P-Selectin, A Granule Membrane Protein of Platelets and Endothelial Cells, Follows the Regulated Secretory Pathway in AtT-20 Cells

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Abstract. P-selectin (PADGEM, GMP-140, CD62) is a transmembrane protein specific to α granules of platelets and Weibel-Palade bodies of endotheial cells. Upon stimulation of these cells, P-selectin is translocated to the plasma membrane where it functions as a receptor for monocytes and neutrophils. To investigate whether the mechanism of targeting of P-selectin to granules is specific for megakaryocytes and endothelial cells and/or dependent on von Willebrand factor, a soluble adhesive protein that is stored in the same granules, we have expressed the cDNA for P-selectin in AtT-20 cells. AtT-20 cells are a mouse pituitary cell line that can store proteins in a regulated fashion. By double-label immunofluorescence, P-selectin was visible as a punctate pattern at the tips of cell processes. This pattern closely resembled the localization of ACTH, the endogenous hormone produced and stored by the AtT-20 cells. Fractionation of the transfected cells resulted in the codistribution of P-selectin and ACTH in cellular compartments of the same density. Immunoelectron microscopy using a polyclonal anti-P-selectin antibody demonstrated immunogold localization in dense granules, morphologically indistinguishable from the ACTH granules. Binding experiments with radiolabeled monoclonal antibody to P-selectin indicated that there was also surface expression of P-selectin on the AtT-20 cells. After stimulation with the secretagogue 8-Bromo-cAMP the surface expression increased twofold, concomitant with the release of ACTH. In contrast, the surface expression of P-selectin transfected into CHO cells, which do not have a regulated pathway of secretion, did not change with 8-Br-cAMP treatment. In conclusion, we provide evidence for the regulated secretion of a transmembrane protein (P-selectin) in a heterologous cell line, which indicates that P-selectin contains an independent sorting signal directing it to storage granules.

SECRETORY proteins in eukaryotic cells can follow an intracellular route that leads directly to the cell surface (constitutive secretion) or a route that leads to specialized granules that can store the protein in a highly concentrated form (regulated secretion). The latter pathway is characterized by the release of the protein through fusion of the granule membrane with the cell membrane when the cell is stimulated. Regulated secretion requires a storage-competent cell type, such as exocrine or endocrine secretory cells, and an as yet unidentified "targeting signal" on the protein itself (Burgess and Kelly, 1987). Constitutive secretion represents the bulk flow of proteins and is independent of external signals. The mechanisms underlying the sorting between the two pathways have been studied by introducing cDNAs encoding secretory proteins and mutated forms thereof into heterologous cells. These studies have not yet led to a general understanding of intracellular sorting. In the case of prosomatostatin (Sevarino et al., 1989; Stoller and Shields, 1989) and von Willebrand factor (vWF)[1] (Wagner et al., 1991), the propolypeptide seems to be important for storage. Cleavable sequences have also been implicated as targeting signals for the import of proteins to the rough endoplasmic reticulum, yeast vacuoles, and mitochondria (Verner and Schatz, 1988). The propeptides of trypsinogen (Burgess et al., 1987) or insulin (Powell et al., 1988), on the other hand, do not seem to contain sorting information; rather self-association of hormone precursors may lead to storage in secretory granules. A mutation in residue 10 of the proinsulin B chain (His to Asp) results in loss of hexamer formation and this has been suggested as a cause of impaired sorting (Gross et al., 1989). In a recent report Quinn et al. (1991), however, found no correlation between sorting of proinsulin and its ability to form hexamers. Secretogranin II, a secretory gran-

1. *Abbreviations used in this paper*: endo H, endoglycosidase H; vWF, von Willebrand factor.

ule protein of endocrine and neuronal cells, aggregates at millimolar concentrations of calcium (Gerdes et al., 1989), which may be a key step in its sorting. The low pH in certain intracellular compartments could also play a role in aggregation and/or storage (Gerdes et al., 1989; Moore et al., 1983*a*; Wagner et al., 1986).

Little is known about the targeting of transmembrane proteins. Although a role has been suggested for the cytoplasmic domains in targeting of membrane proteins to lysosomes (Peters et al., 1990; Klionsky and Emr, 1990; Williams and Fukuda, 1990), it is not known how membrane proteins of secretory granules are sorted. P-selectin (according to new nomenclature for the selectin group of cell adhesion molecules as proposed by Bevilacqua et al., [1991]) (PADGEM, GMP-140, CD62), a member of the selectin family of adhesion receptors (Springer, 1990), is a transmembrane protein found in α granules of platelets (Stenberg et al., 1985; Berman et al., 1986) and Weibel-Palade bodies of endothelial cells (Bonfanti et al., 1989; McEver et al., 1989). When platelets are stimulated by thrombin, the contents of the α-granules are released and P-selectin becomes exposed on the surface, where it serves as an adhesion protein, binding the activated platelets to monocytes and neutrophils (Larsen et al., 1989; Hamburger and McEver, 1990). Similarly, activation of endothelium leads to redistribution of P-selectin and adhesion of neutrophils (Hattori et al., 1989; Geng et al., 1990). Weibel-Palade bodies are endothelial cell-specific storage granules that store vWF (Weibel and Palade, 1964; Wagner et al., 1982). P-selectin is the only other protein constituent identified in these organelles. It is possible, therefore, that a relationship exists between the sorting of vWF and P-selectin. An example of such a relationship was found (Rosa et al., 1989) when secretogranin I was coexpressed together with an antibody directed against it. This resulted in the diversion of the antibody from the constitutive pathway to the regulated pathway. Independent expression of vWF and P-selectin in a storage-competent cell line should provide insight into this question. The mouse anterior pituitary cell line AtT-20 has proved to be capable of correctly processing and storing secretory proteins (Moore et al., 1983*b*). vWF, when expressed in AtT-20 cells, induces the formation of storage granules resembling Weibel-Palade bodies in the absence of P-selectin (Wagner et al., 1991). We have undertaken the present study to see whether this approach can also be used to study the targeting of P-selectin, a transmembrane protein. We show that P-selectin is targeted to the endogenous storage granules of AtT-20 cells and thus contains its own sorting signal.

