Differential Trafficking of Soluble and Integral Membrane Secretory Granule-associated Proteins

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Abstract. The posttranslational processing enzyme peptidylglycine α-amidating monooxygenase (PAM) occurs naturally in integral membrane and soluble forms. With the goal of understanding the targeting of these proteins to secretory granules, we have compared the maturation, processing, secretion, and storage of PAM proteins in stably transfected AtT-20 cells. Integral membrane and soluble PAM proteins exit the ER and reach the Golgi apparatus with similar kinetics. Biosynthetic labeling experiments demonstrated that soluble PAM proteins were endoproteolytically processed to a greater extent than integral membrane PAM; this processing occurred in the regulated secretory pathway and was blocked by incubation of cells at 20°C. 16 h after a biosynthetic pulse, a larger proportion of soluble PAM proteins remained cell-associated compared with integral membrane PAM, suggesting that soluble PAM proteins were more efficiently targeted to storage granules. The nonstimulated secretion of soluble PAM proteins peaked 1–2 h after a biosynthetic pulse, suggesting that release was from vesicles which bud from immature granules during the maturation process. In contrast, soluble PAM proteins derived through endoproteolytic cleavage of integral membrane PAM were secreted in highest amount during later times of chase. Furthermore, immunoprecipitation of cell surface-associated integral membrane PAM demonstrated that very little integral membrane PAM reached the cell surface during early times of chase. However, when a truncated PAM protein lacking the cytoplasmic tail was expressed in AtT-20 cells, >50% of the truncated PAM-1 protein reached the cell surface within 3 h. We conclude that the trafficking of integral membrane and soluble secretory granule-associated enzymes differs, and that integral membrane PAM proteins are less efficiently retained in maturing secretory granules.

SECRETORY granules are specialized organelles which store peptide hormones in neurons and endocrine cells; the contents of secretory granules are secreted primarily upon stimulation by secretagogue in a calcium-dependent manner. Packaging of proteins into immature secretory granules occurs upon exit from the *trans*-Golgi network (TGN; Orci et al., 1987; Sossins et al., 1990); the mechanisms underlying the sorting of proteins destined for secretory granules from proteins secreted without storage are unknown (Burgess and Kelly, 1987; Arvan and Castle, 1992; Chidgey et al., 1993). Expression of cDNAs encoding soluble prohormones in endocrine cell lines usually yields prohormones that are correctly processed and stored in secretory granules, indicating that neuroendocrine cells share common sorting and processing machinery (Moore et al., 1983; Lapps et al., 1988; Devi et al., 1989; Sevarino et al., 1989; Chevrier et al., 1991). For some proteins, including prosomatostatin and von Willebrand factor, the proprotein contains targeting information (Sevarino et al., 1989;

Wagner et al., 1991); in contrast, the prosequences of trypsin, renin, and insulin are not required for the storage of these proteins in secretory granules (Burgess et al., 1987; Powell et al., 1988; Nagahama et al., 1989; Chu et al., 1990). Data from several experimental systems indicate that the selective aggregation of secretory proteins in the lumen of the TGN plays an important role in their routing to storage granules (Gerdes et al., 1989; Reaves and Dannies, 1991; Chanat and Huttner, 1991; Tooze, 1991; Palmer and Christie, 1992). Based on this model, soluble proteins targeted to secretory granules would share an inherently greater tendency to aggregate in the low pH and high calcium environment of the TGN than other secreted proteins.

Most of the known granule-associated proteins are synthesized without a membrane anchor (e.g., prohormones, prohormone convertases, chromogranins, and carboxypeptidase H) and little is known regarding the trafficking of integral membrane proteins which function in secretory granules. P-selectin is a cell adhesion molecule with a single transmembrane domain and is normally localized in α granules of platelets and Weibel-Palade bodies of endothelial cells. When expressed in AtT-20 cells, P-selectin is targeted

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to secretory granules as a result of a signal in the cytoplasmic domain (Disdier et al., 1992; Koedam et al., 1992).

