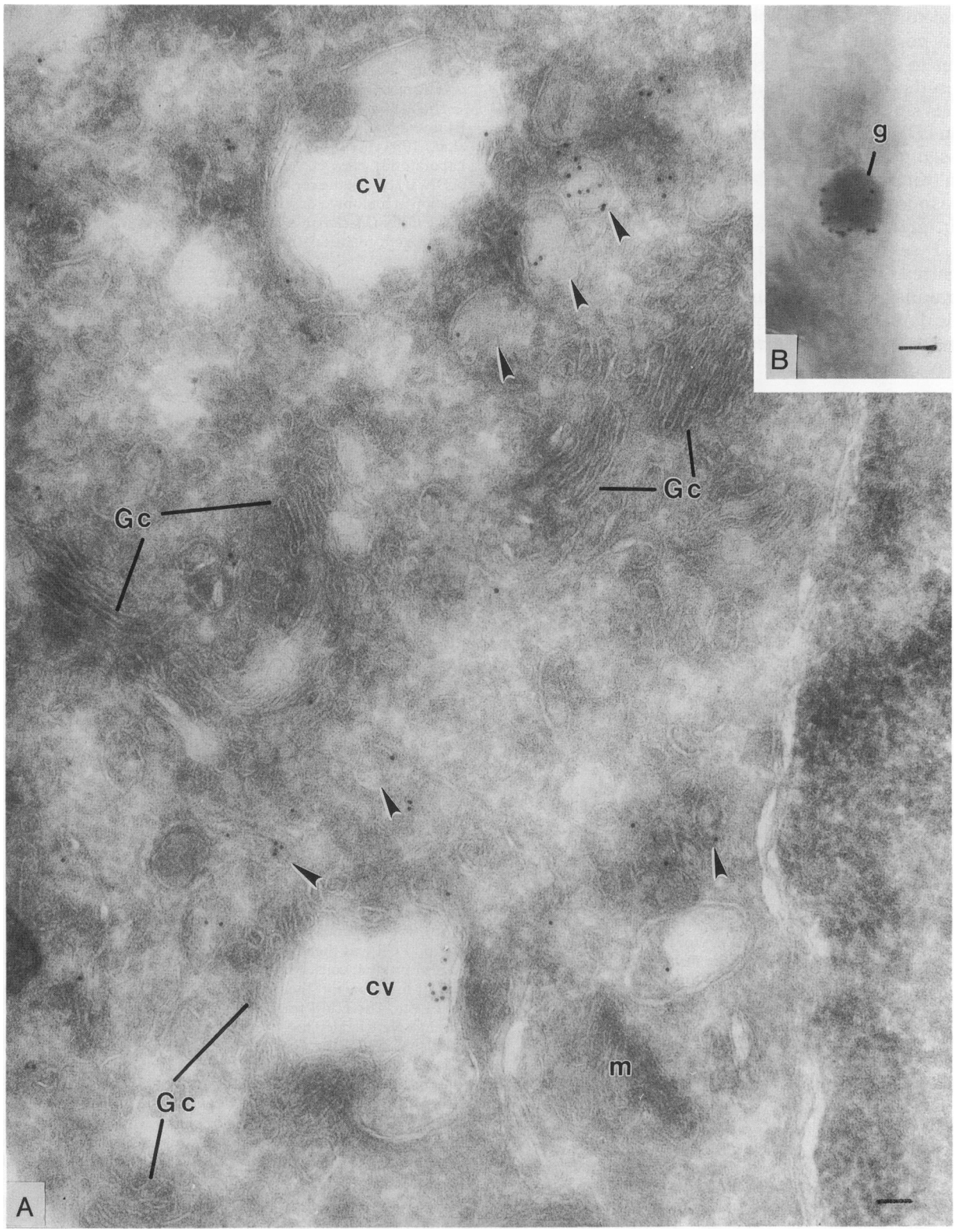

Figure 2. Immunogold labeling of P-selectin on frozen thin sections of AtT20 cells transfected with P-selectin cDNA. (A) In the Golgi region of these cells, labeling is restricted to the condensing vacuoles (CV) near the Golgi complex (Gc) and smaller vesicles (arrowheads) that may represent immature secretory granules. The Golgi stacks (Gc) are not labeled above background. (B) Labeling of P-selectin on the membranes of a mature dense core secretory granule (g). m, mitochondrion. (A) ×67 000; (B) ×71 000. Bars, 0.1 μm.

sosomal compartments (Lobel *et al.*, 1989). It therefore seemed possible that the signals directing membrane proteins to secretory storage granules might also be located in their cytoplasmic domains.

In this study we demonstrate that transfection of P-selectin cDNA into AtT20 cells results in delivery of the expressed protein to granules that store ACTH. In contrast, a truncated form of P-selectin lacking most of the cytoplasmic tail is expressed on the cell surface rather than in granules. Transplantation of the cytoplasmic tail of P-selectin onto a membrane protein normally transported to the cell surface results in delivery to storage granules. These findings indicate that the cytoplasmic domain of P-selectin is both necessary and sufficient for sorting of membrane proteins into the pathway of regulated secretion.

MATERIALS AND METHODS

Cell Culture

AtT20/D16v-F2 cells, a gift from Dr. Jordan Tang (Oklahoma Medical Research Foundation, Oklahoma City, OK), were originally obtained from the American Type Culture Collection (ATCC no. CRL 1795, Rockville, MD). The cells were grown in Dulbecco's modified Eagle's medium with high glucose (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, 4 mM glutamine, 200 U/ml penicillin, and 200 μg/ml streptomycin in a humidified atmosphere with 15% CO_2 at 37°C. COS-7 cells, obtained from the American Type Culture collection (ATCC no. CRL 1651), were grown in the same medium but under 5% CO_2 at 37°C. CHO-K1 cells, obtained from the American Type Culture Collection (ATCC no. CCL 61), were grown in α-minimal essential medium (Whittaker, Walkersville, MD) supplemented with 10% fetal bovine serum, 4 mM glutamine, 200 U/ml penicillin, and 200 μg/ml streptomycin under 5% CO_2 at 37°C.

Transfections

CHO-K1 cells were transfected by the calcium phosphate method (Chen and Okayama, 1987), and permanent transfectants were selected by growth in medium containing 400 μg/ml (active concentration) of G418 (Geneticin, GIBCO). Colonies resistant to G418 were picked after 10 d of selection and were screened for protein expression by enzyme-linked immunosorbent assay (ELISA) of cell lysates. A stable cell line was established after two subclonings performed by limiting dilution.

