

Mannose 6–Phosphate Receptors Are Sorted from Immature Secretory Granules via Adaptor Protein AP-1, Clathrin, and Syntaxin 6–positive Vesicles

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Abstract. The occurrence of clathrin-coated buds on immature granules (IGs) of the regulated secretory pathway suggests that specific transmembrane proteins are sorted into these buds through interaction with cytosolic adaptor proteins. By quantitative immunoelectron microscopy of rat endocrine pancreatic β cells and exocrine parotid and pancreatic cells, we show for the first time that the mannose 6–phosphate receptors (MPRs) for lysosomal enzyme sorting colocalize with the AP-1 adaptor in clathrin-coated buds on IGs. Furthermore, the concentrations of both MPR and AP-1 decline by $\sim 90\%$ as the granules mature. Concomitantly, in exocrine secretory cells lysosomal proenzymes enter and then are sorted out of IGs, just as was previously observed in β cells (Kuliawat, R., J. Klumperman, T. Ludwig, and P. Arvan. 1997. *J. Cell*

Biol. 137:595–608). The exit of MPRs in AP-1/clathrin-coated buds is selective, indicated by the fact that the membrane protein phogrin is not removed from maturing granules. We have also made the first observation of a soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor, syntaxin 6, which has been implicated in clathrin-coated vesicle trafficking from the TGN to endosomes (Bock, J.B., J. Klumperman, S. Davanger, and R.H. Scheller. 1997. *Mol. Biol. Cell.* 8:1261–1271) that enters and then exits the regulated secretory pathway during granule maturation. Thus, we hypothesize that during secretory granule maturation, MPR–ligand complexes and syntaxin 6 are removed from IGs by AP-1/clathrin-coated vesicles, and then delivered to endosomes.

THE TGN is involved in sorting and packaging of cargo and membrane proteins into carrier vesicles for distinct anterograde transport pathways in the cell. The best understood vesicle export from the TGN involves the sorting of lysosomal enzymes. In the Golgi complex, newly synthesized lysosomal enzymes acquire mannose 6-phosphate (M6P)¹ residues that are recognized by either of the two types of M6P receptor (MPR), the cation-dependent (CD) and cation-independent (CI) MPR (Kornfeld and Mellman, 1989; von Figura, 1991). Both

MPRs contain cytosolically exposed information that can be recognized by adaptor protein (AP) complexes that are recruited to membranes of the TGN (Robinson and Kreis, 1992; Stamnes and Rothman, 1993; Traub et al., 1993; Le Borgne et al., 1996), thereby initiating formation of a clathrin coat (Le Borgne and Hoflack, 1997). The subsequently formed clathrin-coated vesicles (CCVs) mediate the delivery of MPR–enzyme complexes to endosomes (Ludwig et al., 1991; Geuze et al., 1992; Klumperman et al., 1993). Syntaxin 6, a soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) protein with homology both to syntaxin 1 and synaptosomal-associated protein of 25 kD (SNAP-25) (Bock et al., 1996), is thought to be incorporated into these endosome-bound vesicles and is implicated in their docking and/or fusion (Bock et al., 1997).

A second vesicle pathway originating from the TGN, the constitutive secretory pathway, involves protein incorporation into small transport vesicles that rapidly convey their contents to the plasma membrane. Cells differentiated for a third TGN-derived exit route, the regulated

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1. *Abbreviations used in this paper:* AP, adaptor protein; CCV, clathrin-coated vesicle; CD-MPR, cation-dependent MPR; CI-MPR, cation-independent MPR; CPE, carboxypeptidase E; IG, immature granule; M6P, mannose 6-phosphate; MPR, mannose 6–phosphate receptor; ProB, procathepsin B; SNARE, soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor.

secretory pathway, also contain secretory granules, which undergo fusion with the cell surface only at low levels under resting conditions, and at amplified levels when a stimulus for exocytosis is received.

Proteins stored in secretory granules tend to share the biophysical property of precipitating under conditions of mild acidity and high calcium to form large, electron-dense, insoluble protein complexes (for review see Arvan and Castle, 1992). Protein condensation can begin to occur in the Golgi stack (Rambourg et al., 1988), the condensing vacuole subcompartment of the TGN (Tooze et al., 1987), or in immature granules (IGs) (Kuliawat and Arvan, 1992; Tooze et al., 1991a; Huang and Arvan, 1994), i.e., early post-TGN compartments that are already capable of signal-mediated exocytosis and in which prohormone processing occurs most extensively. Development of IGs into rather uniformly sized mature granules involves multiple biochemical reactions that take place over a few hours within the organelle lumen and in the delimiting membrane (Arvan and Castle, 1992).

From studies of both endocrine and exocrine secretory cells, it has been known for some years that in addition to the TGN, IGs also exhibit "fuzzy" coated patches (Geuze and Kramer, 1974; Novikoff et al., 1977; Farquhar and Palade, 1981) that contain clathrin (Orci et al., 1985; Tooze and Tooze, 1986; Kuliawat et al., 1997). The clathrin coat on IGs is typically found on membrane evaginations. Interestingly, recent *in vitro* studies also indicate that bovine brain AP-1 can be recruited to (rat) PC12 cell-derived IGs (Dittie et al., 1996). Taken together with the fact that CCVs uncoat before they fuse with a target membrane (for review see Schmid, 1997), the presence of clathrin-coated buds on IGs suggests the formation of CCVs that are involved in the removal of components from maturing granules.

To gain insight into the mechanisms underlying granule biogenesis, it is important to try to determine the destination and cargo of CCVs originating from the IG compartment. Several different destinations have been hypothesized (Arvan and Castle, 1992; Dittie et al., 1996). First, IG-derived CCVs might recycle components to less mature granules or the TGN (Geuze and Kramer, 1974; Kelly, 1985; Dittie et al., 1997). Second, they might mediate an anterograde traffic step in the constitutive-like secretory pathway (i.e., involving vesicular transport from maturing granules under unstimulated conditions), ultimately leading to release of small amounts of secretory protein at the cell surface (for review see Arvan and Castle, 1992; Grimes and Kelly, 1992). Third, they might fuse with endosomes (see below). It should be noted that the latter hypothesis may encompass both of the former alternatives.

The presence of lysosomal enzymes in secretory granules has long been established (Taugner et al., 1985, 1986; Im et al., 1989; Watanabe et al., 1989; Tooze et al., 1991b), but recently we showed that in endocrine pancreatic β -cells, lysosomal proenzymes containing a proper M6P recognition signal are sorted out of IGs and then delivered to lysosomes (Kuliawat and Arvan, 1994; Kuliawat et al., 1997). These data suggested that, like TGN-derived CCVs, IG-derived CCVs participate in the normal intracellular transport of newly synthesized lysosomal proenzymes. In the

present study, we endeavor to solidify the link between the presence of clathrin-coated buds on IGs and the vesicular egress of specific protein components from this compartment. At the same time, we have attempted to generalize our model of IG-based sorting to endocrine as well as exocrine systems. With this in mind, we provide the first comprehensive *in situ* localization of MPRs and AP-1 in both endocrine pancreatic cells, as well as in exocrine parotid and pancreatic cells, indicating that these molecules are removed from granules as they mature. The removal of MPRs in AP-1/clathrin-coated buds represents selective sorting, since we demonstrate that the membrane protein phogrin is not removed from maturing granules. A rationale for these findings in exocrine cell types is provided by new observations suggesting that lysosomal proenzymes do indeed enter IGs, to be sorted out later. Moreover, we have made the first demonstration that the clathrin-coated buds emerging from IGs contain syntaxin 6, the same SNARE protein implicated in vesicle traffic from the TGN to endosomes (Bock et al., 1997), which is lost during granule maturation in parallel with the loss of AP-1 and MPRs. From these data, we have proven that IGs actively participate in membrane and luminal protein sorting as part of the general mechanism underlying the *de novo* generation of mature secretory granules.

Materials and Methods

Materials and Antibodies

RIA-grade bovine serum albumin, carbachol, DTT, iodoacetamide, and luminol were from Sigma Chemical Co. (St. Louis, MO), soybean trypsin inhibitor was from Worthington Biochemical Corp. (Malvern, PA), [³⁵S]met/cys mixture was from NEN Life Science Products (Express; Boston, MA). Three antisera against rat cathepsin B were used. The first was a rabbit antiserum raised against recombinant rat pro-cathepsin B (ProB) (Rowan et al., 1992). The second was a rabbit antiserum raised against recombinant mature rat cathepsin B. These sera were generously provided by J. Mort (Shriner's Hospital, Montreal, Canada). A third rabbit antiserum raised against biochemically purified rat cathepsin B was obtained from A. Saluja (Beth Israel Hospital, Boston, MA). Horseradish peroxidase-conjugated anti-rabbit serum (Cappel Laboratories, Malvern, PA, and Worthington Biochemical Corp., respectively) was used as a secondary reagent for Western blotting by enhanced chemiluminescence with luminol. Zysorbin-protein A was from Zymed Labs, Inc. (South San Francisco, CA). An affinity-purified polyclonal anti-gamma- α -adapin antibody (AP-1) was kindly provided by M. Robinson (University of Cambridge, Cambridge, UK) and an affinity-purified rabbit antibody directed against phogrin was obtained from J. Hutton (University of Colorado Health Sciences Center, Denver, CO). To immunolocalize syntaxin 6 and proinsulin, we used the monoclonal antibodies 3D10 (Bock et al., 1997) and GS9A8 (O. Madsen, Hagedorn Research Institute, Copenhagen, Denmark), respectively, in combination with a secondary rabbit anti-mouse antibody (DAKOPATTS, Copenhagen, Denmark). The affinity-purified polyclonal antibodies against the CD-MPR (MSC1) and CI-MPR, raised by G. Lienhard (Dartmouth Medical School, Hanover, NH), have been used previously (Klumperman et al., 1993). A rabbit anti-serum raised against the cytosolic tail of the human CI-MPR (III/4) was kindly forwarded by Von Figura (Georg-August University, Göttingen, Germany).