Because it occurs naturally in integral membrane and soluble forms, the posttranslational processing enzyme peptidylglycine α-amidating monooxygenase (PAM)[1] provides a unique model for comparing the maturation and routing of soluble and integral membrane proteins. Naturally occurring forms of PAM with (PAM-1 and -2) and without (PAM-3 and -4) a single transmembrane domain arise as a result of alternative mRNA splicing (see Fig. 1). PAM-1, -2, and -3 contain two catalytic domains within a single precursor protein; peptidylglycine α-hydroxylating monooxygenase (PHM) and peptidyl-α-hydroxyglycine α-amidating lyase (PAL) act in sequence to catalyze the generation of COOH-terminal α-amides from peptidylglycine substrates (for review see Eipper et al., 1992 and 1993). Alternative splicing results in the presence (PAM-1) or absence (PAM-2 and PAM-3) of a noncatalytic linker region (exon A) between PHM and PAL; this domain is important in the endoproteolytic processing of PAM (Milgram et al., 1992). Use of an alternate poly A addition site yields monofunctional PAM-4 which contains exon A but lacks the PAL domain.

In our initial studies we demonstrated that when expressed in AtT-20 cells, integral membrane and soluble PAM proteins were endoproteolytically processed to generate PAM proteins which were secreted from AtT-20 cells in a regulated manner (Milgram et al., 1992). PAM proteins accumulated in the peripheral processes of AtT-20 cells in a pattern overlapping that of the endogenous hormone; however, cells expressing integral membrane PAM also exhibited intense punctate staining in the vicinity of the Golgi apparatus, a pattern which was not observed in cell lines expressing soluble PAM (Milgram et al., 1992, 1993). These data suggested that the mechanisms mediating the targeting of soluble and integral membrane secretory granule-associated proteins differed.

The targeting of full length integral membrane PAM and a truncation mutant containing the transmembrane domain but lacking most of the cytoplasmic tail (PAM-1/899) were compared in neuroendocrine cells (Milgram et al., 1993). A smaller percentage of the PAM-1/899 protein was found to reside in a stimulatable compartment and much of the truncated protein appeared on the plasma membrane. The fact that intense perinuclear staining was also observed when integral membrane PAM proteins were expressed in nonneuroendocrine hEK-293 cells, suggests that the cytoplasmic domain signal is not a secretory granule targeting signal per se, but rather may be important for some other aspect of integral membrane PAM trafficking (Tausk et al., 1992). The cytoplasmic domain of integral membrane PAM also contains information mediating the rapid internalization of this protein from the cell surface; the relationship between internalization and targeting to secretory granules is unknown (Milgram et al., 1993).

With the goal of understanding the trafficking of integral membrane proteins within the regulated secretory pathway, we have directly compared the trafficking of soluble and inte-

gral membrane PAM proteins. We have used biosynthetic labeling and immunoprecipitations to study the maturation, endoproteolytic cleavage, secretion and surface expression of integral membrane and soluble PAM proteins. These studies have led to the development of a model of protein targeting in neuroendocrine cells in which the post-TGN trafficking of soluble and integral membrane secretory granule-associated proteins differs.

Materials and Methods

Cell Lines

Stable AtT-20 cell lines expressing PAM-1, -2, -3, and -4 were established and characterized previously using enzyme assays, Northern analysis, and Western blotting (Milgram et al. 1992, 1993). Transfected cells were maintained in DME-F12 containing 10% fetal calf serum, 10% Nu Serum, and G418; cells were passaged weekly and remained stable over several months.