Materials and Methods

Materials

Restriction endonucleases and DNA polymerase I large fragment (Klenow) were from New England BioLabs, Inc. (Beverly, MA). T4 DNA ligase was from Bethesda Research Laboratories (Gaithersburg, MD). Alkaline phosphatase and endoglycosidase H (endo H) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). G418 (Geneticin), DME, penicillin/streptomycin, and trypsin/EDTA were from Gibco Laboratories (Grand Island, NY). L-[^{35}S]cysteine (1,300 Ci/mmol) was from Amersham Corp. (Arlington Heights, IL) and Na[^{125}I] (17 Ci/mg) was from DuPont Co. (Wilmington, DE). The following reagents were obtained from Sigma Chemical Co. (St. Louis, MO): 8-bromoadenosine 3′:5′ cyclic monophosphate (8-Br-cAMP), Percoll, Density Marker Beads, PMSF, leupeptin, soybean trypsin inhibitor, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, *o*-phenylenediamine dihydrochloride (OPD), protein A-Sepharose 4B, BSA, and ovalbumin.

Plasmid Construction

The expression vector (pCMV3) that contains the cytomegalovirus IE promotor followed by a BglII restriction site and the SV-40 small t antigen polyadenylation and transcriptional termination signals was kindly provided by Dr. Richard Goodman (University of Oregon, Portland, OR). The full-length P-selectin cDNA (Larsen et al., 1990) was excised from the cloning vector pSP65 (Promega Biotec, Madison, WI) by digestion with AccI. The insert was purified from a gel, blunt ended with Klenow fragment and subcloned into the pCMV3 vector by blunt-end ligation. The correct orientation of the P-selectin cDNA was confirmed by mapping of restriction sites.

Cell Culture and Transfection

AtT-20 cells (a cell line derived from a mouse anterior pituitary tumor) were grown in DME supplemented with 10% FBS and 100 U/ml penicillin/streptomycin. The cells were transfected with 40 μg pCMV3-P-selectin and 2 μg pRSVneo using calcium phosphate, followed by selection in 300 μg/ml G418 (Wagner et al., 1991). G418-resistant clones were picked and screened for the expression of P-selectin by immunofluorescence staining. AtT-20 cells expressing the mature subunit of vWF were described previously (Wagner et al., 1991). COS-1 (monkey kidney) cells were grown and transfected under the same conditions as AtT-20 cells, with the omission of cotransfection with pRSVneo and selection in G418. CHO cells expressing full-length P-selectin on their cell surface were a kind gift of Dianne Sako and Glenn Larsen (Genetics Institute, Cambridge, MA). HL60 cells were maintained as described (Larsen et al., 1989). Binding of HL60 cells to COS cells transfected with P-selectin cDNA was performed as described (Larsen et al., 1990).

Immunofluorescence Staining

AtT-20 cells expressing P-selectin were grown on glass coverslips, fixed in 3.7% formaldehyde and permeabilized in 0.5% Triton X-100 in PBS. The cells were stained for P-selectin using the monoclonal antibody AC1.2 (Larsen et al., 1989) at a 1:200 dilution of ascites followed by rhodamine-conjugated goat antibody to mouse IgG (Organon Teknika-Cappel, Malvern, PA) at 1:500 and subsequently for ACTH using a polyclonal antibody (a gift from the National Hormone and Pituitary Program, University of Maryland School of Medicine, College Park, MD) at 1:500 followed by fluorescein-conjugated goat antibody to rabbit IgG (ICN ImmunoBiologicals, Lisle, IL) at 1:200. All incubations with antibodies were for 30 min at 37°C.

Immunoprecipitation and Gel Electrophoresis

Anti-P-selectin IgG (subclass IgG1) was purified from AC1.2 ascites on a Protein A-Sepharose 4B column using the Affi-Gel Protein A Monoclonal Antibody Purification System (Bio-Rad Laboratories, Richmond, CA). IgG was coupled to CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) at 3 mg IgG/ml bedvolume. Cells expressing P-selectin were metabolically labeled for 2 d with 30 μCi/ml [^{35}S]cysteine and lysed with 1% Triton X-100 in 0.1 M Tris, pH 8.3, containing 4 mM EDTA and 4 mM PMSF. The lysate was incubated for 2 h with 25 μl of AC1.2 Sepharose, followed by washing of the beads in 0.1% SDS, 0.5% deoxycholate, 0.5% NP-40, 5 mg/ml ovalbumin, 2 mM EDTA, and 2 mM PMSF in 0.1 M Tris, pH 8.3. Finally, the beads were boiled in electrophoresis sample buffer (Laemmli, 1970) containing DTT and the supernatant was analyzed on a 7% SDS-polyacrylamide gel. The gels were treated with EN^{3}HANCE (New England Nuclear, Boston, MA) and fluorographs (XRP-1 film; Eastman Kodak Co., Rochester, NY) were scanned with a Bromma ultroscan XL laser densitometer (LKB Instruments, Inc., Gaithersburg, MD). Removal of high mannose carbohydrate was performed by incubating the immunoprecipitate with endo H (Wagner et al., 1986).

Cell Fractionation

AtT-20 cells were fractionated on Percoll density gradients using an adaptation of a protocol for the isolation of Weibel-Palade bodies from endothelial cells (Ewenstein et al., 1987). Cells (five 10-cm dishes) were washed once with HBSS and detached with trypsin/EDTA. An equal volume of 1 mg/ml soybean trypsin inhibitor in homogenization buffer (20 mM Tris, pH 7.2,

0.2 M sucrose, 1 mM EDTA) was added to the cell suspension and the cells were washed once in homogenization buffer without trypsin inhibitor and resuspended in 1 ml. In later experiments, scraping of cells in ice-cold homogenization buffer was used instead of trypsinization. This method gave identical results. The suspension was subjected to 20 strokes in a Dounce homogenizer (size 19; Kontes Class Co., Vineland, NJ) at 4°C. Nuclei and cellular debris were removed by centrifugation for 10 min at 600 *g* and the supernatant was centrifuged at 10,000 *g* for 5 min to collect membrane and granule fractions. The pellet was resuspended in 200 μl homogenization buffer and layered on Percoll.