Tissue Preparations

Pancreatic and parotid glands were dissected from 200-g Sprague-Dawley rats that had been subjected to an overnight fast with water available *ad libitum*. Parotid or pancreatic tissue was dissected and cleaned of connective tissue as described (Arvan and Castle, 1987), and then lobules were prepared as described by a modification of Scheele and Palade (1975). For electron microscopy, male Wistar rats were anaesthetized and then sub-

jected to perfusion fixation as described in Slot et al. (1991). The fixative solution consisted of 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4.

Immunoelectron Microscopy

Tissue fragments were washed briefly in PBS and then immersed for at least 4 h at 4°C in 2.3 M sucrose. Ultrathin cryosectioning was performed as described, using the improved pick-up method with a mixture of sucrose and methyl cellulose (Liou et al., 1996). (Multiple) Immunolabeling was performed according to the protein A–gold method, as described previously (Slot et al., 1991). Mouse-derived antibodies (proinsulin and syntaxin 6) were indirectly marked by protein A–gold via a rabbit anti–mouse IgG bridging antibody. To establish the relative distribution patterns of CD-MPR, AP-1, phogrin, and syntaxin 6 (see Table I), areas of the grid were selected that contained cells exhibiting well-preserved ultrastructure. Then, at a magnification of 20,000, the gold particles were counted randomly by scanning along fixed tracks, and each encountered gold particle was attributed to the compartment over which it was located, based on gold particles that were within a distance of ~30 nm from a membrane. The number of gold particles found over each of these compartments was finally expressed as a percentage of the total number of gold particles counted. In pancreatic β -cells, quantitations were performed in sections that were double-immunogold labeled for proinsulin and CD-MPR, AP-1 or phogrin, which allowed us to identify IGs by the presence of proinsulin label (Kuliawat et al., 1997). By this definition, 20–25% of all granules were considered immature in different experiments. In these cells, 525 gold particles were counted for CD-MPR, 699 for AP-1, and 504 for phogrin, respectively. In exocrine cells, morphological criteria were used to distinguish newly formed granules (moderately dense content, irregularly shaped, and with considerable size variation), from mature granules (dense content, uniformly sized, lacking membrane buds, and with a characteristic oval or round appearance). In exocrine pancreas, of all secretory granules, ~20% were immature by this definition. In these cells, 500 gold particles were counted for CD-MPR and 1,287 for AP-1. In our preparation of parotid cells, ~35% of all secretory granules were considered immature. In these cells, 485 gold particles were counted for syntaxin 6 and 504 for AP-1. To establish the degree of colocalization for syntaxin 6 and AP-1, double immunogold-labeled grids were randomly searched for syntaxin 6-positive membranes with a morphologically apparent clathrin coat. Each coated, syntaxin 6-positive membrane, i.e., TGN membranes and vesicles as well as IGs, was then scored as positive or negative for AP-1. In both β -cells and parotid, 300 syntaxin 6- and clathrin-positive membranes were analyzed this way.

The Golgi complexes of the cell types under study were well developed and bordered a cytoplasmic area with many clathrin-coated membranes. All membranes present in the Golgi area that could not be distinguished as IGs were considered part of the TGN. Unlike endocrine cells, the TGN of exocrine cells showed attached condensing vacuoles. Evidently, in ultrathin sections these condensing vacuoles could not always be unambiguously discriminated from free IGs. Endosomes were defined in this study as tubulovacuolar compartments with a variable number of internal vesicles.

Pulse-Chase Protocol

Parotid or pancreatic tissue was washed three times for 5–10 min each in oxygenated Eagle's modified minimal essential medium plus 0.1% bovine serum albumin and 0.01% soybean trypsin inhibitor, pH 7.4. The tissue was then preincubated for 10 min in methionine- and cysteine-free DME (Sigma Chemical Co.) with the same additives at 37°C. After pulse labeling with [³⁵S]met/cys for 30 min, the tissue was washed three times in a large vol of complete DME chase medium with the above additives. The bathing media from subsequent defined chase periods were then collected. All bathing media were freshly oxygenated before use, since this was found necessary to maintain secretory performance. To examine the presence or absence of labeled lysosomal enzyme in the regulated secretory pathway at specified chase times, equal amounts of pulse-labeled lobules were incubated in the presence or absence of stimulation with a secretagogue. For parotid lobules, the secretagogue used was 30 μ M of D,L-isoproterenol. For pancreatic lobules, this secretagogue was comprised of a mixture of 25 μ M of carbachol, 1 mM 8Br-cAMP, and 130 nM phorbol 12-myristate, 13-acetate. When the cells were to be analyzed, the lobules were lysed by sonication as described (Arvan and Castle, 1987) and then diluted into immunoprecipitation buffer (Kuliawat et al., 1997). To confirm a stimulated secretory response, equal vol aliquots of unstimulated or stimulated chase media were analyzed by SDS 8%-PAGE and then fluorography.

Biochemical Analysis of Cathepsin B in Exocrine Tissues

Both media and cell lysates were precleared by a brief incubation with Zysorbin followed by spinning in a microfuge (Beckman Instruments, Stanford, CA) before further analysis. Immunoprecipitation was performed using rabbit anti–rat recombinant ProB (refer to *Materials and Antibodies*). For immunoblotting of cathepsin B, samples analyzed by SDS 12%-PAGE were electrotransferred to nitrocellulose and then blotted with an equal mixture of both rabbit antisera to recombinant cathepsin B (refer to *Materials and Antibodies*) at a dilution of 1:1,000, and then detected with a peroxidase-conjugated secondary antibody by enhanced chemiluminescence.

Results

Immunolocalization of CD-MPR and AP-1 in Endocrine Pancreatic β Cells

Recently, we showed that soluble lysosomal hydrolase precursors, such as ProB, abundantly enter IGs of β cells (Kuliawat and Arvan, 1994; Kuliawat et al., 1997). This entry into IGs is not inhibited when the normal carbohydrate-dependent mechanism of ProB sorting is partially or completely blocked, but efficient sorting out of maturing granules requires a high affinity for MPRs. We reasoned that if lysosomal enzymes exit the regulated secretory pathway via a similar mechanism to that operative in the TGN, then one should predict the presence of MPRs and AP-1 in IGs. In β cells, immunoreactivity with an antibody against a proinsulin cleavage site (Arg31–Arg32) is one criterion that can be used to discriminate IGs from mature granules (Orci et al., 1985; Kuliawat et al., 1997). In the present study, we used this antiproinsulin antibody in double-immunogold labeling studies to perform a detailed morphological analysis of the subcellular distribution of CD-MPR and AP-1 in β cells.

Immunoelectron microscopy of ultrathin cryosections established both CD-MPR (Fig. 1) and AP-1 (Fig. 2, A and B) immunogold labeling over the limiting membrane of proinsulin-positive IGs, and in clathrin-coated buds emerging from these compartments (Fig. 1 C and Fig. 2 B). Additional CD-MPR label in the TGN area was found over CCVs (Fig. 1 C) and over small, electron-dense MPR transport vesicles that previously have been identified in cells lacking a regulated secretory pathway (Fig. 1 C) (Bleekemolen et al., 1988; Klumperman et al., 1993). AP-1 was also found to be associated with clathrin-coated membrane buds emerging from TGN tubules (Fig. 2 A), suggesting that the clathrin-mediated biosynthetic pathway from the TGN, analogous to that identified in constitutive secreting cells (Kornfeld and Mellman, 1989), continues to be operational in regulated secretory cells as well.

Because a significant fraction of ProB and other lysosomal proenzymes are sorted out of IGs of β cells (Kuliawat et al., 1997), the concentration of MPRs should be expected to decrease upon granule maturation. Indeed, semiquantitative analysis of the CD-MPR and AP-1 distributions revealed that the percentage of immunolabeling for these antigens declined approximately threefold from IGs to mature granules (Table I). Importantly, in these cells we determined that the abundance of IGs was one-third to one-fourth that of mature granules. Together, these data indicate that in β cells, ~90% of the CD-MPRs in IGs are removed during granule maturation.