AtT20 cells were plated on 10-cm dishes for permanent transfections and on glass cover slips placed in 3.5-cm dishes for transient expression. The cells were grown to 80–90% confluence before transfection. Equal volumes of plasmid and lipofectin reagent (BRL, Grand Island, NY), each diluted in OptiMEM medium (BRL), were incubated for 20 min at a final concentration of 15 μg/ml for each reagent. The transfection mixture (5 ml for the 10-cm dishes and 1 ml for the 3.5-cm dishes) was then added to the cells. After a 5-h incubation at 37°C, an equal volume of complete medium was added, and the incubation was continued overnight. The transfection medium was then replaced by complete medium. Stable clones were established by selection in medium containing 400 μg/ml of G418 and were screened by ELISA or immunofluorescence. Cells analyzed for transient expression were studied 3 d after transfection. COS-7 cells were analyzed for transient expression with the same procedures used for AtT20 cells.

Constructs

Two plasmid expression vectors from Invitrogen (San Diego, CA) were used: pRc/RSV, which contains a Rous sarcoma virus promotor, and pcDNAI/Neo, which contains a cytomegalovirus promotor. Both contain a Neomycin resistance gene for selection of stable transfectants in medium containing G418. Most of the experiments were performed with the pRc/RSV vector, although expression of several constructs was also observed with pcDNAI/Neo.

The cDNA clone encoding full-length human P-selectin (termed E4 in the original reference) has been described previously (Johnston *et al.*, 1989a). The cDNA was excised from the plasmid pIBI20 (IBI Laboratories, New Haven, CT) by digestion with *Sal* I and cloned in pcDNAI/Neo at the compatible site *Xho* I or into pRc/RSV with blunt ligation at a blunted *Bst*XI site. The cDNA clone encoding full-length tissue factor (Mackman *et al.*, 1989) was inserted in the vector pcDNAI (Invitrogen, identical to pcDNAI/Neo but without the Neomycin gene).

Four additional constructs were made using the polymerase chain reaction (PCR). All amplifications were for 30 cycles using the following parameters: denaturation for 1 min at 94°C, annealing for 1.5 min at 50°C, and extension at 72°C for 2.5 min. The nucleotide sequences generated by PCR were confirmed by a double-stranded sequencing protocol (Johnston *et al.*, 1989a). The constructs were prepared as follows.

Construct 1. This construct is a truncated form of P-selectin in which a stop codon was introduced at the junction between the stop-transfer (ST) segment of exon 14 and exon 15. A PCR product, using the P-selectin cDNA as template, was made extending from the *Xba* I site at base 2240 to the stop codon after base 2444, followed by another *Xba* I site. This fragment was then inserted to replace the *Xba* I fragment of the P-selectin cDNA extending from the *Xba* I site in the cDNA to the *Xba* I site in the polylinker of pRc/RSV.

Construct 2. This construct is similar to construct 1, except that a stop codon replaced the codon encoding the fifth amino acid (residue 767) of C1. The PCR product was made extending from the *Xba* I site at base 2240 to the stop codon after base 2456, followed by another *Xba* I site.

Construct 3. This construct joins the extracellular and transmembrane domains of tissue factor to the cytoplasmic tail of P-selectin. An *Eco*RI fragment of tissue factor extending from the 5′ polylinker region to the *Eco*RI site at nucleotide 775 (numbered according to Morrissey *et al.*, 1987) was excised from pcDNAI. Construction of the remainder of the hybrid molecule was performed by generation of two separate PCR products, one for each half of the hybrid product. To each of these products was added a cloning site incorporated into one of the primer oligonucleotides. The first product, using the tissue factor cDNA as template, included the *Eco*RI site beginning at nucleotide 775 of tissue factor and extended to base 855 of tissue factor. The second product, using the P-selectin cDNA as template, included bases 2424 to 2534 of P-selectin followed by an *Xba* I site. The two inside oligonucleotide primers (antisense for tissue factor and sense for P-selectin) overlapped. The two products were gel purified and mixed. A second PCR was then performed using the two outside primers. The hybrid product consisted of an *Eco*RI site followed by bases 775 to 855 of tissue factor, then bases 2424 to 2534 of P-selectin, then an *Xba* I site. The *Eco*RI fragment of tissue factor and the hybrid PCR product were ligated together in the plasmid pGEM7Zf(+) (Promega, Madison, WI) at the *Eco*RI and *Xba* I sites. This sequence was then excised with *Hin*dIII and *Xba* I and cloned into the corresponding sites of both pRcRSV and pcDNAI/Neo.

Construct 4. This construct, which used construct 3 as template for the PCR reactions, replaced the proximal ST sequence of the cytoplasmic domain of P-selectin in construct 3 with the corresponding sequence of L-selectin, a receptor delivered to the plasma membrane after synthesis (Tedder *et al.*, 1989). The first PCR product began at the *Hpa* I site at base 723 of tissue factor and extended to base 855 of tissue factor. The second product began with overlapping sequence in the sense primer for nucleotides 1157 to 1174 of L-selectin (encoding the amino acids RRLKKG), followed by the P-selectin sequence extending from base 2448 to base 2534, followed by the *Xba* I site. After annealing and amplification, the hybrid PCR product was ligated into

the *Hpa* I and *Xba* I sites of construct 3 to replace the original *Hpa* I-*Xba* I segment.

Immunofluorescence

Wild-type or permanently transfected COS-7, CHO-K1, or AtT20 cells were grown on sterile glass coverslips for several days before immunofluorescence analysis. In the case of cells expressing proteins transiently, wild-type cells were transfected directly on the slides and then examined by immunofluorescence 3 d after transfection.

Cells were washed once with Hanks' balanced salt solution (HBSS) and then fixed at room temperature with 0.5% paraformaldehyde for 5 min, except for immunofluorescence staining with antibodies to ACTH when the cells were fixed with 4% paraformaldehyde for 10 min. The cells were washed three times with HBSS at room temperature and then incubated for 30 min at 37°C with HBSS containing 1% bovine serum albumin (BSA) (HBSS/BSA). When cells were permeabilized, 0.2% saponin was added to the HBSS/BSA buffer; saponin was also included in the buffers of all subsequent incubations.

Primary antibodies were diluted in HBSS/BSA and incubated with cells for 30 min at 37°C. Two murine monoclonal antibodies against P-selectin, S12 and W40 (Johnston *et al.*, 1989b), were used in combination, each as purified IgG at a concentration of 12.5 μg/ml. Three murine monoclonal antibodies against tissue factor, TF9-1B8, TF9-5B7, and TF8-11D12 (Morrissey *et al.*, 1988), were used in combination as purified IgG, each at a concentration of 0.6 μg/ml. The mouse monoclonal antibody MOPC21 (Organon Teknika-Cappel, Malvern, PA), used as a negative control, was added at a concentration of 12.5 μg/ml. A rabbit antiserum, termed "Kathy," directed against the carboxy-terminal residues 1-39 of ACTH (Mains and May, 1988), was a generous gift of Dr. Richard Mains (Johns Hopkins University, Baltimore, MD) and was used at a dilution of 1:5000.