Centrifugation was carried out in 10-ml Oak Ridge tubes (Nalge Co., Rochester, NY) containing 9 ml 20% Percoll and 0.25 M sucrose, pH 7.2, for 60 min at 40,000 *g* in a rotor (model SM24) using a centrifuge (Sorvall RC-5B; DuPont Co., Wilmington, DE). 12 800-μl fractions were collected manually from the top, 1 mM PMSF and 12.5 μg/ml leupeptin were added and the fractions were freeze thawed twice before their contents were assayed.

Assays For Cellular Proteins in Percoll Fractions

The distribution of cellular proteins in the Percoll gradient was determined by TCA precipitation of fractions obtained from fractionation of metabolically labeled cells. The TCA pellet was dissolved in Biofluor cocktail (New England Nuclear) and counted in a scintillation counter. ACTH was measured by radioimmunoassay (INCSTAR Corp., Stillwater, MN). The lysosomal marker *N*-acetyl-β-glucosaminidase was assayed by the release of *p*-nitrophenol from *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sellinger et al., 1960). P-selectin was quantitated by a dot-blot method in which 4-μl aliquots were applied on a nitrocellulose membrane (0.2 μm; Schleicher & Schuell, Inc., Keene, NH). After blocking in 5% nonfat dry milk in 50 mM Tris, pH 8.0, the membrane was incubated with ^{125}I-labeled antibody to P-selectin (see below) at 0.3 μCi/ml, washed and exposed to film (XRP-1, Eastman Kodak Co.) using Cronex intensifying screens (DuPont Instruments). The dots were scanned with a Bromma Ultroscan XL Laser Densitometer (LKB Instruments). vWF antigen was determined by ELISA (Hamer et al., 1986) using IgG purified from Assera vWF antiserum (Diagnostica Stago, Asnières, France) as the coating layer and peroxidase-conjugated anti-vWF (Dako Corp., Santa Barbara, CA) as the developing antibody.

Immunoelectron Microscopy

Cells were fixed with 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 for 1 h at room temperature. They were washed three times in phosphate buffer and embedded in glycol methacrylate. Thin sections were incubated for 1 h with anti-ACTH antibody (1:100), or rabbit anti-P-selectin polyclonal antibody (1:100; kindly provided by Dr. M. C. Berndt, Westmead Hospital, Westmead, Australia). The sections were then extensively washed in TBS and incubated with gold conjugates (goat anti-rabbit IgG coupled to 10-nm gold particles; Janssen Pharmaceutica, Beerse, Belgium) for 1 h. Finally, they were counterstained with uranyl acetate and lead citrate and examined on an electron microscope (model EM 301; Philips Electronic Instruments, Inc., Mahwah, NJ).

Anti-P-Selectin Antibody Binding to Cells

The monoclonal anti-P-selectin antibody AC1.2 (Larsen et al., 1989) was radiolabeled with ^{125}I using Enzymobead radioiodination reagent (Bio-Rad Laboratories) to 0.4–0.8 μCi/μg IgG. Cells (grown on 48-well plates) were washed in HBSS containing 10 mM Hepes (pH 7.4), 1 mM $CaCl_2$, and 1% BSA. They were incubated for 30 min at 37°C in the same buffer containing ^{125}I-labeled antibody (3 μg/ml) in the absence or presence of 5 mM 8-Br-cAMP. Then the cells were washed four times in buffer, lysed with 0.2% SDS, 4 mM EDTA, and counted in a γ counter. To determine the amount of nonspecific binding, a 100-fold excess of unlabeled antibody was added. 80–90% of the radiolabeled antibody bound specifically.

Results

Expression of P-Selectin by Heterologous Cells

P-selectin cDNA (Larsen et al., 1990) was inserted into a mammalian expression vector containing the cytomegalovirus IE promotor. Transient expression of this construct in COS-1 cells resulted in production of biologically active P-selectin on the plasma membrane as judged by the binding of HL60 cells (a cell line with neutrophil-like characteristics) to transfected cells (not shown). To study targeting of P-selectin to storage granules, a cell line with a regulated pathway of secretion was chosen for stable expression of P-selectin. The mouse pituitary cell line AtT-20, which stores the hormone ACTH, was cotransfected with the pCMV–P-selectin plasmid and pRSVneo, a plasmid that encodes resistance to the neomycin analogue G418. G418-resistant clones were screened for P-selectin expression by immunofluorescent microscopy. To characterize the recombinant protein, cells were metabolically labeled and lysed. P-selectin was isolated from the lysate by immunoprecipitation (Fig. 1). Two species of P-selectin were observed with an apparent relative molecular mass of 120,000 and 140,000. Treatment of the immunoprecipitates with endo H resulted in a shift of the lower molecular weight band to 95,000 M_r. The 120,000-M_r species therefore appears to be a precursor containing N-linked carbohydrates of the high-mannose type. HEL cells (Johnston et al., 1989*b*) and endothelial cells (McEver et al., 1989) are also known to contain this precursor. Scanning of the autoradiogram revealed that ~7% of P-selectin in AtT-20 cells was endo H sensitive. Therefore the majority of the protein is present in a post-medial Golgi compartment. A pulse–chase experiment in AtT-20 cells demonstrated that the precursor was converted to its mature endo H–resistant form in 30–60 min from the onset of synthesis (not shown). Three potential sites for the localization of the mature form of P-selectin are the Golgi apparatus, the cell surface, or a storage compartment.

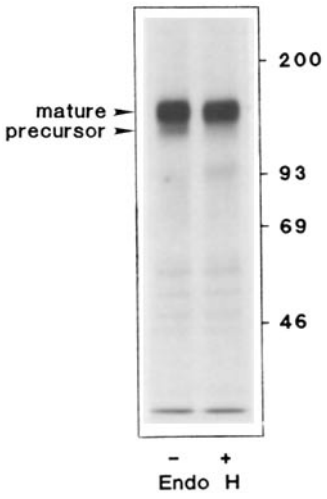

Figure 1. Molecular forms of P-selectin detected in AtT-20 cells. AtT-20 cells expressing P-selectin were metabolically labeled with [^{35}S]cysteine for 2 d. After immunoprecipitation from the cell lysates with a monoclonal antibody to P-selectin and treatment with (+) or without (−) endo H, the protein was analyzed on a 6% polyacrylamide gel under reducing conditions followed by fluorography. The migration of the lower band was increased after endo H treatment.