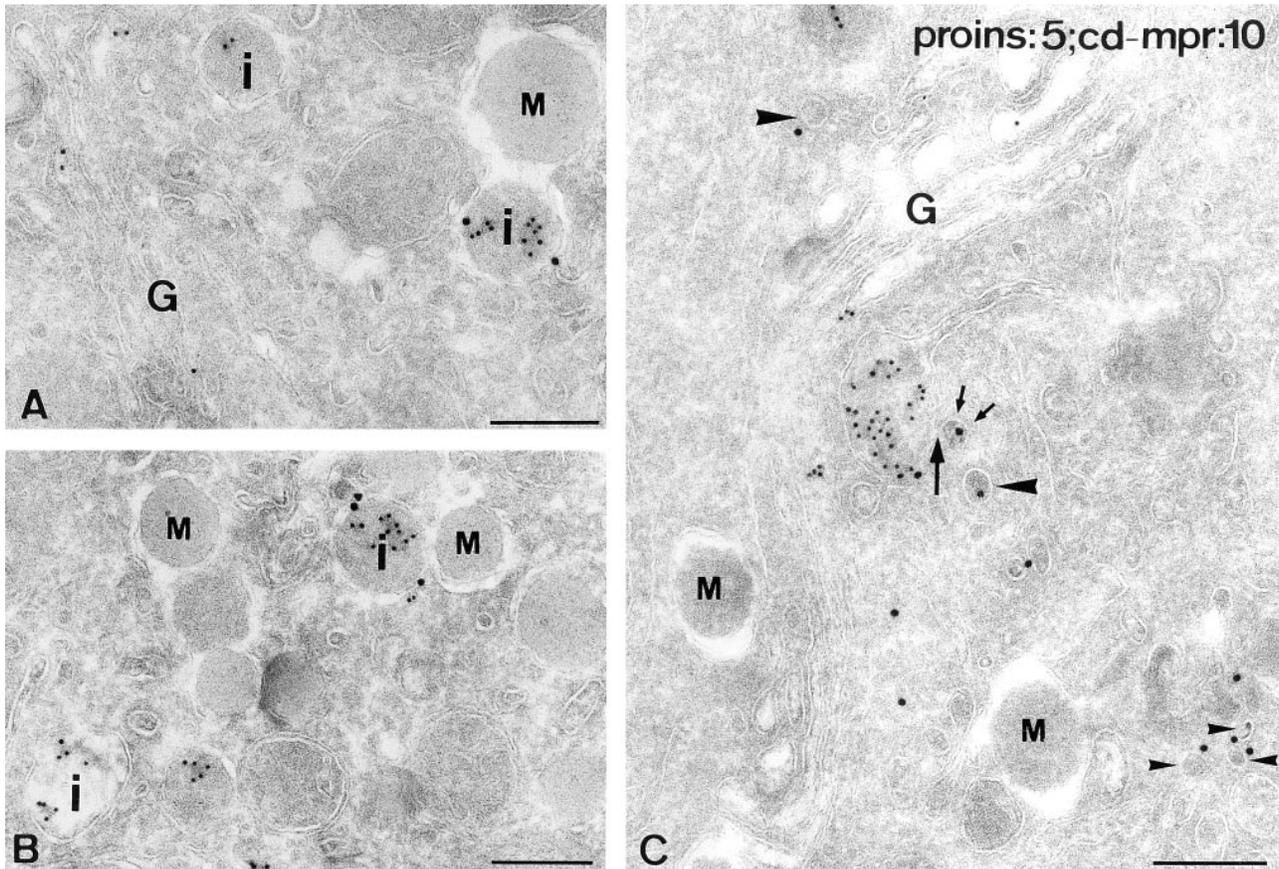


Figure 1. Ultrathin cryosections of endocrine pancreatic β cells that were double-immunogold labeled for proinsulin (5-nm gold) and CD-MPR (10-nm gold). Granules that were positively stained for proinsulin were marked as IGs (*i*), whereas proinsulin-negative granules were considered to be matured (*M*). Note also that mature β granules have the classically described appearance of an electron lucent halo between their content and limiting membrane. (*A* and *B*) CD-MPR label is present in the limiting membrane of IGs, but absent from mature granules. (*C*) An example of a proinsulin-positive condensing vacuole. The arrow points to a clathrin-coated (the coat is indicated by *small arrows*) membrane bud containing CD-MPR label. Additional CD-MPR staining is found over clathrin-coated TGN vesicles (*large arrowheads*) and small transport vesicles (*small arrowheads*). *G*, Golgi. Bars, 200 nm.

To unequivocally establish that MPR removal from IGs represents a specific protein sorting event, we compared this distribution to that of the secretory granule membrane protein phogrin (Kawasaki et al., 1996; Wasmeier and Hutton, 1996). Immunogold labeling of β cells for phogrin resulted in a specific labeling over both immature and mature secretory granules (Fig. 2 *C*). Notably, unlike the relative distribution of CD-MPR and AP-1, the immunolabeling of IGs did not decline at all as a function of granule maturation; indeed, 83% of all phogrin label was present on the mature granules (Table I). The concentration of phogrin in the plasma membrane and endosomes was below detection level, consistent with relatively rapid reuptake and recycling to the biosynthetic pathway after exocytosis. However, the major point from these data is that the exit of CD-MPRs from the regulated secretory pathway, in parallel with the disappearance of AP-1, is a protein-selective process mediated by CCVs.

MPRs Are Sorted out of IGs in Exocrine Cells

In our previous study in endocrine β cells we noticed that

the amount of ProB that appeared to enter the stimulus dependent-release pathway differed between individual tissues (Kuliawat et al., 1997). These findings might signify differences in the protein traffic patterns within an individual tissue that is exposed to different levels of secretory stimulation, or even unexplored differences among different regulated secretory tissues. With this in mind, we extended our examination of intragranular protein sorting to exocrine tissues, i.e., rat parotid and pancreatic acinar cells, which are some of the best characterized regulated secretory systems.

Importantly, given the maturation-dependent progression of protein condensation that has been established for exocrine granules, a range of secretory granules can be identified along the spectrum of maturity, based on the electron density of their luminal contents. Using this as a guide, Fig. 3 shows ultrathin cryosections of parotid cells in which we found both CI-MPR and AP-1 labeling associated with secretory granules with less condensed contents that were found in close proximity to the Golgi complex (i.e., IGs), whereas there were relatively few gold particles over granules with dense contents (i.e., those at later