After incubation with the primary antibodies, the cells were washed three times with HBSS/BSA and then incubated for 30 min at 37°C with labeled secondary antibodies. The Texas Red conjugated reagent (Molecular Probes, Eugene, OR) was an affinity-purified goat polyclonal antibody against mouse IgG (heavy and light chains) adsorbed against IgGs of several different species. The fluorescein isothiocyanate (FITC)-labeled antibodies (Chemicon International, Temecula, CA) were a donkey anti-rabbit IgG and a goat anti-mouse IgG; these antibodies were all affinity purified and adsorbed against IgGs from several different species. The antibodies were all diluted in HBSS/BSA at 1:400 for the Texas Red reagent and 1:100 for the FITC reagents.

After incubation with the secondary antibodies, the cells were washed twice with HBSS/BSA and once with HBSS at room temperature. After drying, the glass coverslip was turned upside down on the microscope slide and sealed with one drop of mounting medium, a polymer solution of polyvinyl alcohol (Air Product and Chemical, Allentown, PA) made as described previously (Yumura *et al.*, 1984). Fluorescence images were acquired with a Zeiss (Thornwood, NY) Axiophot microscope equipped with a 75 W xenon epifluorescence illuminator. A 100× (n.a. = 1.30) Plan-Neofluar (Oberkochen, FRG) objective was used. FITC fluorescence was observed with an Omega Optical (Brattleboro, VT) FITC filter. Texas Red fluorescence was observed with a Zeiss filter (model 48 79 00). Both filters were set for dual-wavelength observation; no FITC fluorescence was detected by the Zeiss Texas Red filter and no Texas Red fluorescence was detected by the Omega FITC filter. A Hamamatsu (Bridgewater, NJ) C2400 Silicon Intensified Target TV camera, interfaced to a Zeiss IBAS 2000 Image Analyzer, was used to digitize the images. Sixteen images were averaged for the final image.

Electron Microscopy

AtT20 cells growing in flasks were washed free of serum with RPMI and fixed at 4°C for 2 h in 4 or 8% paraformaldehyde in phosphate buffer (pH 7.4). They were then scraped from the flask and infiltrated in a solution of 2.1 M sucrose and 13% polyvinylpreprolene (~10 000 mol wt) and frozen in liquid nitrogen. Frozen thin sections of AtT20 cells transfected with P-selectin cDNA were cut and incubated with rabbit anti-human P-selectin serum at 1:50 dilution (Figures 2 and 3A). The sections were then incubated with goat anti-rabbit gold-10 (GAR-10, Amersham, Arlington Heights, IL) and subsequently were stained with uranyl acetate as previously described (McEver *et al.*, 1989). A double-label experiment was performed using the rabbit antiserum to ACTH (1:200) and a mixture of the following monoclonal antibodies to P-selectin, W40, G1 and G3 (Geng *et al.*, 1990), and G553 (Geng and McEver, unpublished data) at 5 μg/ml; gold labeling was with goat anti-rabbit gold-10 (GAR-10) and goat anti-mouse gold-5 (GAM-5), respectively (Figure 3B). Frozen thin sections of AtT20 cells transfected with construct 3 were labeled with the anti-tissue factor monoclonal antibodies TF9-9B4 and TF9-8E8 (Morrissey *et al.*, 1988) at 5 μg/ml and goat anti-mouse 10 (GAM-10) (Figure 8, A and C). Double labeling of these cells was performed using the same monoclonal antibodies to tissue factor and goat anti-mouse gold-5 (GAM-5) plus the rabbit antiserum to ACTH and goat anti-rabbit gold-10 (GAR-10) (Figure 8B). Controls consisted of a primary incubation with normal rabbit or mouse serum.

^{125}I-Labeled Monoclonal Antibody Binding to Cells

The monoclonal antibodies S12 (to P-selectin) or TF8-5G9 (to tissue factor) were labeled with Na^{125}I with the iodogen reagent (Pierce Chemical, Rockford, IL). Labeled antibody was separated from free iodide by gel filtration on a PD10 column (Pharmacia, Piscataway, NJ), dialysed against 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 0.1 M NaCl, pH 7.4, and then centrifuged at 89 000 × *g* for 30 min at 4°C to remove aggregates. The protein concentration was determined with the Micro BCA method (Pierce) with a standard curve derived from unlabeled monoclonal antibody. Specific activities of the labeled antibodies ranged from 2000 to 5000 cpm/ng. After labeling, the antibodies were stored in buffer supplemented with 0.1% BSA and 0.02% sodium azide at 4°C. Before each binding experiment, the labeled antibody was centrifuged for 10 min at 4°C at 10 000 × *g*.

Cells were grown in 24-well plates for binding studies. In the case of cells transiently expressing protein, binding was measured 3 d after transfection. Cells were washed and fixed as described for the immunofluorescence studies. In the case of permeabilized cells, 0.2% saponin was included in the buffers in all incubations. The fixed cells were incubated for 30 min at room temperature with increasing concentrations of ^{125}I-labeled antibody and were diluted in HBSS/BSA in the presence or absence of a 50-fold excess of unlabeled antibody. The cells were washed twice with HBSS/BSA and then with 25 mM HEPES, 0.1 M NaCl, pH 7.4. The cells were lysed in 0.1% Triton X-100 in 25 mM HEPES, 0.1 M NaCl, pH 7.4, and aliquots were counted in a gamma counter. Specific binding was calculated by subtracting the radioactivity measured in the presence of unlabeled antibody from that measured in its absence. Nonspecific binding was always <0.5% of input radioactivity; at saturable concentrations of antibody, nonspecific binding was typically <10% of specific binding. The percentage of surface binding (measured on nonpermeabilized cells) relative to total binding (measured on permeabilized cells) was calculated at each concentration of antibody where saturable specific binding was noted for both nonpermeabilized and permeabilized cells. Typically, several points were measured at saturation, and a mean value was calculated for each experiment. The percentage of intracellular binding was determined by subtracting the percentage of surface binding from 100. The data shown in Table 1 are the mean values of independent experiments performed on different days; the standard deviations for the means from independent experiments were <7%.