Biosynthetic Labeling and Immunoprecipitations

Cells were plated on 12-mm culture dishes coated with 0.1 mg/ml poly-L-lysine and grown a minimum of 36 h before experiments were begun. Biosynthetic labeling with 0.2–0.4 mCi [^{35}S]methionine or [^{35}S]methionine/cysteine labeling mix (1–2 μM methionine; Amersham Corp., Chicago, IL) was performed as described previously (Milgram et al., 1993). The length of the labeling period was 15 or 30 min and is given in each figure legend. At the end of the labeling period, the cells were rinsed once with complete serum free medium (CSFM; Milgram et al., 1992) and then either extracted or incubated for varying periods of time in 300 μl CSFM. For stimulation experiments, the secretagogue used was 15 nM phorbol, 12-myristate-13 acetate diluted into CSFM from a 1 mg/ml stock in DMSO (Calbiochem Corp., La Jolla, CA). Chase media were collected, centrifuged at 300 g to pellet non-adherent cells and stored at −80°C following the addition of protease inhibitors (30 μg/ml PMSF, 2 μg/ml leupeptin, and 16 μg/ml benzamidine). Labeled cells were extracted in the presence of protease inhibitors as described previously (Milgram et al., 1992). Immunoprecipitation of cell extracts and media was performed using PHM or PAL rabbit polyclonal antisera; immune complexes were isolated by incubation for 1–2 h with 20 μl protein A-Sepharose (Sigma Chemical Co., St. Louis, MO) (Yun et al., 1993; Milgram et al., 1993). Samples were fractionated by SDS-PAGE on 8 or 10% polyacrylamide gels. Gels were fixed in 30% isopropanol, 10% acetic acid, and then impregnated for 30 min with Amplify (Amersham Corp.) before drying and fluorography. Densitometric analyses were performed using an Abaton Scan 300/GS linked to an Apple Macintosh IIci and NIH Image 1.35 software (National Institute of Mental Health) as described (Milgram et al., 1993).

Analysis of Cell Surface-associated PAM Proteins

Cells expressing PAM-1, PAM-2, or PAM-1/899 were grown on 12-mm culture dishes until 75% confluent. Metabolic labeling was performed for 30 min at 37° and cells were chased at 37°C for 1, 2, or 3 h. After the chase period, the cells were rinsed twice in CSFM which lacked sodium bicarbonate and contained Hepes (CSFM-AIR) at 4°C and incubated for 90 min in CSFM-AIR containing a 20-fold dilution of PHM antiserum. The cells were then rinsed 3 times with CSFM-AIR at 4°C. To quench any unbound antibodies still present after the rinses, the cells were incubated for 45 min at 4°C in CSFM containing high levels of soluble PAM collected from hEK-293 cells expressing PAM-3 (Husten et al., 1993). After quenching, cells were rinsed again with CSFM-AIR and extracted in 300 μl of TES/mannitol/Triton X-100 as described (Milgram et al., 1992). Cell surface-associated PAM was immunoprecipitated after the addition of an excess of protein A-Sepharose. To determine the total amount of PAM present in whole cell extracts, 16% of the supernatant from the first incubation with protein A was incubated with an excess of PHM antiserum and immune complexes were collected after incubation with protein A. Samples were analyzed by SDS-PAGE and fluorography. Control experiments included incubation of intact cells expressing PAM-3 with PHM antiserum. No surface-associated PAM proteins were detected in these cells at any time of chase (not shown).

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1. *Abbreviations used in this paper:* CSFM, complete serum free medium; PAL, peptidyl-α-hydroxyglycine α-amidating lyase; PAM, peptidylglycine α-amidating monooxygenase; PHM, peptidylglycine α-hydroxylating monooxygenase.

Endoglycosidase H Digestion of PAM Proteins

Immunoprecipitated proteins were released from protein A–Sepharose beads by boiling in 50 mM Na phosphate, pH 5.5, 0.1% SDS, and 50 mM β-mercaptoethanol. Samples were diluted fivefold in 50 mM Na phosphate buffer, pH 5.5, containing 50 mM β-mercaptoethanol and 200 μg bovine serum albumin. One half of each sample was digested overnight at 37°C with 2 mU endoglycosidase H (Boehringer Mannheim, Mannheim, Germany); control samples were incubated in the absence of added enzyme. Samples were fractionated on 6 or 8% SDS-PAGE gels and prepared for fluorography.