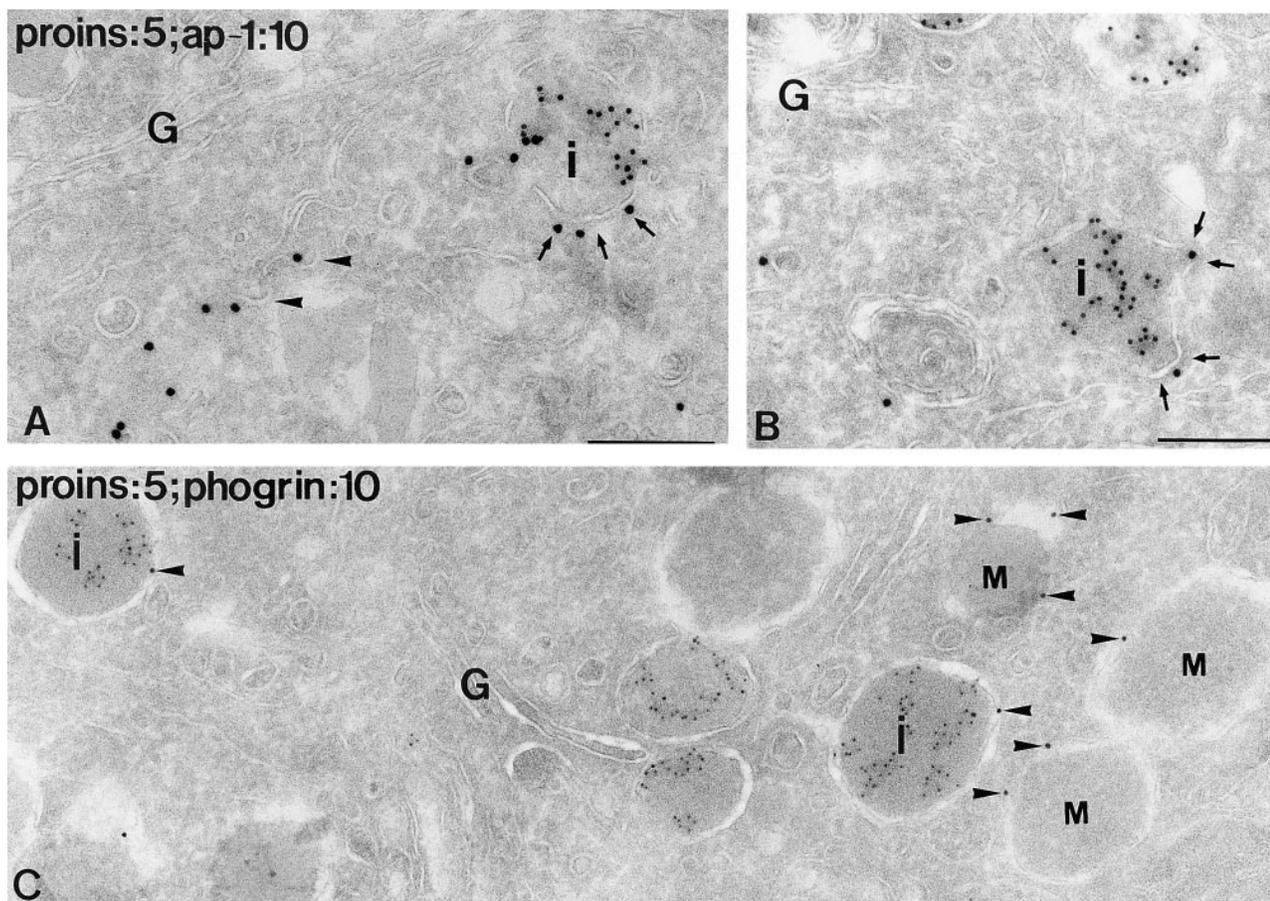


Figure 2. Ultrathin cryosections of endocrine pancreatic β cells. (A and B) Double-immunogold labeling for proinsulin (5-nm gold) and AP-1 (10-nm gold). AP-1 is present in clathrin-coated (arrows) membrane buds of proinsulin-positive IGs (*i*) and in clathrin-coated membrane buds evolving from TGN membranes (A, arrowheads). (C) Double-immunogold labeling for proinsulin (5-nm gold) and phogrin (10-nm gold). Gold particles indicating the presence of phogrin (arrowheads) are found in both IGs as well as mature (*M*) granules. *G*, Golgi. Bars, 200 nm.

stages of maturation). Indeed, by semiquantitative analysis, we confirmed that AP-1 labeling declined as a function of granule maturation (Table I), although occasional label could be found on coated membranes that appeared to bud from secretory granules with somewhat denser contents. In these cells, we focused on CI-MPR since labeling for CD-MPR appeared low, and both MPRs exhibit similar distributions in the TGN region (Klumperman et al., 1993). The CI-MPR was found over noncoated as well as clathrin-coated membrane areas of IGs (Fig. 3 A), whereas AP-1 localized predominantly to the clathrin-coated membranes (Fig. 3 B). Labeling of CI-MPR and AP-1 was also observed over TGN vesicles and tubules and, like in endocrine cells, AP-1 was found to be associated with clathrin-coated membrane buds emerging from TGN tubules (Fig. 3 B).

In exocrine pancreatic cells, CI-MPR (Fig. 4 C) and AP-1 (Fig. 4, B and C) exhibited similar distribution patterns as those observed in the parotid. Further, CD-MPR exhibited a localization pattern identical to that of CI-MPRs (Fig. 4 A). In pancreatic acinar cells, MPRs were often found in clathrin-coated buds emerging from IGs (Fig. 4 A) and double-immunogold labeling unequivocally established colocalization of AP-1 and CI-MPR in clathrin-

Table I. Relative Distributions of CD-MPR, AP-1, and Phogrin in Pancreatic β Cells, CD-MPR and AP-1 in Exocrine Pancreas, and Syntaxin 6 and AP-1 in Parotid

	Endocrine pancreas			Exocrine pancreas		Parotid	
	CD-MPR	AP-1	Phogrin	CD-MPR	AP-1	Syntaxin 6	AP-1
Golgi	0	0	0	0	0	12	0
TGN	66	70	2	48	64	65	72
IG	10	12	15	18	16	12	15
MG	3	4	83	9	10	3	1
End/lys	4	0	0	4	0	3	0
CCVs	3	11	0	4	8	0	11
NCVs	13	3	0	14	3	6	0
PM	1	0	0	3	0	0	0

The numbers represent the percentage of total gold labeling assigned to each compartment. In the first group, note the decrease in labeling over mature relative to immature granules for CD-MPR and AP-1, whereas the granule membrane protein phogrin remains associated with maturing granules. The differences in labeling density are even more apparent when taking into account that only a modest fraction (20–25% in different experiments) of granules are immature. In the second group of exocrine pancreas cells, in which ~20% of the secretory granules were considered immature, a similar decrease in CD-MPR and AP-1 labeling over maturing granules occurred. In the third group in parotid, in which ~35% of the granules was considered immature, the labeling densities of AP-1 as well as the SNARE protein syntaxin 6 also decrease upon maturation of the granules. *TGN*, trans-Golgi network; *IG*, immature granule; *MG*, mature granule; *end*, endosomes; *CCV*, clathrin-coated vesicles; *NCV*, vesicles in which a clathrin coat was not visible, located outside the TGN area; *PM*, plasma membrane.

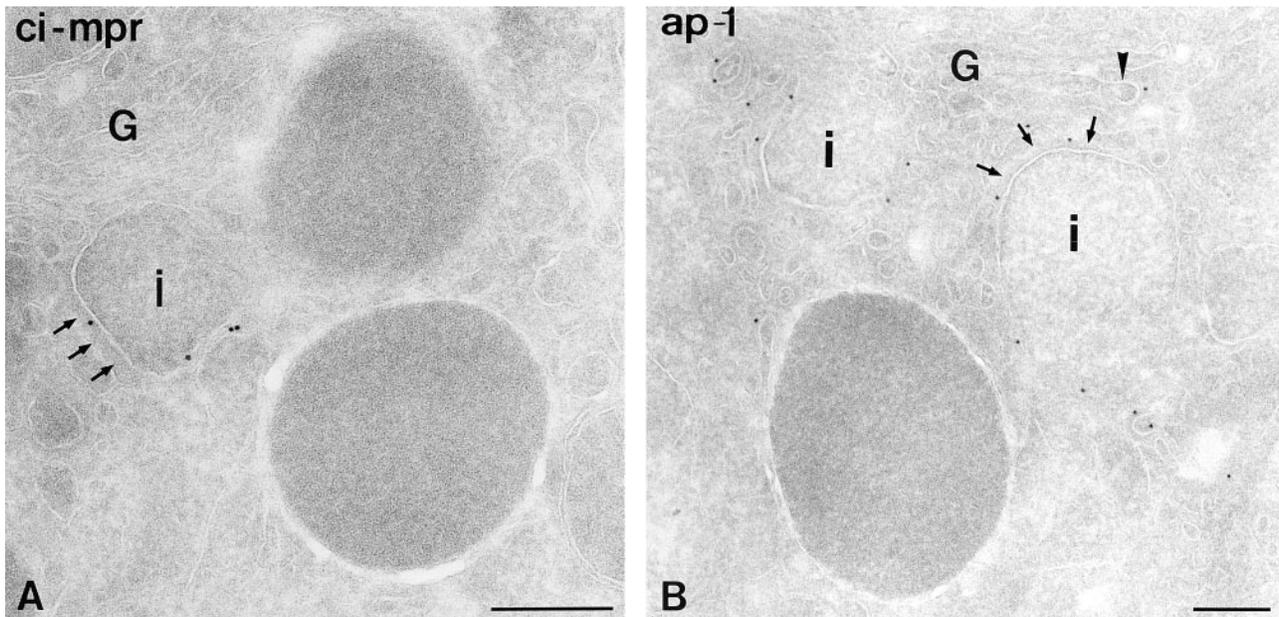


Figure 3. Immunogold localization of CI-MPR (A) and AP-1 (B) in the Golgi (G) region of rat parotid acinar cells. (A) CI-MPR is present in IGs (i) with less electron-dense contents. At some sites, MPR-positive granule membranes display the electron-dense coating characteristic for the presence of clathrin (arrows). (B) The presence of AP-1 in clathrin-coated (arrows) membrane areas of IGs with less electron-dense contents. AP-1 label is also associated with vesicles budding from TGN tubules (arrowheads). Bars, 200 nm.

coated membrane areas of IGs (Fig. 4 C). A semiquantitative analysis of the subcellular distribution of CD-MPR revealed that 18% of total CD-MPR label was associated with IGs, and only 9% with mature granules (Table I). We also determined that there are only approximately one-fourth as many IGs as mature granules in these cells, in agreement with earlier studies (Muller and Gerber, 1985). Together, these data indicate that ~90% of the MPRs of IGs are removed as a consequence of granule maturation in exocrine tissues. AP-1 showed a comparable decrease in labeling density between IGs and mature granules (Table I).

Sorting of ProB out of IGs of Exocrine Cells

The observation that MPRs enter and are subsequently retrieved from the regulated pathway of exocrine cells raised the question if in these cells, like in endocrine cells, newly synthesized lysosomal enzymes might enter IGs as part of normal trafficking en route to the endosomal-lysosomal system. To test this question, we began by immunogold localization of cathepsin B in rat parotid acinar cells. This tissue was of particular interest because it has been previously reported that using this technique, secretory granules fail to label with an antibody raised against rat liver cathepsin B (Tooze et al., 1991b), whereas lysosomes in these cells were positively stained. By contrast, we found that immunolabeling revealed a high number of gold particles representing cathepsin B in granules with a less dense content (i.e., IGs), whereas there were relatively few gold particles over granules with dense contents (Fig. 5 A). Furthermore, occasional cathepsin B immunoreactivity was detected in clathrin-coated evaginations that appeared to be budding from forming granules (Fig. 5 B).