To measure activation-induced changes in surface expression of P-selectin, cells stably transfected with P-selectin cDNA were grown in 24-well dishes until 80% confluent. The cells were then incubated with increasing concentrations of [^{125}I]S12 in medium for 2 h at 37°C in the presence or absence of 10 mM 8-Br-cAMP (Sigma, St. Louis,

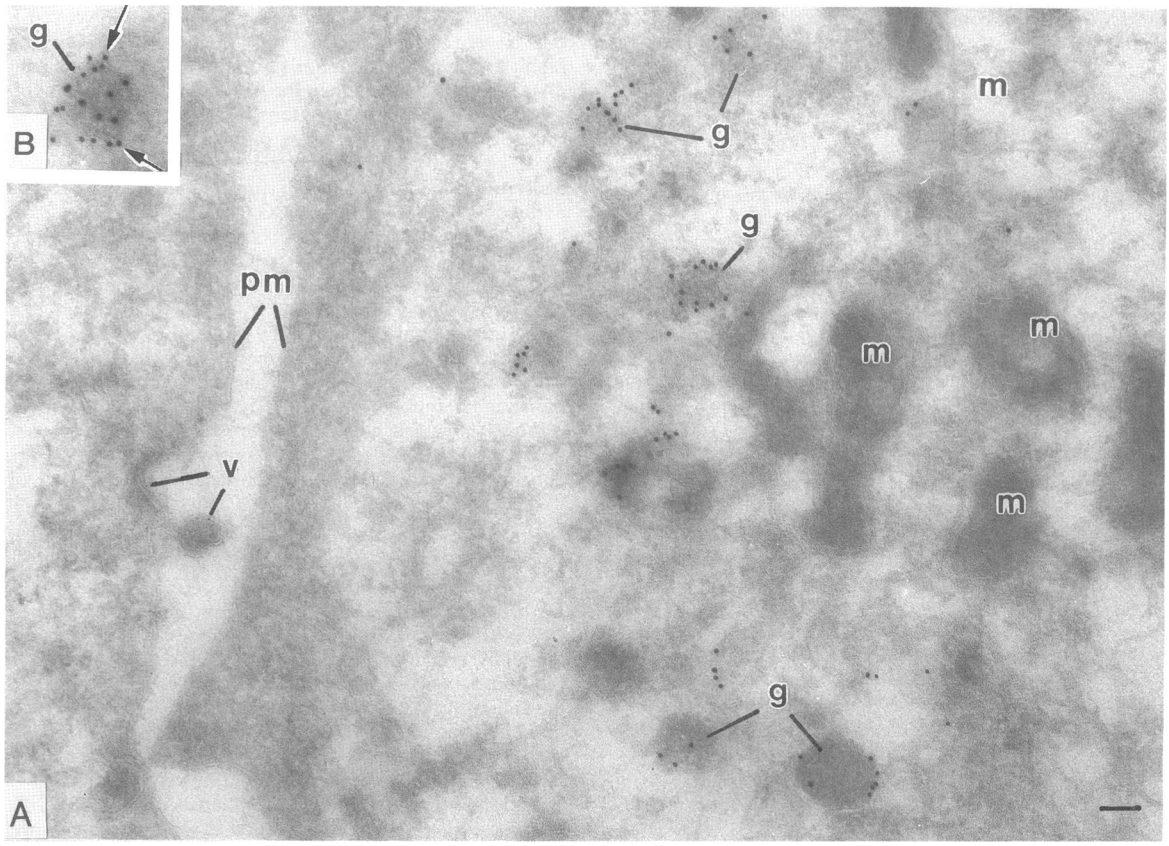

Figure 3. Immunogold labeling of frozen thin sections of AtT20 cells transfected with P-selectin cDNA. (A) Labeling for P-selectin. As seen in the periphery of the cell, the dense core granules (g) tend to accumulate near the plasma membrane (pm). Note that the gold particles are distributed near the membrane of the granules. Some granules are cut in grazing sections. The plasma membrane, endogenous viruses of the cell (v), and mitochondria (m) are not labeled. (B) Double labeling of a dense core granule with antibodies to ACTH and P-selectin. Label for ACTH in the matrix is detected with the large gold (GAR-10), and label for P-selectin near the membrane (arrows) is detected with the small gold (GAM-5). (A) ×60 000; (B) ×80 000. Bar, 0.1 μm.

MO). As a control, parallel sets of cells were incubated with increasing concentrations of the monoclonal antibody 28-8-6S directed against the plasma membrane H-2K^{b}D^b antigen (Ozato and Sachs, 1981). The 28-8-6S antibody (ATCC no. HB51), obtained as purified IgG from Dr. Paul Kincade (Oklahoma Medical Research Foundation, Oklahoma City, OK), was radiolabeled in the same manner as S12. For each concentration of radiolabeled antibody, nonspecific binding was determined by the addition in a parallel well of a 50-fold excess of unlabeled antibody. After incubation, the cells were washed twice with medium, lysed in HBSS containing 1% Triton X-100, and counted in a gamma counter for 1 min. Nonspecific binding was subtracted from total binding to give specific binding.

Tissue Factor Assay

The distributions of surface and intracellular tissue factor were measured by a clotting assay of cells disrupted by freeze/thawing as previously described (Drake *et al.*, 1989). Briefly, stably transfected cells were incubated with a mixture of three monoclonal antibodies that block the coagulation cofactor activity of tissue factor, TF8-11D12, TF9-9B4, and TF9-6B4, at concentrations of 10 μg/ml each, or as a control, with an isotype-matched control monoclonal antibody, HB3, at a concentration of 30 μg/ml. Incubation was for 15 min at 37°C in the presence of 10 mM sodium azide. The cells were then washed, scraped from the dishes, disrupted by at least five cycles of freeze/thawing, and assayed for tissue factor activity.

RESULTS

P-Selectin is Sorted into Granules that Store ACTH in AtT20 Cells

To determine whether P-selectin could be sorted into the regulated secretory pathway of heterologous cells, we transfected a cDNA encoding human P-selectin into mouse pituitary AtT20 cells. As controls, we transfected the cDNA into COS-7 and CHO-K1 cells, which do not have a regulated secretory pathway. The distribution of expressed protein was examined by immunofluorescence microscopy. Figure 1, A and C, indicates that nonpermeabilized transfected COS-7 and CHO-K1 cells exhibited strong surface staining with monoclonal antibodies to P-selectin. After permeabilization, the cells demonstrated immunofluorescence staining in the perinuclear region (Figure 1, B and D), consistent with dis-