Results

Measurement of Endoplasmic Reticulum to Golgi Transport Rates

PAM-1, -2, and -3 contain a single N-linked oligosaccharide in the PAL domain (Fig. 1); the Asn-Pro-Thr site in exon A is not N-glycosylated but O-linked sugars are present in PAM proteins retaining exon A (Tausk et al., 1992; Yun et al., 1993). To compare the rates of transport of integral membrane and soluble PAM proteins from the ER to the Golgi apparatus, cells were labeled with [^{35}S]methionine for 15 min and chased in CSFM for increasing lengths of time. Immunoprecipitated PAM proteins were digested with Endoglycosidase H (Endo H) and analyzed after SDS-PAGE; resistance to digestion with Endo H is diagnostic of processing to complex-type oligosaccharides which occurs in the *medial*-Golgi complex (Lodish et al., 1983). Immediately after the pulse, all of the newly synthesized PAM proteins were sensitive to digestion with Endo H (Fig. 2). During the chase PAM-1 increased in size from ~113 to 120 kD, reflecting the addition of both N- and O-linked oligosaccharide, and PAM-2 increased from 102 to 105 kD. Both integral membrane PAM proteins acquired Endo H resistance with a $t_{1/2}$ of ~60 min; by 150 min all of the newly synthesized
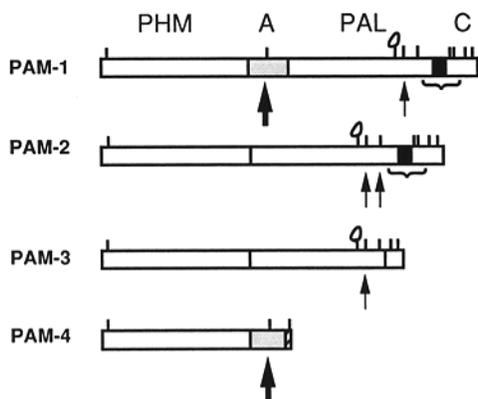

Figure 1. Integral membrane and soluble PAM proteins. PHM and PAL domains are as labeled; the transmembrane domain is represented by the black box. Short vertical lines represent paired basic amino acid residues, the oval within the PAL domain is the single N-linked glycosylation site which is used, and the hatched box after exon A in PAM-4 represents 20 amino acids unique to this form of PAM. The signal sequence (amino acids 1–25) is absent; the first vertical tic mark represents the end of the PAM propeptide (amino acids 26–35). Heavy arrows denote efficient sites of endoproteolytic processing while thinner arrows represent less efficient cleavage sites. The bracket indicates exon B which is lacking in PAM-3. *A*, exon A; *C*, COOH-terminal domain.
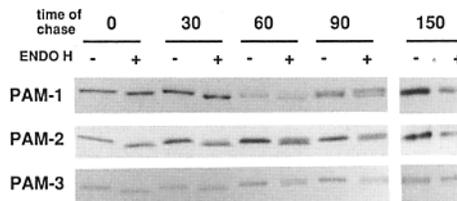

Figure 2. Sensitivity of newly synthesized PAM proteins to digestion with Endo H. AtT-20 cells expressing PAM-1, -2, or -3 were incubated for 15 min in medium containing 400 μCi [^{35}S]methionine and then chased in CSFM for the times indicated. Cell extracts were immunoprecipitated with PHM antiserum; the immunoprecipitated proteins were recovered and incubated with or without Endo H. Samples were fractionated on 6% (PAM-1 and -2) or 8% (PAM-3) SDS-PAGE gels. Similar results were obtained in 2–4 separate experiments using similar times of chase.

PAM-1 and -2 were resistant to digestion with Endo H. The transport of soluble PAM-3 from the ER to Golgi apparatus followed similar kinetics. Initially synthesized as a 92-kD protein, PAM-3 increased 3–4 kD in size during the chase. Approximately 50% of the PAM-3 was resistant to digestion with Endo H after 60 min of chase; by 150 min all of the newly synthesized PAM-3 had become resistant to digestion with Endo H (Fig. 2). Therefore, soluble and integral membrane PAM proteins display similar ER to Golgi transport rates.