To examine the distribution of P-selectin in AtT-20 cells, the cells were first evaluated by indirect immunofluorescent light microscopy. A weak perinuclear staining of P-selectin was observed. In addition, a punctate pattern of P-selectin distribution was observed at the tips of processes extending from the cells (Fig. 2, *A* and *C*). ACTH appeared to codistribute with P-selectin as determined by double label immunostaining (Fig. 2, *A* and *B*). The rhodamine and fluorescein conjugates did not interfere with each other (Fig. 2, *C–F*). ACTH-containing secretory granules have been shown to accumulate at process tips (Tooze and Burke, 1987; Mat-

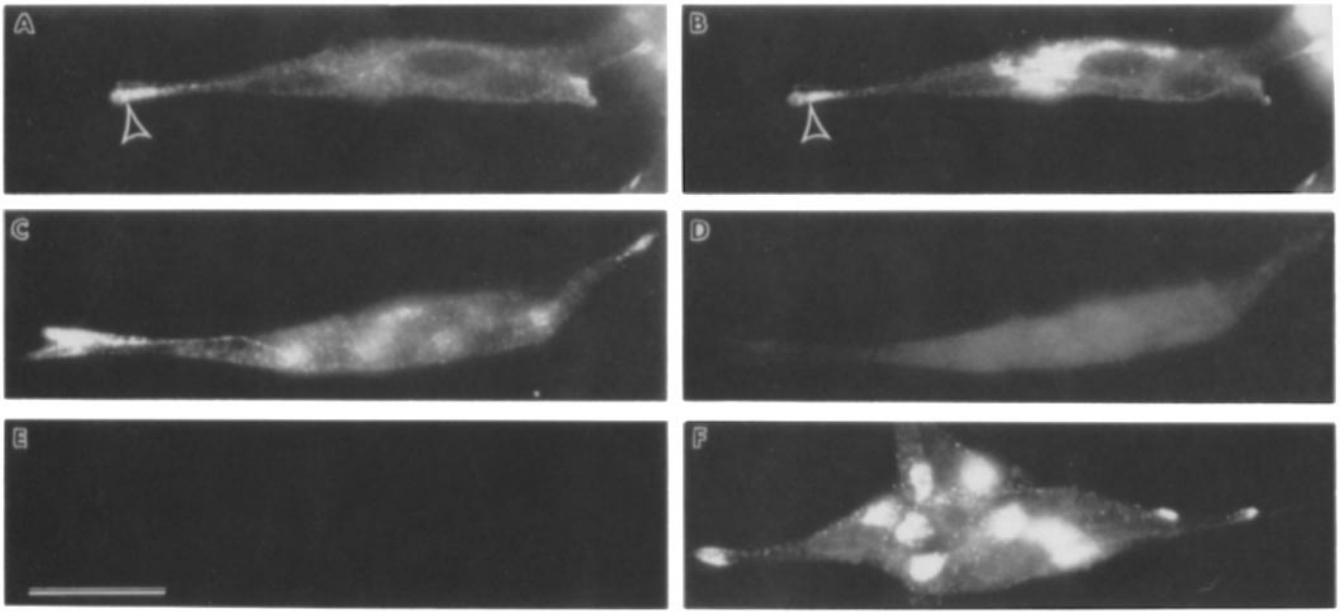

Figure 2. Distribution of P-selectin and ACTH in AtT-20 cells. Fixed and permeabilized cells were stained by double label fluorescence for P-selectin (*A*, *C*, and *E*) and ACTH (*B*, *D*, and *F*). In each pair, identical fields are shown. The top pair shows the similar distribution of P-selectin (*A*) and ACTH (*B*). Arrowheads point to the tips of processes containing granules that are positive for both P-selectin and ACTH. The middle and lower pairs show cells in which the anti-ACTH (*D*) and the anti-*P*-selectin antibodies (*E*), respectively, were omitted. Panels *C* and *F* were stained for P-selectin and ACTH, respectively. Bar, 20 μm.

suuchi et al., 1988). Various soluble proteins that are secreted by the regulated pathway in transfected AtT-20 cells such as trypsinogen, insulin, and growth hormone colocalize with ACTH in these areas (Burgess et al., 1987; Orci et al., 1987; Rivas and Moore, 1989). However, mitochondria and lysosomes appear to accumulate in these processes as well (Matsuuchi et al., 1988). Therefore, the apparent similarity in distribution of P-selectin and ACTH does not unequivocally demonstrate that P-selectin follows the same secretory pathway as ACTH.

Fractionation of AtT-20 Cells

To further study the compartmental distribution of P-selectin in AtT-20 cells, we fractionated cell homogenates on Percoll density gradients (Fig. 3). ACTH served as an endogenous marker for the secretory granules and was recovered from the gradient as two well separated bands (Fig. 3 *B*). The band of higher density, representing the intact granule fraction, contained 60% of the total amount of hormone (ranging 33–72% in four separate experiments). The band of lower density coincided with most of the cellular protein (Fig. 3 *A*) and likely consisted of incompletely processed hormone in the RER and Golgi apparatus. P-selectin was quantitated by a dot-blot procedure using ^{125}I-labeled monoclonal antibody to P-selectin and densitometric scanning of the autoradiograph. The P-selectin distribution coincided with ACTH (Fig. 3 *B*), with a significant portion (~20%) being recovered from the granule peak. The presence of P-selectin in the dense fraction further suggested the existence of a pool of P-selectin within the storage granules. The profile of a lysosomal marker, *N*-acetyl-β-glucosaminidase (Fig. 3 *B*), overlapped with the secretory granule peak, but we consistently observed the lysosome peak at a higher density than the granule peak. Using similar conditions, others (Hutton et al., 1982) also found that lysosomes were more dense than insulin granules.