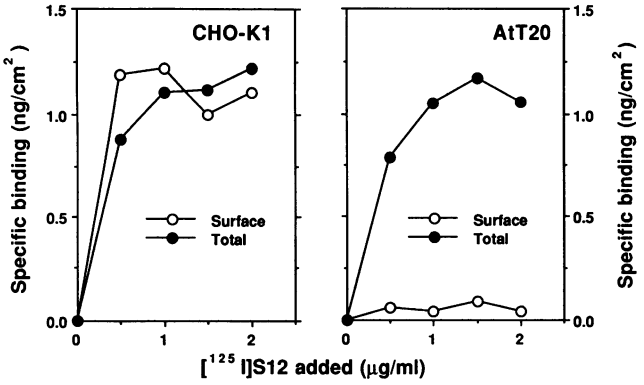

Figure 4. Specific binding of [^{125}I]S12, a monoclonal antibody to P-selectin, to CHO-K1 and AtT20 cells transfected with P-selectin cDNA. The open circles represent surface binding to nonpermeabilized cells, and the closed circles represent total binding to permeabilized cells. This experiment was performed with permanently transfected cells, but similar results were found with cells transiently expressing P-selectin.

tribution of newly synthesized protein in the Golgi complex. Little punctate intracellular immunoreactivity was noted. In contrast, nonpermeabilized transfected AtT20 cells demonstrated only faint surface staining (Figure 1E), but permeabilized transfected cells had strong perinuclear as well as punctate cytoplasmic immunoreactivity (Figure 1F). The fluorescent cytoplasmic vesicles were typically small and were often concentrated in the tips of long cellular projections.

The preferential diversion of P-selectin into intracellular vesicles only in the AtT20 cells suggested that these structures were secretory granules. The endogenous soluble hormone ACTH is stored in dense core granules of AtT20 cells (Tooze and Tooze, 1986; Burgess and Kelly, 1987; Tooze and Burke, 1987; Mains and May, 1988). Dual-label immunofluorescence studies indicated that there was general codistribution of intracellular staining for P-selectin and for ACTH. To determine the precise nature of the organelles containing P-selectin in transfected AtT20 cells, we used electron microscopy to examine frozen thin sections of these cells labeled with immunogold. The most abundant label for P-selectin was found in the typical electron-dense secretory granules of this cell type, with lesser amounts noted in vacuoles and vesicles in the Golgi region. Quantitation of the distribution of gold in various organelles was difficult because of the polarized nature of the cells and possible differential efficiency of labeling on frozen thin sections (Griffiths and Hoppeler, 1986). Nevertheless, the presence of antigen in storage granules could be determined. As shown in Figure 2A, the stacked Golgi cisternae and ER were not labeled above background. Label was found, however, in adjacent large condensing vacuoles and in smaller vesicles. On frozen thin sections, the condensing material was only partially preserved

Table 1. Distribution of surface and intracellular protein in transfected cells

Cell line	Protein	Distribution of protein (%) Surface	Intracellular	n
CHO-K1	P-selectin	83	17	3
AtT20	P-selectin	15	85	4
	Tissue factor	65	35	1
		93[a]	7[a]	3
	Constructs			
	1	79	21	3
	3	16	84	6
	4	17	83	3

The amount of expressed protein was determined by measuring specific binding of ^{125}I-labeled monoclonal antibodies to fixed transfected cells. Surface binding was measured on intact cells, and total binding was measured on permeabilized cells. Binding on the inside of the cells was calculated by subtracting surface binding from total binding. The percentages shown are the mean values of the indicated (n) number of independent experiments. The standard deviation was always <7%.

[a] Independent measurements of surface and intracellular protein by a functional assay for tissue factor as described in MATERIALS AND METHODS.

in these vacuoles, as previously noted (Tooze and Tooze, 1986). Figure 2B illustrates labeling of a mature dense core granule with most of the immunogold distributed near the granule membrane. Similarly labeled secretory granules were found scattered throughout the cytoplasm, although the main accumulation was in the periphery of the cell, sometimes in a small tip-like process. Figure 3A shows immunolabeling of several dense core

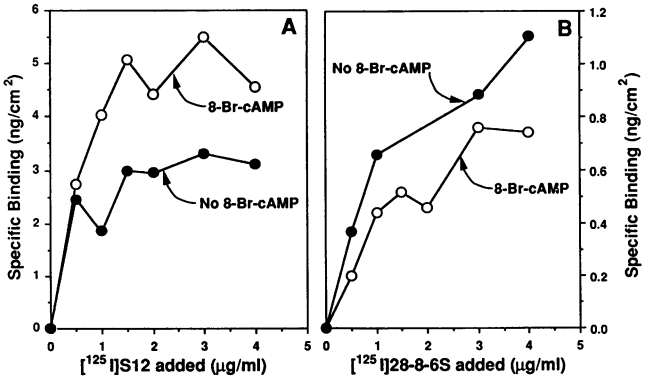

Figure 5. Effect of 8-Br-cAMP on the surface expression of P-selectin in AtT20 cells. Cells permanently transfected with P-selectin cDNA were incubated with radiolabeled S12, a monoclonal antibody to P-selectin (A), or with 28-8-6S, a control monoclonal antibody to a plasma membrane H2 antigen (B), in the presence or absence of the agonist 8-Br-cAMP. After a 2-h incubation at 37°C, specific binding of antibody was measured. Shown is a single experiment representative of four performed with similar results.