Kinetics of Endoproteolytic Processing and Storage of PAM Proteins in AtT-20 Cells

All of the PAM proteins expressed in AtT-20 cells are subjected to endoproteolytic processing by resident AtT-20 processing enzymes (Milgram et al., 1992, 1993); these cleavages are unique to neuroendocrine cells and are not observed when PAM is expressed in fibroblasts (Tausk et al., 1992; Eipper et al., 1993). To compare the endoproteolytic processing of soluble and integral membrane PAM proteins, cells were incubated with [^{35}S]methionine for 15 min and chased for 0, 0.5, 1, 2, or 3 h. In cell extracts prepared immediately after the pulse or after 30 min of chase, all of the PAM proteins were uncleaved, full length proteins (Fig. 3). Both PAM-1 and PAM-4 contain exon A and can be cleaved to yield 44–46 kD PHM proteins. Processing of PAM-4 to generate 44–46 kD PHM was evident after 1 h of chase and proceeded nearly to completion by 2 h (Fig. 3). Integral membrane PAM-1 was processed less efficiently than soluble PAM-4 and only ~40% had been cleaved after 3 h of chase (Fig. 3). Longer exposures of the autoradiograms demonstrated that a small amount of monofunctional PHM was also generated from PAM-1 within 1 h of chase (not shown and Fig. 8); however, by comparison to soluble PAM-4, processing of PAM-1 occurred at a slower rate.

PAM-2 and -3 lack exon A; endoproteolytic processing of both was less efficient than that observed for PAM-1 and PAM-4. Processing of soluble PAM-3 between PAL and the COOH-terminal domain generated a bifunctional 75-kD protein (Fig. 3). Cleavage of 95 kD PAM-3 was first observed after 2 h of chase; ~50% of the PAM-3 protein was cleaved by 3 h. Integral membrane PAM-2 was processed slowly to generate bifunctional PAM proteins (83 and 75 kD). Cleavage of PAM-2 was first detectable after 2 h of
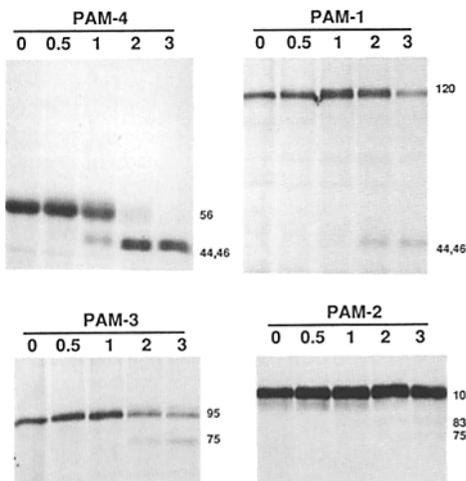
Figure 3. Endoproteolytic processing of newly synthesized PAM proteins. Cells were incubated for 15 min in medium containing 300 μCi [^{35}S]methionine and then chased for the times indicated in the figure. Cell extracts were immunoprecipitated with PHM antiserum and fractionated on 8% SDS-PAGE gels. Apparent molecular masses are in kD. Similar results were obtained in two separate experiments comparing the processing of PAM-1 and -4 and in a single additional experiment comparing PAM-2 and PAM-3.

Figure 4. Storage of integral membrane and soluble PAM proteins. AtT-20 cells expressing PAM-1, -2, -3, or -4 were labeled for 15 min with 300 μCi [^{35}S]methionine and either immediately extracted or chased in basal medium for 4, 8, or 16 h. Cell extracts were immunoprecipitated with PHM or PAL antiserum and the immunoprecipitates were fractionated on 8% SDS-PAGE gels; apparent molecular masses are in kD. The protein band marked by the arrow in the PAL immunoprecipitation from cells expressing PAM-1 is not related to PAM. For PAM-2 and -3, immunoprecipitation with PAL antiserum yielded identical results (not shown). Similar results were obtained in two separate experiments.

chase and was very inefficient; <5% of the newly synthesized PAM-2 was processed by 3 h of chase (Fig. 3).