Since electron microscopic identification of cathepsin B

immunoreactivity in secretory granules cannot distinguish mature B (Tooze et al., 1991b) from ProB, we next biochemically examined the intracellular contents and stimulus-dependent secretion of cathepsin B from rat parotid tissue. By Western blotting, the mature, ~31-kD lysosomal form of cathepsin B clearly predominated in the steady state (Fig. 6 E), although small amounts of ProB were occasionally detected (data not shown). After synthesis, intracellular conversion of newly synthesized ProB to mature B was also apparent, but even up to 2 h of chase, a predominant fraction of the new enzyme was still in the precursor form (Fig. 6 A), suggesting that this fraction had not yet been delivered to lysosomes. Interestingly, in medium samples collected during the first chase hour, unstimulated release of ProB was apparent, suggesting the possibility of transport through constitutive and/or constitutive-like secretory pathways. Exposure to isoproterenol (a secretagogue) during either the first or second chase hour clearly demonstrated stimulus-dependent release of newly synthesized amylase, the secretory granule marker from this tissue (Fig. 6 B). In addition, ProB release was detectably increased upon isoproterenol stimulation (Fig. 6 C, left two lanes). In the second chase hour, although the unstimulated secretion of ProB was much less than before, stimulated exocytosis of ProB upon secretagogue exposure was still clearly demonstrated (Fig. 6 C, right two lanes). Although later chase times were not examined intensively along with stimulated amylase release, stimulated secretion of a diminished fraction of newly synthesized lysosomal proenzyme remained apparent, providing no evidence for formation of mature cathepsin B within stimulatory granules, at times up to 5 h of chase (Fig. 6 D). Taken together, the morphological and biochemical data in Figs. 5 and 6 strongly suggest that ProB enters IGs in

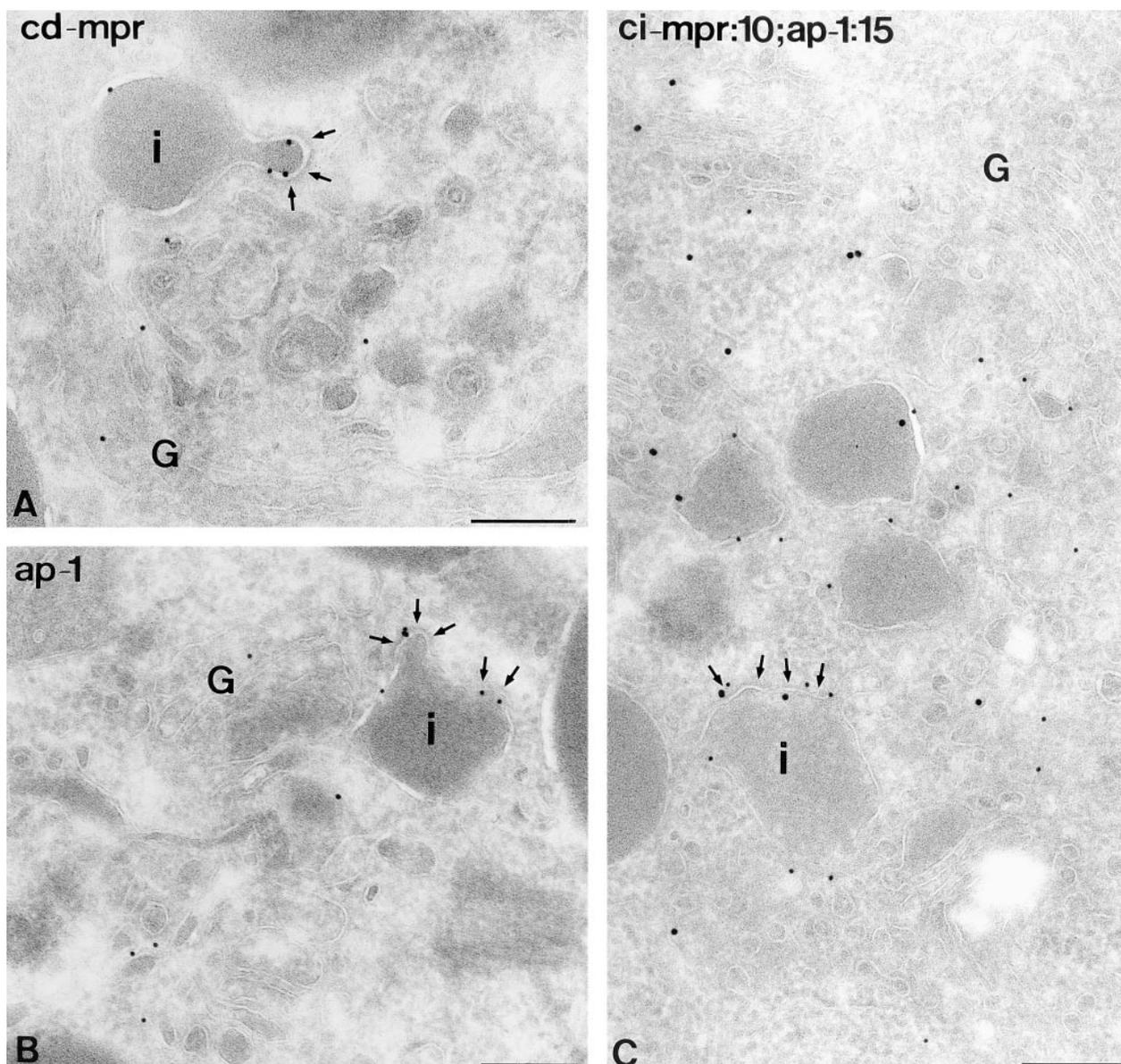


Figure 4. Ultrathin cryosections of rat pancreatic acinar cells illustrating immunogold labeling in the TGN region for CD-MPR (A), AP-1 (B), and AP-1 + CI-MPR (C). Note the irregular shape of IGs (*i*). (A) CD-MPR is clearly present in clathrin-coated (*arrows*) membranes budding from an IG. (B) AP-1 associated with clathrin-coated (*arrows*) membranes budding from an IG. (C) Double-immunogold labeling showing colocalization of CI-MPR (10-nm gold) and AP-1 (15-nm gold) in clathrin-coated (*arrows*) membrane areas of an IG. G, Golgi. Bars, 200 nm.

the rat parotid, but that intragranular concentrations of the hydrolase eventually decline, while the lysosomal form of cathepsin B appears gradually after a delay, ultimately achieving accumulation of mature B that is apparent upon detection at steady state. Thus, as in endocrine pancreatic β cells (Kuliawat et al., 1997), ProB behaves as a transient resident of the regulated secretory pathway.

In the rat exocrine pancreas, mature cathepsin B rather than the unprocessed, mannose-6-phosphorylated precursor has been reported to enter secretory granules (Tooze et al., 1991b). Such a conclusion would suggest that secretory granules in this tissue do not serve primarily as an intermediate in the biosynthetic pathway of lysosomal cathepsin B, but rather as an endpoint. By contrast, consti-

tutive-like secretion from the unstimulated rat exocrine pancreas lasts up to 7 h of chase, and therefore one might predict that ProB enters IGs in this tissue, with conversion to the mature form occurring at a much slower time scale. Unfortunately, we were unable to obtain a specific immunoelectron microscopic labeling for cathepsin B in exocrine pancreatic cells. Nevertheless, it was of interest to examine whether a clathrin-mediated escape route from maturing granules might also be operational in this tissue. In agreement with Tooze et al. (1991b), upon Western blotting of freshly prepared pancreatic tissue, the predominant steady-state form of intracellular cathepsin B was the mature, ~31-kD lysosomal form (Fig. 6 H), although trace amounts of ProB could be detected. However, in