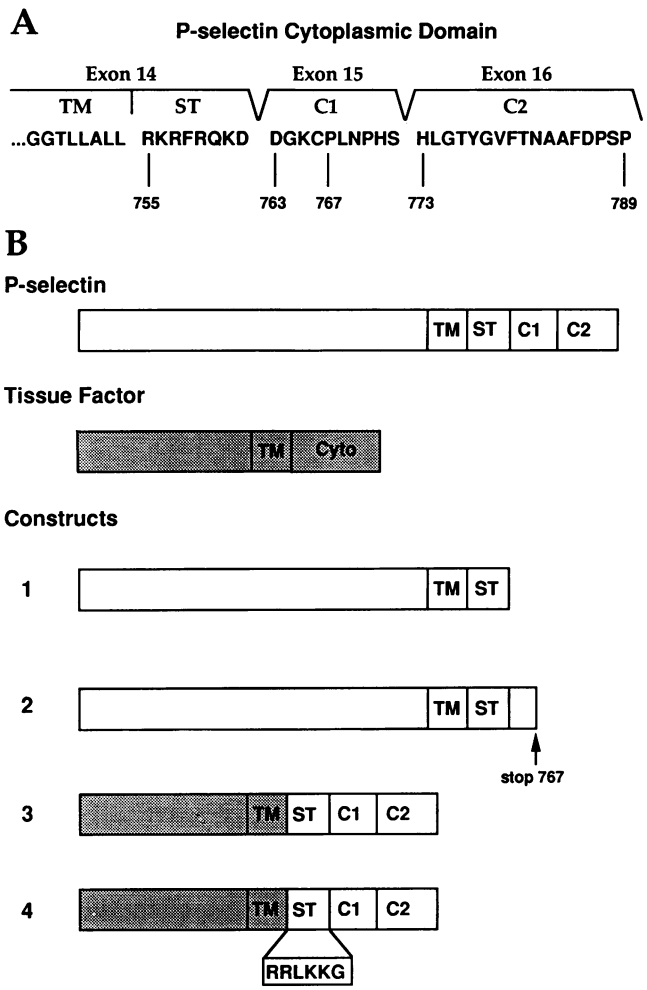

Figure 6. Amino acid sequence of the cytoplasmic domain of P-selectin and schematics of the cDNA constructs. (A) The cytoplasmic domain of P-selectin contains 35 residues. Exon 14, which also encodes the transmembrane (TM) domain, encodes the first eight amino acids that include a putative stop-transfer (ST) sequence. Exon 15 encodes the next 10 residues of the cytoplasmic domain (C1), and exon 16 encodes the last 17 residues (C2). (B) At the top are diagrams of wild-type P-selectin and tissue factor. Below these are schematics of the mutants and chimeric constructs used. The cytoplasmic domains, but not the extracellular or transmembrane domains, are drawn to scale.

granule membranes but not of the plasma membrane, forming viruses, or mitochondria. Double-label experiments demonstrated the colocalization of ACTH in the matrix with P-selectin on the membrane of dense core granules (Figure 3B). These results indicate that a significant portion of the P-selectin found inside AtT20 cells is in secretory granules that store ACTH.

To quantitate the relative amounts of P-selectin expressed on the cell surface or inside the cell, we measured specific binding of [^{125}I]S12, a monoclonal antibody to P-selectin, to fixed, permeabilized, or nonpermeabilized cells transfected with P-selectin cDNA. Intracellular antibody binding was calculated by subtracting surface binding (to nonpermeabilized cells) from total binding (to permeabilized cells). Figure 4 demonstrates that radioiodinated S12 bound specifically to a saturable number of sites on both permeabilized and nonpermeabilized CHO-K1 and AtT20 cells transfected with P-selectin cDNA. In CHO-K1 cells, >80% of the antibody bound to the cell surface, whereas in AtT20 cells, >80% of the antibody bound to intracellular sites. Table 1 summarizes the surface and intracellular protein distributions for P-selectin and for other constructs used in this study.

When platelets or endothelial cells are stimulated by agonists that induce degranulation, P-selectin is redistributed to the plasma membrane (Stenberg *et al.*, 1985; Berman *et al.*, 1986; McEver *et al.*, 1989; Hattori *et al.*, 1989). To determine whether agonist-induced degranulation of transfected AtT20 cells would lead to increased surface expression of P-selectin, cells were incubated with [^{125}I]S12 in the presence or absence of an agonist, 8-Br-cAMP, that induces secretion of ACTH from storage granules (Gumbiner and Kelly, 1982). At saturating concentrations of antibody, the stimulated cells exhibited nearly a twofold increase in S12 binding, consistent with movement of some of the intracellular protein to the cell surface (Figure 5A). In contrast, binding of 28-8-6S, a monoclonal antibody to a constitutive plasma membrane H2 antigen, did not increase after activation of the cells (Figure 5B).

Sorting Signal is Located in the Cytoplasmic Domain of P-Selectin

Because cytoplasmic domains have been implicated in targeting membrane proteins to the ER (Jackson *et al.*, 1990) and lysosomes (Peters *et al.*, 1990; Williams and Fukuda, 1990), it seemed possible that this region of P-selectin might mediate sorting to the regulated secretory pathway. As shown in Figure 6A, the cytoplasmic domain of human P-selectin contains 35 residues encoded by three separate exons (Johnston *et al.*, 1989a, 1990). The first eight residues, a charged sequence that includes a putative ST signal, are encoded by exon 14, which also encodes the hydrophobic transmembrane domain. The next 10 residues are encoded by exon 15 (C1) and the final 17 amino acids by exon 16 (C2). To test the role of the cytoplasmic domain of P-selectin in sorting to the regulated pathway, we made a series of constructs (Figure 6B) and measured the cellular distribution of the constructs after transfection into AtT20 cells (Table 1).

We first made a truncated form of P-selectin (construct 1) by introducing a stop codon after the eight-residue ST sequence of the cytoplasmic domain, at the junction between exons 14 and 15. When transfected into AtT20 cells, ~80% of the truncated molecules were expressed