If the dense peak of P-selectin truly represents a storage compartment, a protein that is not stored should be absent from this region of the Percoll gradient. To test this assumption, we fractionated homogenized AtT-20 cells that expressed the mature subunit of vWF. This protein, which lacks the propeptide that is required for storage of vWF, is secreted exclusively by the constitutive pathway from AtT-20 cells (Wagner et al., 1991). Mature vWF was found in the upper portion of the gradient (Fig. 3 *C*), but not in the ACTH peak at higher density, which further supported the conclusion that the latter peak represented the storage pool.

Ultrastructural Localization

The experiments described above indicated that P-selectin was stored in granules indistinguishable from the ACTH-containing granules by light microscopy and density centrifugation. To further confirm this interpretation, we performed electron microscopy of immunogold-labeled sections. Electron-dense granules were found in AtT-20 cells and were shown to contain ACTH (Fig. 4, *A* and *C*). Of ~1,000 granules counted, 75% displayed positive staining for ACTH. This observation has been previously documented by others (Tooze and Tooze, 1986; Burgess et al., 1987). The electron-dense granules also labeled for P-selectin (Fig. 4, *B* and *D*). About 18% of the granules contained gold particles. Less than 2% of the granules were labeled in control untransfected cells incubated with the same antibodies. In contrast to the ACTH label, which was seen randomly distributed throughout the granules, P-selectin–associated gold particles were preferentially confined to the granule membrane. No P-selectin

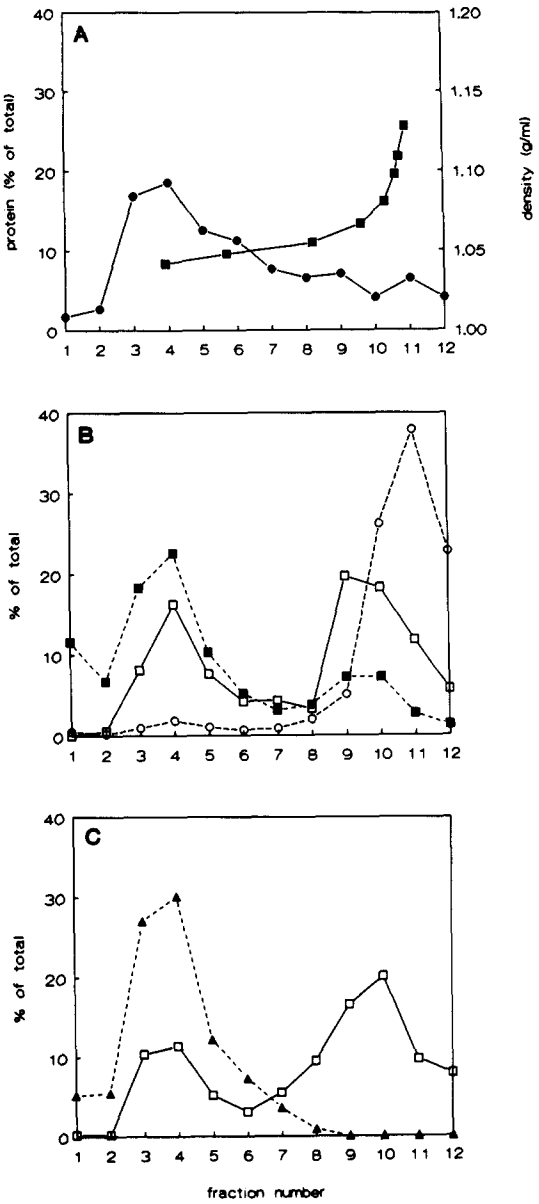

Figure 3. Density gradient fractionation of AtT-20 cells. Trypsinized AtT-20 cells were homogenized and the 10,000-*g* pellet was layered on 20% Percoll in 0.25 M sucrose. After centrifugation for 60 min at 40,000 *g*, 800-μl fractions were collected from the top and analyzed (see Materials and Methods). (*A* and *B*) AtT-20 cells expressing P-selectin; (*C*) AtT-20 cells expressing mature vWF. (*A*) (●) total protein; and (■) density; (*B*) (□) ACTH; (■) *P*-selectin; and (○) *N*-acetyl-β-glucosaminidase (lysosomal marker); (*C*) (□) ACTH; and (▲) mature vWF. P-selectin was present in the denser second peak of ACTH, which likely represents the ACTH-storage granules (Gumbiner and Kelly, 1981), but mature vWF, a nonstored protein (Wagner et al., 1991), was absent from this peak. Similar profiles were obtained from fractionation of cells collected by scraping rather than by detaching with trypsin/EDTA (not shown).

labeling was found in lysosomes, mitochondria, nuclei, or other unidentified organelles. Table I provides a further illustration of the specificity of the granular staining of P-selectin and ACTH: although granules occupy <5% of the surface area of a cell, 64% of the gold particles was found on the granules. Fig. 5 shows the granule-rich tip of a process from an AtT-20 cell expressing P-selectin. Numerous granules displayed P-selectin labeling but very few gold particles were found on the plasma membrane (Fig. 5 *B*). Although the presence of P-selectin in the light fractions from the Percoll gradient (Fig. 3) suggested localization in compartments such as the plasma membrane, ER, or Golgi apparatus, we found little labeling in these structures (Table I). A relatively low density of P-selectin in these areas, the affinity of the polyclonal antibody, and the sensitivity of this technique may all contribute to this result.

Release from AtT-20 Cells

The cAMP analogue 8-Br-cAMP has been widely used as a secretagogue for AtT-20 cells. We observed an approximately threefold increase in the release of ACTH from 8-Br-cAMP–treated cells over the amount secreted by untreated cells during an incubation for 30 min at 37°C (Fig. 6). To determine if P-selectin is concomitantly translocated to the plasma membrane from the storage granules, we used a ^{125}I-labeled monoclonal anti–P-selectin antibody to detect surface expression of P-selectin (Fig. 6). The binding was specific for P-selectin–expressing cells, as no detectable binding was observed to control AtT-20 cells expressing mature vWF instead of P-selectin. These control cells secreted ACTH in response to 8-Br-cAMP in a similar fashion as the P-selectin–expressing cells. As shown in Fig. 6, binding of the antibody to the P-selectin–expressing cells was enhanced twofold in the presence of the secretagogue. Thus, AtT-20 cells can redistribute P-selectin to the plasma membrane upon stimulation, indicating that this transmembrane protein follows the regulated pathway of secretion in AtT-20 cells. As a control, P-selectin–expressing CHO cells (a cell line that does not exhibit a regulated pathway of secretion) were used. P-selectin was present on the surface of these cells and its surface expression, as determined by antibody binding, did not increase after treatment with 8-Br-cAMP (Table II).