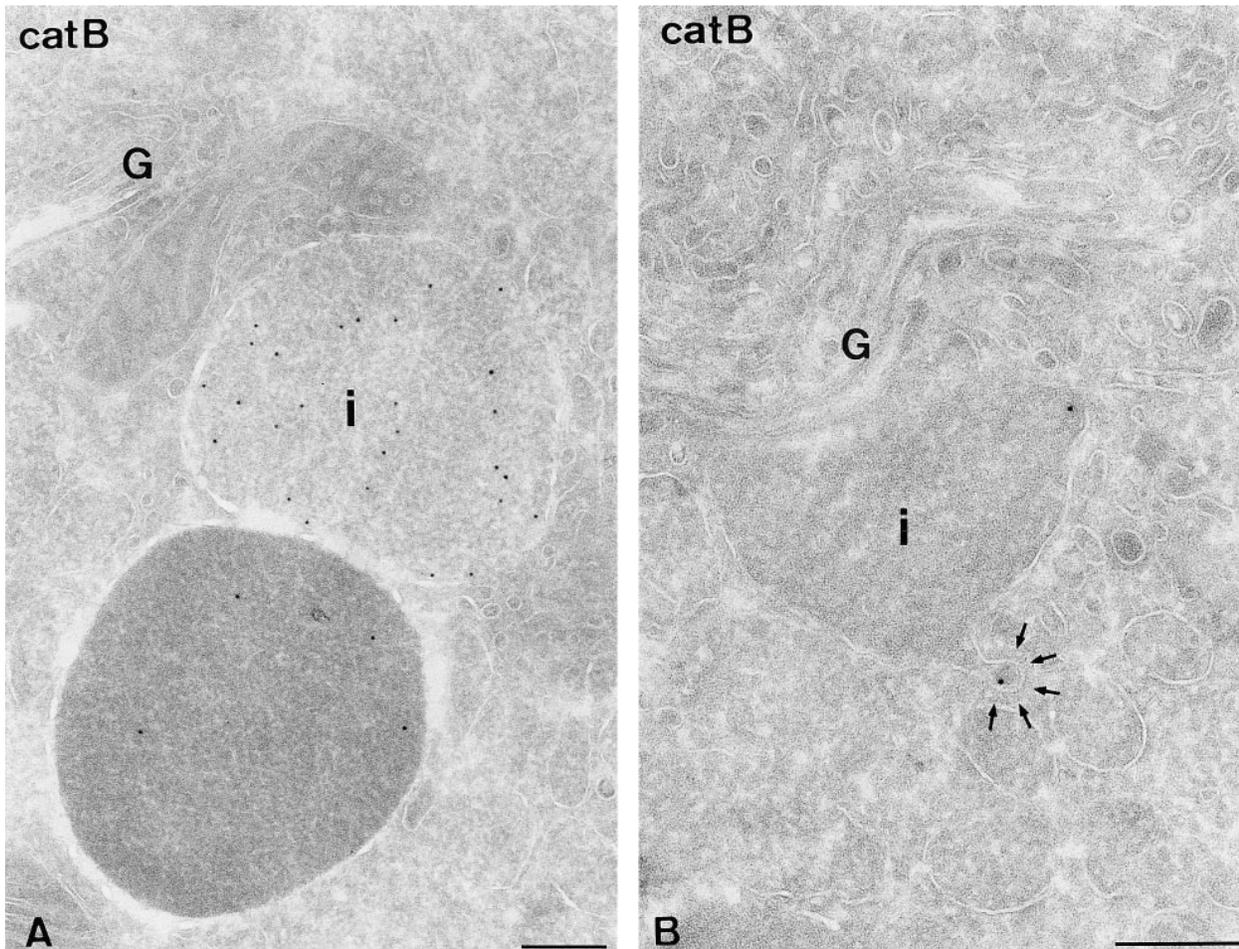


Figure 5. Ultrathin cryosections of rat parotid acinar cells showing immunogold labeling for cathepsin B (*catB*) in secretory granules. (A) Two secretory granules that markedly differ in the electron densities of their protein cores. Note that the labeling density for cathepsin B tends to be significantly higher in the IG (*i*) with the least dense contents. (B) Occasional cathepsin B labeling in membrane buds emerging from a forming secretory granule. Note on the lower bud the presence of a clathrin coat (*arrows*). *G*, Golgi. Bars, 200 nm.

pulse-chase experiments up to 3 h, little or no newly synthesized ProB had yet reached lysosomes, based on conversion to the mature form. At chase times >3 h, ongoing conversion was detected (data not shown), but such long chase experiments were not routinely feasible in our hands, since a significant decline in tissue performance (i.e., stimulus-dependent secretion) was observed at these later times. However, upon secretagogue exposure during the chase period from 1–3 h, there was a modest (10–15%) stimulus-dependent release of newly synthesized amylase and other granule proteins (Fig. 6 *F*) along with clear stimulus-dependent release of ProB (Fig. 6 *G*, left two lanes). An immunoreactive band with a molecular mass of ~30 kD was also immunoprecipitated in the stimulated secretion, but if this band is mature B, it must have been created after secretion, since the intracellular B up to this chase time had not yet been converted from the precursor form (Fig. 6 *G*, right two lanes). Thus, we cannot state with certainty the kinetics and site(s) of ProB conversion in this tissue from these data. However, unlike the results of Tooze et al. (1991*b*), these data clearly indicate that ProB rather than mature B enters the IGs of the rat exocrine pancreas.

Loss of the SNARE Protein Syntaxin 6, in Parallel with MPR and AP-1, from Maturing Granules in Endocrine and Exocrine Cells

Secretory granules have been reported to be rich in certain members of the SNARE docking and fusion machinery, specifically syntaxin 3 (Gaisano et al., 1996*a*), vesicle-associated membrane protein (VAMP)-2 and cellubrevin (Gaisano et al., 1996*b*; Wheeler et al., 1996), synaptotagmin 1 (Levius et al., 1997), and the recently described syncollin (Edwardson et al., 1997). By contrast, syntaxin 6, a mammalian homologue of yeast Pep12, has been implicated in the delivery of TGN-derived AP-1/clathrin-coated vesicles to endosomes (Bock et al., 1997). Since IGs serve at least in part as a functional extension of the TGN (Arvan and Castle, 1992), the AP-1/clathrin-coated vesicles budding from IGs might contain this particular SNARE protein. Indeed, in both endocrine β cells and exocrine parotid cells, syntaxin 6 labeling was found over TGN and IG membranes, whereas mature granules were nearly devoid of label (Figs. 7 and 8). In β cells, 25% of all syntaxin 6 label was found in membranes exhibiting a morphologically apparent clathrin coat. Of these coated membranes positive for

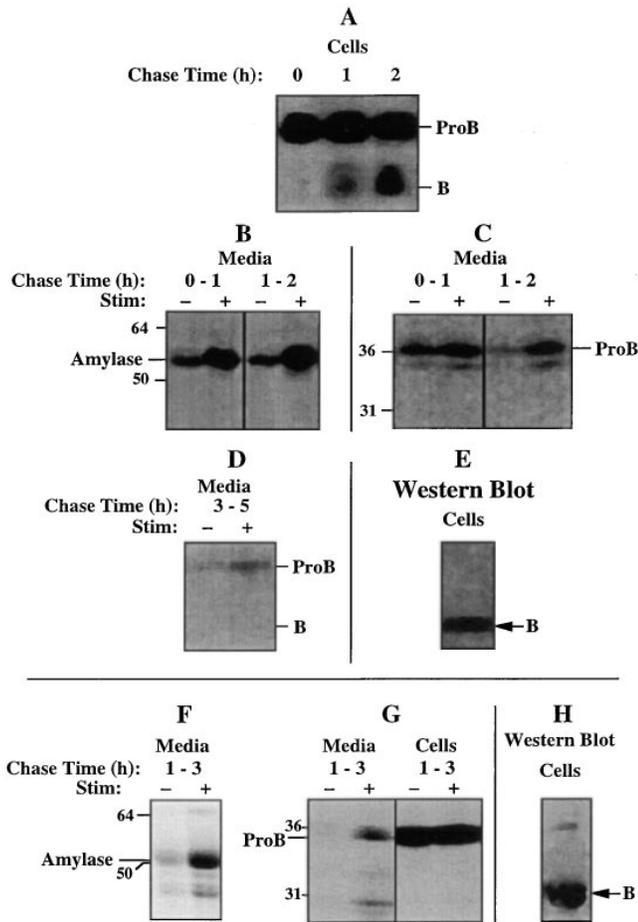


Figure 6. Releasable and intracellular forms of cathepsin B in the rat exocrine tissues. (A–E) Parotid; (F–H) pancreas. (A–D and F–G) Lobules were pulse labeled with ^{35}S -amino acids for 30 min; chase media were collected from lobules bathed either in the absence (–) or presence (+) of secretagogue, and the media (C, D, and G) and cell lysates (A and G) were immunoprecipitated with an antibody that recognizes both ProB and mature cathepsin B (refer to Materials and Methods). Equal aliquots of chase media were analyzed by SDS-PAGE/fluorography. (B and F): Equal aliquots of chase media were analyzed by SDS-PAGE without previous immunoprecipitation, and the secretion of newly synthesized amylase was identified on fluorographs based on band mobility relative to Coomassie-stainable amylase and molecular weight standards (*left*). (C and G) Additional aliquots of chase media analyzed in B and F were immunoprecipitated for cathepsin B and then analyzed by SDS-PAGE/fluorography. Note the stimulus-dependent secretion of the lysosomal prohydrolase. (C) The small amount of a ~35-kD band directly beneath ProB does not represent mature B, was not a consistent finding, and most likely represents partial proteolysis in the medium samples. (G) The small stimulated release of a ~30-kD band directly beneath ProB most likely represents proteolysis of ProB in the medium sample rather than mature cathepsin B, because mature cathepsin B still had not appeared intracellularly at the 3-h chase time. (D) Immunoprecipitation of cathepsin B yielded only a modest stimulus-dependent secretion of labeled ProB during the 3–5 h chase period. (E and H) Immunoblot of parotid and pancreatic tissue, respectively, with anti-cathepsin B. The mature, lysosomal form predominates at steady state in both tissues.

syntaxin 6, nearly one-third were buds emerging from IGs (Fig. 7 A). By double-immunogold labeling, approximately half (49%) of the coated, IG-associated buds positive for syntaxin 6 were also labeled for AP-1. CD-MPR also colocalized with syntaxin 6 in IGs (Fig. 7 B). The degree of syntaxin 6/AP-1 colocalization in the coated membranes of the TGN (53%) was similar to that observed in IGs.