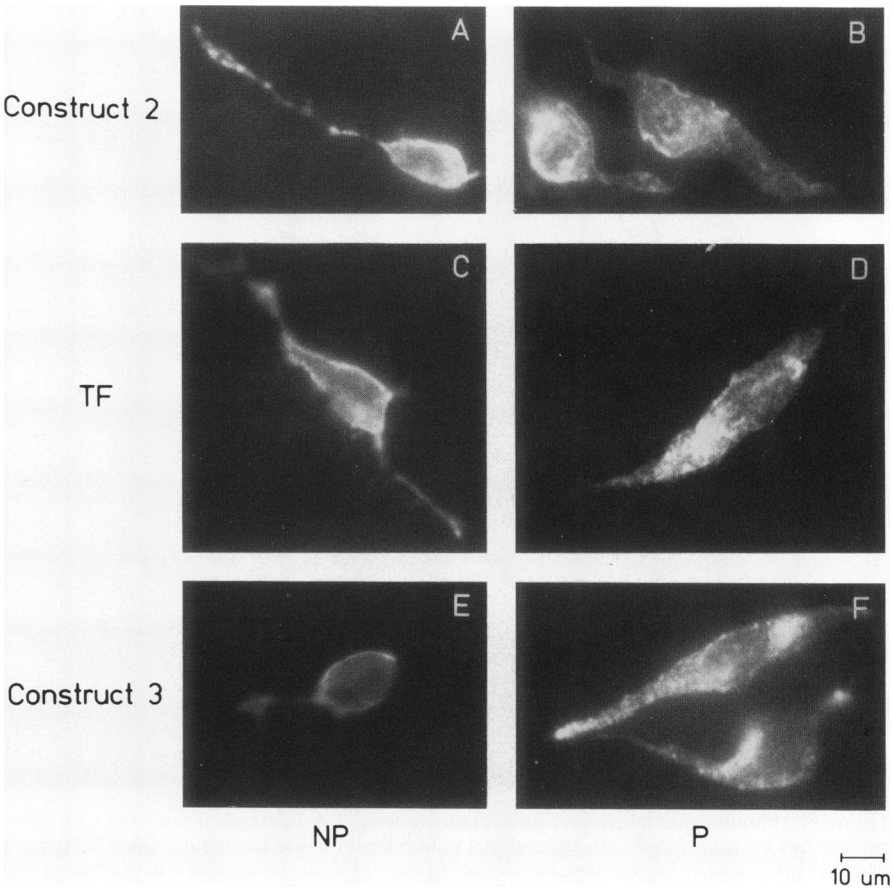

Figure 7. Immunofluorescence of transfected AtT20 cells expressing a truncated form of P-selectin (construct 2), native tissue factor (TF), or a chimeric form of tissue factor in which the cytoplasmic domain of the protein was replaced with the corresponding domain of P-selectin (construct 3). Cells were stained with monoclonal antibodies to epitopes on the extracytoplasmic domain of P-selectin or tissue factor, followed by FITC- or Texas Red-conjugated anti-mouse IgGs. Cells in A, C, and E were not permeabilized (NP), whereas those in B, D, and F were permeabilized (P). Nonpermeabilized cells expressing construct 2 or tissue factor had strong surface staining (A and C); staining of permeabilized cells was patchier but clearly was located on the surface as determined by focusing on different levels of the cell (B and D). In contrast, nonpermeabilized cells expressing construct 3 (E) had faint surface staining that was detected only in the absence of screening filters, whereas permeabilized cells (F) exhibited strong intracellular staining that was visualized even in the presence of screening filters.

at the cell surface (Table 1). Surface immunofluorescence staining was uniformly observed with antibodies to P-selectin, but little intracellular immunofluorescence was noted in permeabilized cells. Construct 2 had a stop codon introduced after the first four residues of C1 encoded by exon 15; when expressed in AtT20 cells, this truncated molecule also appeared predominantly on the cell surface as assessed by immunofluorescence (Figure 7, A and B). These results suggest that at least part of the C-terminal 23 residues of the cytoplasmic domain is required for targeting of P-selectin to intracellular granules.

The diversion of truncated P-selectin to the cell surface could mean that the deleted sequence normally interacts with cytoplasmic components to mediate delivery into granules. Alternatively, deletion of this region might alter the conformation of other areas of the molecule that participate directly in sorting. To distinguish between these possibilities, we replaced the 21-residue cytoplasmic domain of tissue factor, a coagulation protein cofactor normally transported directly to the plasma membrane (Drake *et al.*, 1989), with the 35 amino acids in the cytoplasmic tail of P-selectin (construct 3). The surface and intracellular distribution of native tissue factor or the chimeric protein was quantitated by binding of monoclonal antibodies directed against the extracytoplasmic domain of tissue factor and by a functional assay for tissue factor that distinguishes between surface and intracellular protein (Table 1). These data and immunofluorescence analysis (Figure 7, C and D) indicated that tissue factor was expressed predominantly at the cell surface of transfected AtT20 cells. In contrast, construct 3, like the native P-selectin molecule, was sorted efficiently into intracellular vesicles (Table 1 and Figure 7, E and F).

To confirm that the chimeric molecule was sorted into secretory granules, we performed immunogold analysis on frozen thin sections of transfected AtT20 cells. Immunogold labeling for the tissue factor component of the chimeric protein was found in dense core granules (Figure 8A) as well as in numerous small and large vesicles, some multivesicular bodies (Figure 8, A and C), and occasionally on the plasma membrane. Colocalization of ACTH in the matrix with tissue factor on the membranes was observed in the dense core granules (Figure 8B). These data confirm that the chimeric construct 3 is sorted into dense core granules that store ACTH and indicate that the cy-

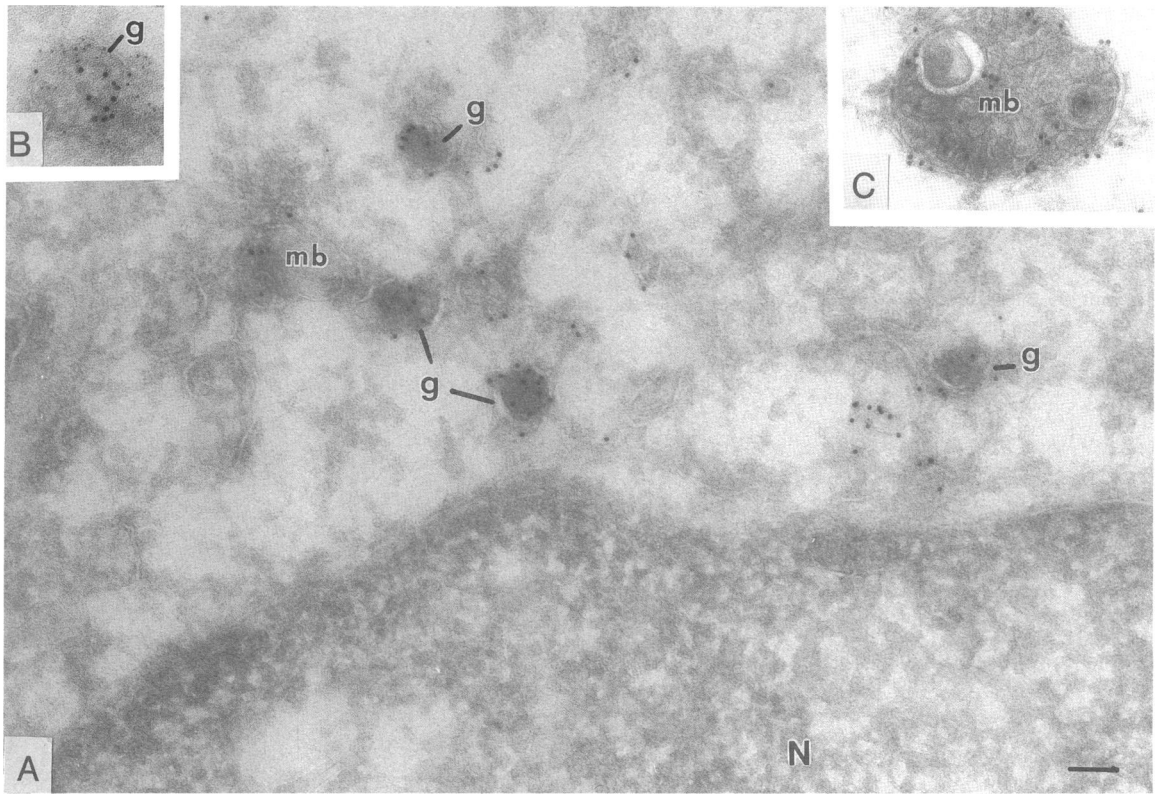

Figure 8. Immunogold labeling of frozen thin sections of AtT-20 cells transfected with cDNA encoding construct 3. (A and C) The chimeric protein has been localized with monoclonal antibodies to tissue factor plus GAM-10 (large gold). Note the presence of label in dense core granules (g) as well as in multivesicular bodies (mb). (B) Double labeling of a dense core granule with antibodies to ACTH and to tissue factor. Label for ACTH in the matrix is detected with the large gold (GAR-10), and label for tissue factor near the membrane (marking the chimeric protein) is detected with the small gold (GAM-5). N, nucleus. (A) ×71 000; (B) ×88 000; (C) ×71 000.

toplasmic domain of P-selectin is both necessary and sufficient for sorting of membrane proteins into secretory granules.