Discussion

When expressed by AtT-20 cells, P-selectin was sorted to the regulated pathway together with ACTH, the endogenous hormone of these cells. Several lines of evidence led us to this conclusion. First, by immunofluorescence, P-selectin was found to accumulate at the tips of processes that protrude from the AtT-20 cell (Fig. 2). Rivas and Moore (1989) found that vesicular stomatitis virus G protein, which is a membrane protein transported through the constitutive pathway, is inserted into the plasma membrane of the cell body (i.e., not at the tips of processes). This resulted in a different immunofluorescent staining pattern as compared to regulated secretory proteins such as insulin, growth hormone, and ACTH, which accumulate at process tips (Orci et al., 1987; Rivas and Moore, 1989; Tooze and Burke, 1987; Matsuuchi et al., 1988). Second, P-selectin sedimented together with ACTH on Percoll gradients (Fig. 3). In contrast, vWF with its prosequence deleted, which is secreted constitutively (Wagner et al., 1991), was absent from the secretory granule peak. Third, by EM, P-selectin was present in electron-dense granules that were morphologically indistin-

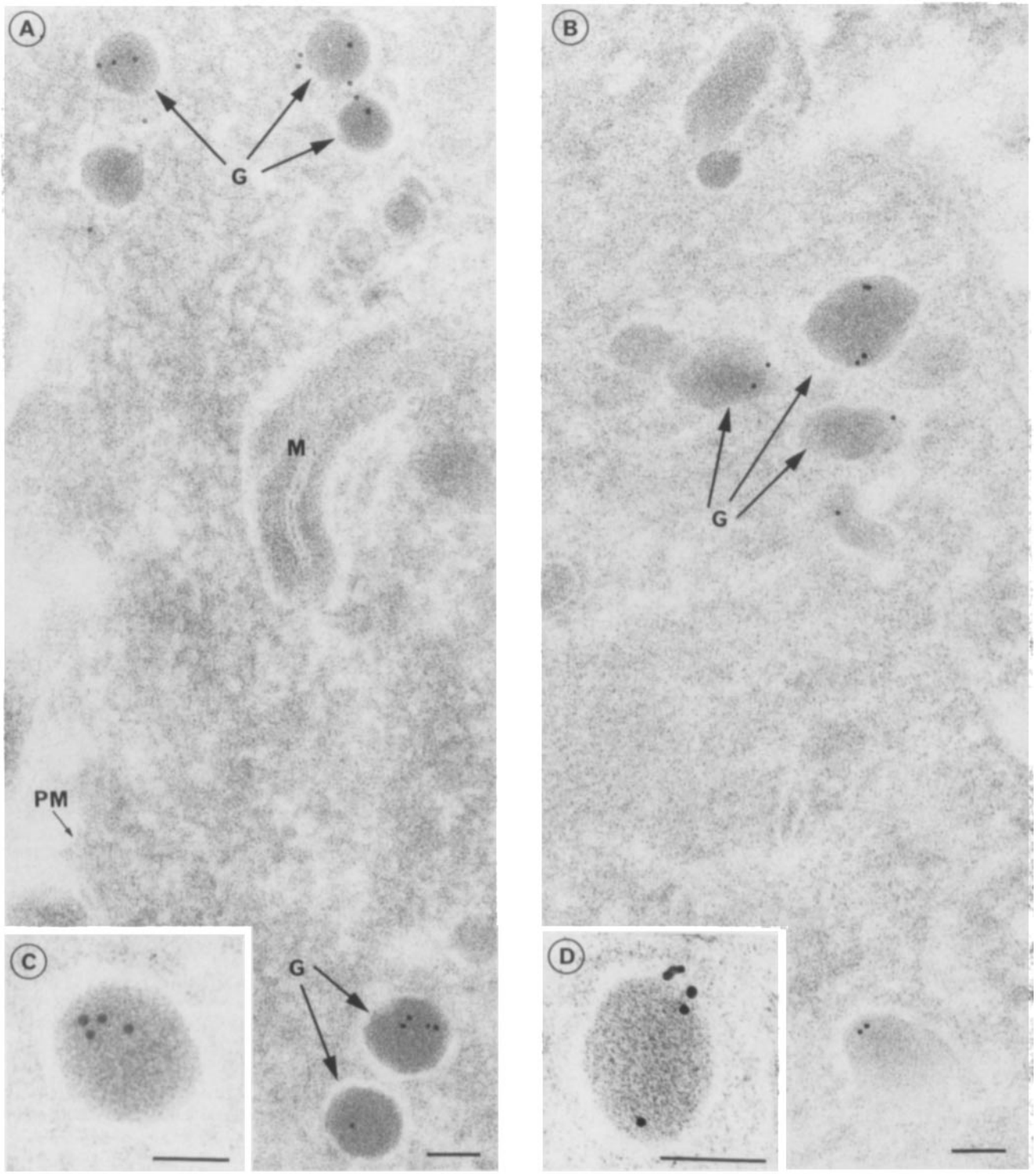

Figure 4. Electron micrograph of unstimulated AtT20 cells that express P-selectin. *A* and *C* show immunogold labeling for ACTH; *B* and *D* for P-selectin of AtT-20 cells transfected with P-selectin cDNA. Labeling of both proteins was restricted to the dense core granules (*G*). Anti-ACTH antibody revealed a matricial labeling of the secretory granules. In contrast, P-selectin labeling was rather found near the granule membrane. *M*, mitochondria; *PM*, plasma membrane. Bars, 100 nm.

guishable from the ACTH-containing granules (Figs. 4 and 5). As might be expected for a transmembrane protein, P-selectin was found near the periphery of the granules. Fourth, secretagogue treatment of the AtT-20 cells resulted in translocation of P-selectin to the cell surface. Release of stored proteins upon stimulation of the cell is an important hallmark of the regulated pathway of secretion. We found a two-fold increase in surface-expressed P-selectin in the presence of 8-Br-cAMP, a secretagogue that induces the release of ACTH (Fig. 6). Since there is also specific binding of the