In rat parotid cells, 15% of total syntaxin 6 label was found over membranes with a morphologically apparent clathrin coat. Of these coated membranes positive for syntaxin 6, 28% were buds emerging from IGs. By double-immunogold labeling, 41% of the coated, syntaxin 6-positive IG-associated buds and 40% of the coated, syntaxin 6-positive TGN membranes, were also positive for AP-1 (Fig. 8, *inset*). Further semiquantitative analysis showed that 12% of total syntaxin 6 label was associated with IGs, and only 3% with mature granules (Table I). Based on the numbers of IGs and mature granules in these cells, we could estimate that syntaxin 6 labeling density declined ~88% as a consequence of granule maturation. Taken together, the data indicate a high degree of colocalization of syntaxin 6 and AP-1 in the IG-derived clathrin-coated buds of both endocrine and exocrine cells, strongly suggesting that syntaxin 6, together with MPR, is sorted out of the regulated secretory pathway via AP-1/clathrin-coated vesicles.

Discussion

Two different kinds of models, which are not mutually exclusive, have been proposed to explain the sorting of regulated secretory proteins. The “sorting for entry” model hypothesizes a selective delivery system in the TGN, such that only regulated secretory proteins can enter IGs whereas other proteins are excluded. However, in regulated secretory cells, the high flow rate into IGs may limit the sorting efficiency for certain kinds of proteins in the TGN. For such molecules, an extended sorting opportunity provided by IGs might lead to increased sorting efficiency. With this in mind, the “sorting by retention” model postulates that selective sorting of soluble proteins may not be required at the entry point into forming granules, but as IG maturation progresses, the condensation of regulated proteins improves the efficiency of their retention, whereas other luminal proteins can be sorted out efficiently (by receptors) (Kuliawat and Arvan, 1994) or inefficiently (by fluid-phase transport) (Kuliawat and Arvan, 1992).

We now present the first direct demonstration that a sorting pathway out of maturing granules is a common feature in regulated secretory cells, involving MPRs (at least in part), and mediated by AP-1/clathrin-coated vesicles. First, we have now provided powerful immunoelectron microscopic evidence (Figs. 1–4 and Table I) that MPRs are present in both endocrine and exocrine IGs, that MPRs colocalize with AP-1 in clathrin-coated evaginations that bud from these IGs, and that the densities of CD-MPR and AP-1 decrease in parallel, ~90%, upon maturation of secretory granules. The selectivity of MPR removal from maturing granules is proven by the fact that phogrin, a membrane protein of insulin granules, exhibits no retrieval from IGs. Second, immunoelectron microscopy

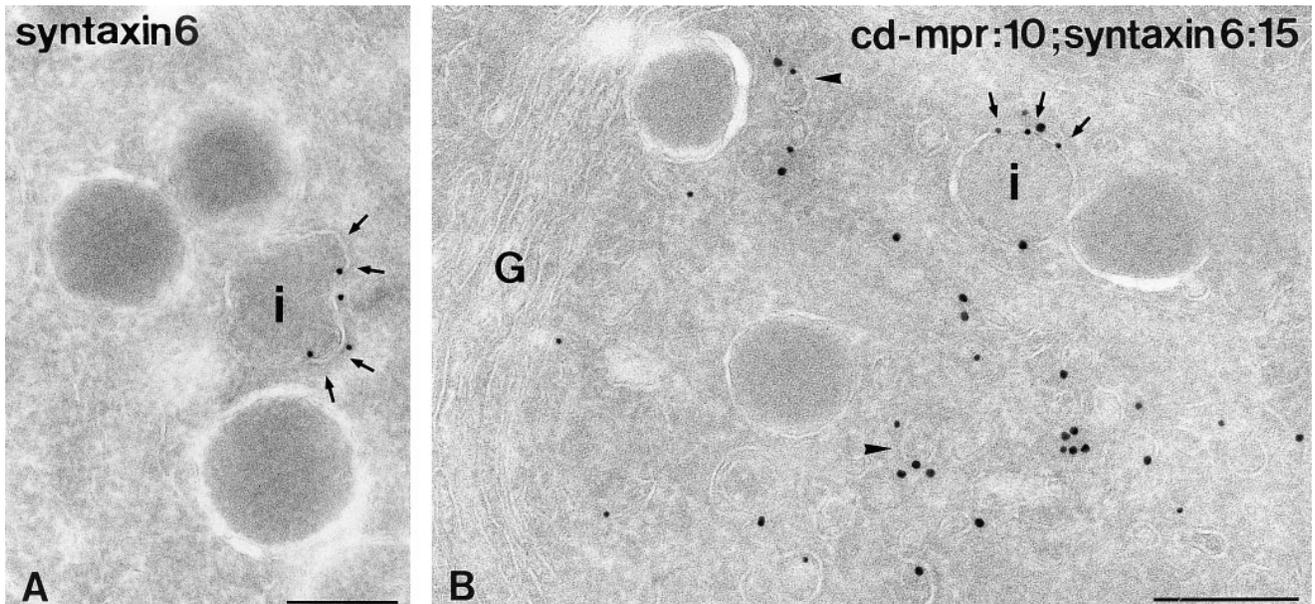


Figure 7. Golgi (*G*) region of endocrine pancreatic β cells immunogold labeled for the SNARE protein syntaxin 6. (*A*) Syntaxin 6-labeling is concentrated in clathrin-coated (*arrows*) membrane buds of an IG and absent from granules with a mature morphology. (*B*) Colocalization of CD-MPR (10-nm gold) and syntaxin 6 (15-nm gold) on IGs (*arrows*) and TGN vesicles (*arrowheads*). Bars, 200 nm.

of rat parotid tissue indicates the presence of cathepsin B immunoreactivity in IGs of this exocrine tissue, including finding the enzyme in IG-associated clathrin-coated buds, whereas few gold particles are observed at later stages of granule maturation (Fig. 5). Moreover, our biochemical data establish unequivocally that in exocrine cells of both the parotid and pancreas, a significant fraction of newly synthesized procathepsin B molecules initially enter the stimulus-dependent secretory pathway, just as has been observed in the endocrine pancreatic β cells (Fig. 6; Kuliawat and Arvan, 1994; Kuliawat et al., 1997). Importantly, these data overturn the previous hypothesis that mature B can be formed in the TGN of exocrine secretory tissues (Tooze et al., 1991*b*), although extracellular activation of ProB after secretion (especially from the exocrine pancreas) must be viewed as a distinct possibility (Fig. 6). The finding that, under physiological conditions, mature exocrine secretory granules have low levels of cathepsin B-immunogold label, whereas mature cathepsin B is the predominant form detected at steady state (by Western blotting), is consistent with the scenario that lysosomal proenzymes and MPRs independently enter IGs, whereafter MPR-ligand complexes are routed out of the regulated secretory pathway by AP-1/clathrin-coated vesicles during granule maturation. At the approximate time of their arrival in lysosomes, we hypothesize that cathepsin B and other hydrolases are converted to their mature forms, except under pathological circumstances that may potentially lead to premature enzyme activation (Hirano et al., 1991).

Recently, the membrane-associated form of carboxypeptidase E (CPE), an enzyme involved in prohormone processing, has been proposed to serve as a receptor mediating the transport of regulatory proteins into IGs of neuroendocrine cells (Cool et al., 1997). However, no evidence presented to date has distinguished an action of

CPE in sorting for entry (i.e., at the TGN) versus sorting by retention (i.e., in IGs). Further, proteins that are not thought to interact with CPE have been reported to efficiently enter the regulated secretory pathway of neuroendocrine cells (Burgess et al., 1985; Fennewald et al., 1988; Stahl et al., 1996). Nevertheless, for some regulated secretory proteins, association with other binding proteins may well facilitate the entry step into IGs. However, based on the fact that (*a*) lysosomal proenzymes can abundantly enter IGs in a manner independent of recognition by known receptors (Kuliawat and Arvan, 1994); (*b*) prohydrolase departure from maturing granules occurs by a mechanism whose efficiency is dependent on binding affinity for MPRs (Kuliawat et al., 1997); and (*c*) there is a significant decline of MPR immunolabeling over granules as they mature (this report), we suggest that in both endocrine and exocrine secretory cells, at least a portion of (soluble and membrane) proteins not destined for storage in mature granules may routinely advance from the TGN into IGs, only to be sorted out subsequently.