The preferential surface expression in AtT20 cells of construct 1, a deletion mutant of P-selectin, suggests that the first eight residues in the cytoplasmic tail do not carry sufficient information to direct the protein to secretory granules. This segment, however, might contain sorting information used in conjunction with other portions of the cytoplasmic domain. To address this possibility, we replaced the proximal eight-residue segment in construct 3 with the six-residue proximal cytoplasmic tail sequence of L-selectin, a plasma membrane protein (construct 4). This chimeric molecule was sorted into intracellular vesicles as efficiently as P-selectin or construct 3 (Table 1 and immunofluorescence data). This result suggests that a unique proximal sequence is not required for sorting. However, a less specific sequence in this region might still function in other ways, for example, by positioning a sorting signal a minimal distance from the membrane.

DISCUSSION

P-selectin is targeted efficiently to secretory storage granules in the two cell types in which it is normally expressed: platelets and endothelial cells (Stenberg *et al.*, 1985; Berman *et al.*, 1986; Bonfanti *et al.*, 1989; Hattori *et al.*, 1989; McEver *et al.*, 1989). Our results indicate that this membrane protein is also efficiently transported to storage granules in AtT20 cells, suggesting that the machinery for regulated secretion is conserved across cell types and species. A similar conservation has been noted for the system sorting soluble proteins to storage granules, which has been studied extensively in AtT20 cells (Burgess and Kelly, 1987). However, one striking difference is the relative efficiencies of the sorting processes for soluble and membrane proteins. When transfected into AtT20 cells, only a small percentage of soluble proteins capable of storage in dense core granules are actually sorted to these organelles; most of the proteins are secreted constitutively (Burgess and Kelly, 1987; Wagner *et al.*, 1991). The same inefficiency is some-

times seen in nontransformed cells. For example, only a minority of von Willebrand factor is stored in Weibel-Palade bodies of cultured human endothelial cells, whereas the majority is secreted constitutively (Sporn *et al.*, 1986). In contrast, P-selectin is sorted efficiently to secretory granules when endogenously synthesized in megakaryocytes and endothelial cells or when transfected into AtT20 cells.

Like the targeting signals for transmembrane proteins retained in the ER (Jackson *et al.*, 1990) or sorted to lysosomes (Peters *et al.*, 1990; Williams and Fukuda, 1990), the signal directing P-selectin to secretory storage granules is located in the cytoplasmic domain. Removal of most of this segment results in delivery of the truncated molecule to the cell surface. Addition of this domain to tissue factor, a protein normally delivered to the plasma membrane, promotes efficient sorting to storage granules. The chimeric tissue factor molecule appears to fold properly because it is recognized by several conformationally dependent monoclonal antibodies. Furthermore, the chimeric molecule is fully functional as a cofactor for the Factor VIIa-catalyzed cleavage of Factor X (Table 1 and Morrissey, unpublished observations). Fusion of the P-selectin cytoplasmic tail is therefore unlikely to expose cryptic sorting signals in the extracytoplasmic regions of tissue factor. Instead, the cytoplasmic domain of P-selectin probably interacts directly with sorting machinery in the cytoplasm. Such machinery is presumably present only in cells that have a regulated pathway of secretion, because transfection of P-selectin into COS-7 and CHO-K1 cells, which do not have this pathway, results in delivery to the cell surface.

Although the sorting information for P-selectin has been located to the distal 10-residue C1 and 17-residue C2 segments, the precise features required for sorting remain to be determined. There are no obvious sequence homologies between this region and the cytoplasmic domains of other membrane proteins directed to the regulated secretory pathway (Perin *et al.*, 1988, 1991; Johnston *et al.*, 1989a). Therefore, sorting information may require a three-dimensional structure that can be conferred by more than one combination of amino acids. This would be analogous to some endocytosis signals that can be created by combinations of an aromatic residue and a variety of other amino acids (Collawn *et al.*, 1990). P-selectin itself is internalized after its appearance on the surface of activated endothelial cells (Hattori *et al.*, 1989), and it contains at least two candidate endocytosis signals in the C2 segment, YGVF and FTNAAF, that are similar to previously described internalization motifs (Chen *et al.*, 1990; Canfield *et al.*, 1991). It remains to be determined whether these sequences actually mediate endocytosis and whether these signals are distinct from those required for sorting to storage granules.

The itinerary followed by P-selectin in the cell also requires further study. The immunogold findings in AtT20 cells are consistent with sorting of P-selectin in the trans-Golgi network because label for the protein is observed in this location in condensing vacuoles and mature electron-dense granules. However, it is possible that some or all of the newly synthesized protein reaches the cell surface and must be internalized before it is eventually sorted, as is true for some lysosomal membrane proteins (Braun *et al.*, 1989). Whether P-selectin can be efficiently recycled into storage granules from the plasma membrane of activated cells also remains to be determined. In cells capable of regulated secretion, the movements of P-selectin may be dictated by both sorting and internalization signals.

Finally, our studies have implications for the properties of a soluble form of P-selectin that is synthesized by both megakaryocytes and endothelial cells (Johnston *et al.*, 1989a, 1990). Transcripts encoding this molecule are derived by alternative splicing of precursor RNA, such that exon 14 encoding the transmembrane and ST sequences is removed. Exons 15 and 16, encoding the C1 and C2 sequences that contain the sorting signal, are retained at the C-terminus of the molecule. However, because the soluble molecule should not be anchored in the membrane, the C-terminus should no longer face the cytoplasm of the cell where it can interact with the sorting machinery. The soluble form of P-selectin is therefore predicted to be secreted constitutively. This prediction is consistent with our inability to detect the putative soluble form of P-selectin in the releasate of activated platelets (Moore and McEver, unpublished observations). Furthermore, when the cDNA encoding the alternatively spliced form of P-selectin is transfected into AtT20 cells, the expressed protein is constitutively secreted rather than packaged into granules (Disdier and McEver, unpublished observations). Although the membrane form of P-selectin is a known adhesion molecule for leukocytes, the physiological role of a soluble form of the molecule remains to be determined. The current data indicate that its function should be considered in the context of constitutive secretion from megakaryocytes or endothelial cells rather than acute release after agonist-induced degranulation.

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