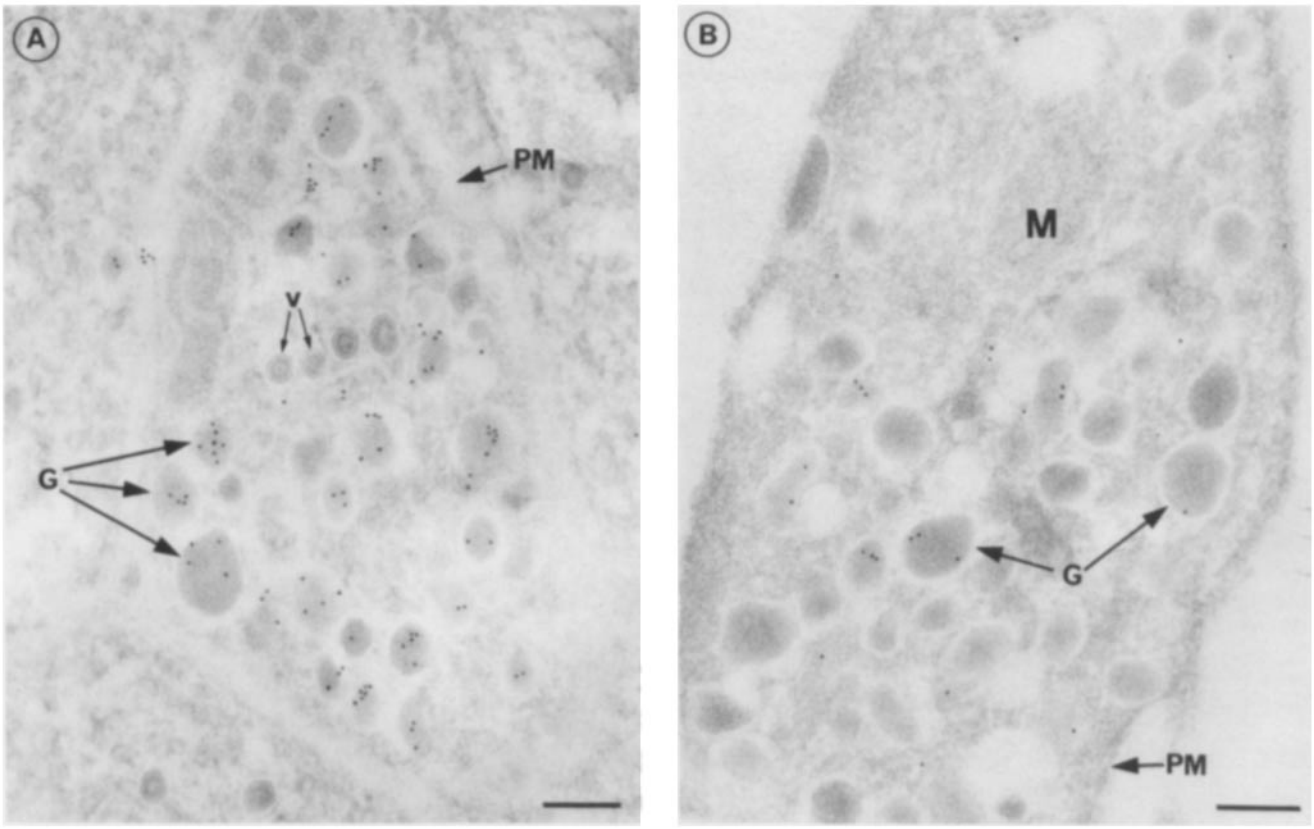

Figure 5. Electron micrograph of tips of AtT-20 cells. AtT-20 cells transfected with P-selectin were labeled for ACTH (*A*) or P-selectin (*B*). Both antigens were found mainly in granules (*G*). Note the absence of labeling on the plasma membrane (*PM*), virus particles (*V*), and mitochondrion (*M*). The immunogold staining confirmed the localization of P-selectin at the tips of processes as seen by fluorescence microscopy (Fig. 2) and further showed that it was present in dense granules rather than on the plasma membrane. Bars, 200 nm.

anti–P-selectin antibody to resting cells, some P-selectin may be constitutively expressed on the cell surface. Similarly, there is release of ACTH from resting cells. The presence of P-selectin on the surface of unstimulated cells could be attributed to inefficient sorting to the regulated secretory pathway in AtT-20 cells (Burgess and Kelly, 1987) or, alternatively, to basal release from the secretory granules (Matsuuchi and Kelly, 1991). Unstimulated cultured endothelial cells also show a low level of surface P-selectin (Hattori et al., 1989). We could not detect significant amounts of cell surface P-selectin in resting AtT-20 cells by immunoelectron microscopy, presumably because the sensitivity of this technique was too low. Likewise, gold particles were not detected on ER and Golgi membranes.

There was very little P-selectin in the endo H–sensitive precursor form (Fig. 1), which indicates that the majority of the protein in the steady state is present in late-Golgi compartments, secretory granules, or on the plasma membrane. Of these compartments only the secretory granules were significantly labeled for P-selectin by immunogold staining (Table I). Our cell fractionation experiments also revealed the presence of P-selectin in a storage compartment (Fig. 3). The peak at lower density, where the majority of P-selectin was found, contained the bulk of the cell protein and membrane compartments such as the plasma membrane, ER, and Golgi apparatus. Also, membrane fragments derived from ruptured secretory granules containing P-selectin were presumably in this peak. When scraping of cells was used rather than trypsinization, the profile was identical (not shown), which confirms that a relatively minor portion of P-selectin is present on the plasma membrane. The fraction of P-selectin found in the peak at high density (20%) is therefore likely an underestimate of the targeting efficiency. ACTH, which is released during rupture of the granules, is washed away and does not contribute to the low-density ACTH pool. This may account for the relatively larger amount of ACTH found in the high density peak in comparison to P-selectin.

Table I. Distribution of Immunogold Labeling for P-Selectin on Sections of Transfected AtT-20 Cells

Localization	Particles	Particles/μm^2
	(% of total)	
Granules	64	45
Plasma membrane	7	–
Other cytoplasmic structures	29	0.5

20 cell profiles were examined and the gold particles counted.