This is the first report of the SNARE protein syntaxin 6 in the regulated secretory pathway, as well as its removal (by AP-1/clathrin-coated buds) from IGs and the TGN, in endocrine and exocrine cells. Indeed, we observed a parallel decline in syntaxin 6 labeling with AP-1 during granule maturation (Figs. 7 and 8 and Table I). According to the SNARE hypothesis, there is a requirement for specific protein interactions in order that specific membrane fusion events may proceed (Sollner et al., 1993). Our data indicate the distinct possibility that it may be necessary to remove certain inappropriate SNARE proteins from the regulated secretory pathway to create the identity of mature secretory granules, thereby preserving their fusion specificity for the cell surface. Furthermore, the relatively high amount of syntaxin 6 in IG and TGN-derived clathrin-coated buds, and its efficient removal from maturing

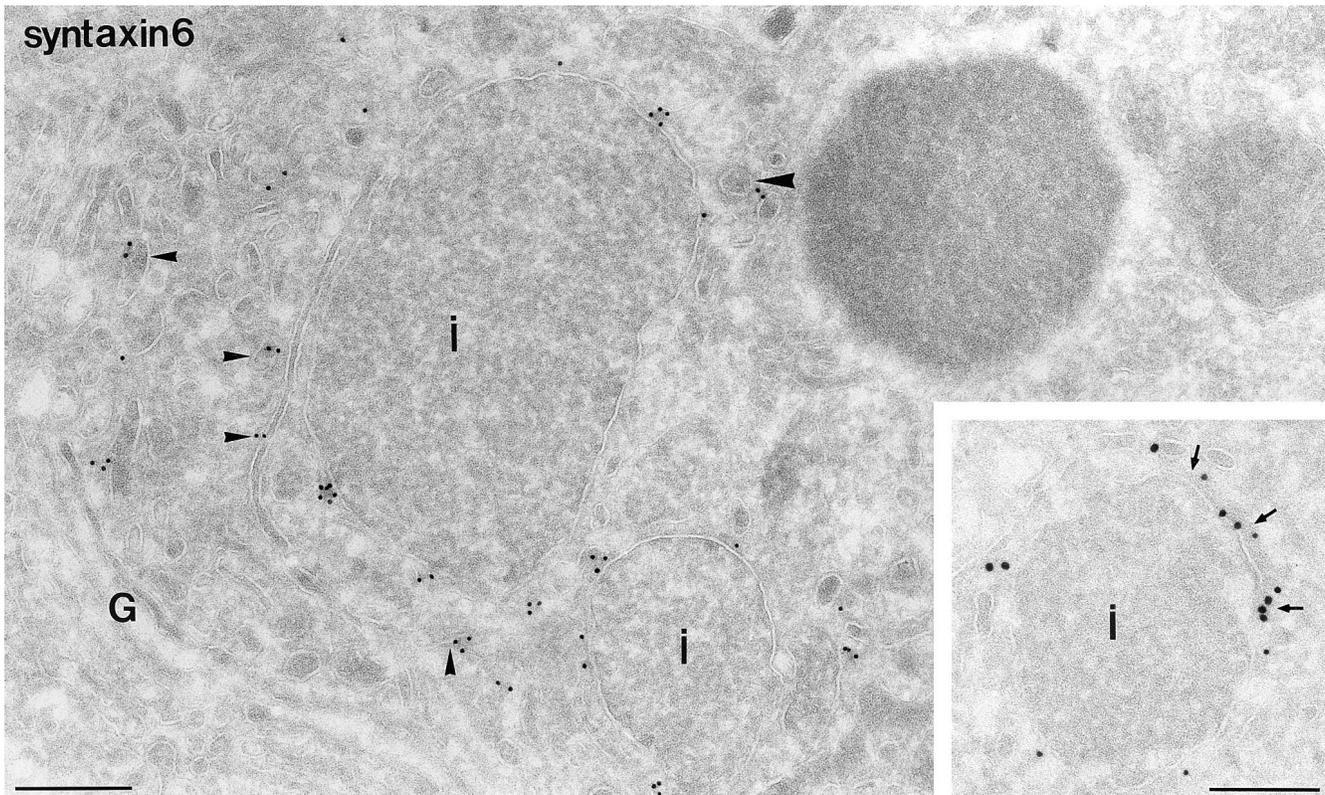


Figure 8. Localization of syntaxin 6 in the Golgi (G) region of a rat parotid acinar cell. Label is present on the limiting membrane of an IG (i) with less dense contents, but absent from those with highly condensed contents. Additional label is seen in a CCV (large arrowhead) as well as in noncoated TGN vesicles and tubules (small arrowheads). *Inset*, Colocalization of AP-1 (10-nm gold) and syntaxin 6 (15-nm gold) in a clathrin-coated (arrows) region of an IG. Bars, 200 nm.

granules (Table I), suggests that syntaxin 6 is actively sorted into CCVs and may function in a SNARE complex that leads to the fusion of these vesicles with membranes of the endosomal system. Current evidence indicates that syntaxin 6, which shares homology with syntaxin 1 and SNAP-25 (Bock et al., 1996), is the mammalian counterpart of the yeast Pep12. Yeast cells expressing mutant Pep12, like certain other class D vacuolar protein sorting (VPS) mutants, accumulate small post-Golgi vesicles that are thought to be involved in the delivery of soluble hydrolyase precursors to the prevacuolar compartment (Becherer et al., 1996). Likewise, syntaxin 6 has been implicated in the targeting/fusion of TGN-derived AP-1/clathrin-coated vesicles with the endosomal system by forming a complex including mVPS45, just as Pep12 forms a complex with yeast VPS45 (Bock et al., 1996; Tellam et al., 1997). Thus, the data in regulated secretory cells (this report) suggest that IG-derived as well as TGN-derived, MPR-positive, AP-1/clathrin-coated vesicles may use syntaxin 6 as part of their mechanism for targeting/fusion with endosomal membranes. However, our data do not formally exclude the possibility that syntaxin 6 could serve its function as a SNARE either before it enters TGN- or IG-derived CCVs, or after its delivery to membranes of the endosomal system. Clearly, further research will be needed to demonstrate syntaxin 6 function as well as its precise site(s) of action.

Use of the terms immature and mature tends to oversimplify granule maturation as if it were a discrete single

event and the immunoelectron microscopic studies in this report should be considered only as an indication of the spectrum of maturational stages. We must remain open to the possibility that vesicle budding kinetics and efficiency might vary between granules or between cells, therefore persisting at low levels at relatively late stages of apparent granule maturation. It is interesting to note that lysosomal proenzymes would not be expected to bind MPRs at the acidic pH normally found in mature endocrine granules, whereas mature exocrine granules are not especially acidic (Arvan et al., 1984; Orci et al., 1987), which would allow prolonged MPR–ligand interaction and favor a more prolonged sorting track in these tissues. Indeed, this possibility might help to reconcile the apparent slow conversion of procathepsin B to the mature form in exocrine tissues (Fig. 6) as well as the detection of cathepsin B immunoreactivity reported in seemingly mature granules (Tooze et al., 1991b). However, we emphasize that procathepsin B normally requires an extremely acidic environment for intramolecular autocatalysis to the mature form (Mach et al., 1994); thus, in contrast to the conclusion of Tooze et al. (1991b), we feel strongly—and our data are consistent with the idea—that the cathepsin B that enters the regulated secretory pathway represents the inactive precursor form.

The concentration of MPRs with exposure of their cytoplasmic tails is one of factors thought to be important for the recruitment (Robinson and Kreis, 1992; Stamnes and

Rothman, 1993; Traub et al., 1993; Le Borgne et al., 1996) and/or stabilization of the AP-1/clathrin coat in the TGN (Le Borgne and Hoflack, 1997), leading to integrated sorting of luminal-, transmembrane-, and cytoplasmic-complexed proteins (Schmid, 1997). Recent studies (Kuliawat and Arvan, 1994; Dittie et al., 1996; Kuliawat et al., 1997) and the data in this report are consistent with a role for MPRs in the formation of AP-1/clathrin-coated vesicles from maturing granules, and for syntaxin 6 in the targeting of those vesicles to endosomes. It seems probable that additional selected membrane proteins are sorted out of IGs by vesicle budding, perhaps involving coat recruitment, whereas other proteins (e.g., phogrin) are destined to be retained. Certain isoforms of peptidylglycine- α -amidating-mono-oxygenase have been proposed to exit maturing granules via vesicle budding (Milgram et al., 1994). Also furin, a membrane-bound endoprotease that resides predominantly in the TGN and actively recycles through multiple compartments, has recently been reported to be present in isolated IGs but not mature granules (Dittie et al., 1997). Even acid phosphatase has been reported to be removed selectively during granule maturation (Novikoff et al., 1977). Future studies will be needed to determine whether each of these membrane proteins exits maturing granules via the same class of clathrin-coated vesicles.

We are grateful to J. Mort, O. Madsen, M. Robinson, R.H. Scheller, J. Hutton, G. Lienhard, and K. Von Figura for generously providing the antibodies used in these studies. We also thank T. van Rijn, M. Niekerk, and R. Scriwanek for excellent preparation of the electron micrographs and G. Posthuma and V. Oorschot (all five from University of Utrecht, Utrecht, The Netherlands) for performing the perfusion fixations for electron microscopy.

This work was supported in part by a National Institutes of Health grant (DK48280) to P. Arvan.

Received for publication 12 December 1997 and in revised form 10 February 1998.

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