When cells were fractionated after secretion was induced by 8-Br-cAMP, there was no significant change in the fractionation profile of either ACTH or P-selectin (not shown), which indicates that only a small portion of the storage pool can be released with this secretagogue. Also the immunofluorescence staining pattern of ACTH granules did not change appreciably. Nevertheless, a significant increase of P-selectin expression on the cell surface was observed after the

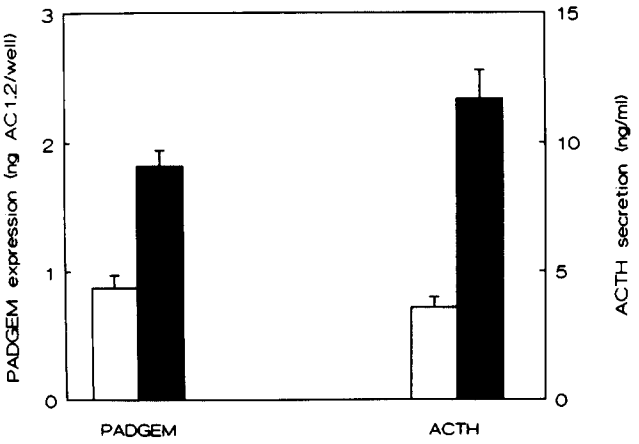

Figure 6. Surface expression of P-selectin and release of ACTH after secretagogue treatment of AtT-20 cells. Specific binding of ^{125}I-labeled AC1.2 monoclonal anti-P-selectin antibody (3 μg/ml) to AtT-20 cells expressing P-selectin in the absence (*open bars*) or presence (*closed bars*) of 5 mM 8-Br-cAMP was determined. In a parallel experiment, the supernatant was collected and assayed for ACTH by radioimmunoassay. The assays were performed in triplicate and the mean ± standard deviation is shown. The experiments were repeated twice with similar results.

AtT-20 cells were stimulated, suggesting either very efficient targeting of P-selectin to the storage granules or a rapid removal of constitutively expressed surface protein. Endothelial cells appear to internalize cell surface P-selectin (Hattori et al., 1989). Although shedding through a proteolytic mechanism has been proposed for L-selectin (gp90^{MEL14}), another member of the selectin family of adhesive proteins (Kishimoto et al., 1989), we found no evidence for release of P-selectin into the supernatant during treatment with 8-Br-cAMP (not shown).

In neuroendocrine cell lines such as AtT-20 and PC12 cells certain membrane proteins are targeted to small synaptic vesicle-like structures (Clift-O'Grady et al., 1990; Cutler and Cramer, 1990). P-selectin did not appear to follow this route, since immunofluorescent staining using the monoclonal antibody SY-38 directed against synaptophysin showed a punctate pattern throughout the cell distinct from that of P-selectin (not shown). Also, the fractions from the Percoll gradient that contained the storage pool did not react with this antibody as determined by dot-blot assay (not shown).

It is of interest that E-selectin (ELAM-1), another member of the selectin class of adhesion molecules (Bevilacqua et al., 1989), is expressed on endothelial cells after stimulation with cytokines (Bevilacqua et al., 1987), but not targeted to storage granules. So, despite similarities in structural domains (Johnston et al., 1989*a*; Bevilacqua et al., 1989), function (Larsen et al., 1989; Bevilacqua et al., 1987), and recognition of carbohydrate ligands (reviewed in Springer and Lasky, 1991), P-selectin and E-selectin follow different intracellular pathways.

P-selectin and vWF colocalize in the Weibel-Palade bodies of endothelial cells (Bonfanti et al., 1989; McEver et al., 1989) and they are the only known constituents of this organelle. The experiments presented here demonstrate that storage of P-selectin is not dependent on vWF. Instead, P-selectin contains its own targeting signal that can be recognized by a heterologous cell line. The mechanism for targeting of P-selectin seems to be different from that of vWF. vWF forms elongated organelles in AtT-20 and RIN5F cells that resemble Weibel-Palade bodies, but these granules are distinct from the ACTH- or insulin-containing endogenous granules and could not be induced to release upon stimulation (Wagner et al., 1991). In contrast, P-selectin is sorted in the AtT-20 cells together with ACTH to releasable storage granules. Another indication that the cellular machinery required for the sorting of P-selectin and vWF is different is found in the gray platelet syndrome, a bleeding disorder which is characterized by a deficiency of normal α granules (Raccuglia, 1971). Gray platelets contain markedly reduced amounts of vWF, although its synthesis in the megakaryocytes of these patients appears to be normal (Cramer et al., 1985). Also, the asymmetrical distribution of vWF in normal α granules is lost in the gray platelets; instead, it is randomly scattered throughout the very small granules found in the patients' platelets (Cramer et al., 1985). In contrast, the intracellular content of P-selectin in gray platelets is similar to that in normal platelets (Rosa et al., 1987). Activation of gray platelets results in redistribution of P-selectin from vesicle membranes to the platelet surface (Rosa et al., 1987). It seems therefore likely that in these patients the storage of vWF and other soluble α granule proteins is disturbed, while the targeting of P-selectin is not affected. While soluble stored proteins must interact with a targeting machinery localized in the lumen of the *trans*-Golgi apparatus, our preliminary results indicate that it is the cytoplasmic tail that directs storage of P-selectin. The expression of P-selectin and mutant variants thereof in AtT-20 cells provides an experimental approach for identification of the specific targeting sequence on this transmembrane protein.

Table II. Surface Expression of P-Selectin on CHO Cells

Cells	AC1.2 binding (ng/well)	
	8-Br-cAMP	
	(−)	(+)
CHO-P-selectin	39.0 ± 0.9	37.3 ± 3.7
CHO	0.49 ± 0.49	0.30 ± 0.47

Specific binding of ^{125}I-labeled AC1.2 monoclonal anti-P-selectin antibody (3 μg/ml) to cells in the absence or presence of 5 mM 8-Br-cAMP was determined. The assays were performed in triplicate and the mean ± standard deviation is shown. The experiment with the CHO-P-selectin cells was repeated with similar results.

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