Longer chase periods were used to determine how efficiently the various PAM proteins were stored in AtT-20 cells. Cells were pulse labeled for 15 min with [^{35}S]methionine and chased for 0, 4, 8, or 16 h. When longer lengths of chase were used, it became evident that soluble PAM proteins were more efficiently stored in AtT-20 cells than integral membrane PAM proteins (Fig. 4). After 8 h of chase, ∼40–50% of the newly synthesized PAM-3 and -4 remained cell-associated. Even 16 h after pulse, 30–35% of the newly synthesized soluble PAM proteins were present in immunoprecipitates of whole cell extracts. After 16 h of chase, only ∼50% of the newly synthesized PAM-3 was endoproteolytically processed to the 75-kD bifunctional form.

In contrast to soluble PAM proteins, the amount of integral membrane PAM-2 decreased dramatically between 8 and 16 h of chase (Fig. 4). While the amount of newly synthesized PAM protein present in immunoprecipitates from cells expressing PAM-3 decreased less than twofold between 8 and 16 h of chase, longer exposures of the autoradiograms demonstrated that the full-length integral membrane PAM-2 decreased over 10-fold. Endoproteolytic processing of integral membrane PAM-1 generates soluble monofunctional PHM and integral membrane 70-kD PAL (Milgram et al., 1992); storage of the two proteins differs significantly. 8 h after a short biosynthetic pulse 20–28% of the newly synthesized PAM-1 was present in cell extracts as monofunctional PHM (Fig. 4); after 16 h there was less than a twofold decrease in the amount of 44–46 kD PHM which was present. Thus soluble monofunctional PHM generated through cleavage of the PAM-1 precursor was stored in a manner similar to soluble PAM proteins. In contrast, while monofunctional integral membrane PAL was visualized in immunoprecipitates after 4 and 8 h of chase, little 70-kD PAL was present after 16 h. Like PAM-2, the membrane-associated integral mem-
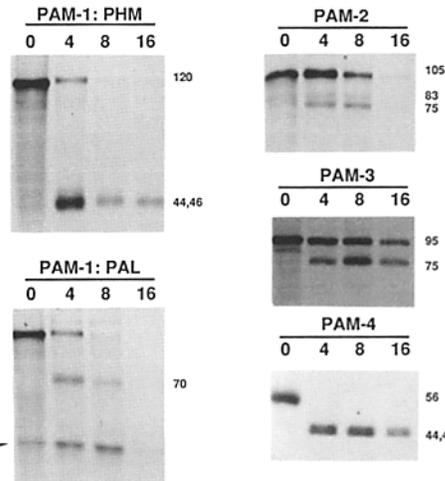
brane 70-kD PAL derived from PAM-1 was not stored in AtT-20 cells as efficiently as the soluble monofunctional PHM derived from PAM-1 (Fig. 4). Between 8 and 16 h of chase, the decrease in 70-kD PAL (derived from PAM-1) and 105-kD PAM-2 could not be totally accounted for by secretion of protein into the chase medium. The addition of leupeptin, ammonium chloride, or methylamine at concentrations sufficient to inhibit lysosomal proteases did not alter the secretion or intracellular accumulation of integral membrane PAM proteins over 16 h of chase (not shown).

Effects of 20°C Temperature Block on the Endoproteolytic Processing of PAM

When cells are cooled to 20°C, protein export from the TGN is blocked and secretory proteins accumulate in this compartment (Saraste and Kuismanen, 1984; Griffiths et al., 1985). To compare the intracellular sites where endoproteolytic processing of soluble and integral membrane PAM occurs, cells were labeled for 15 min at 37°C and then chased for 3 h at either 37 or 20°C; PAM proteins in cell extracts and medium were compared after immunoprecipitation with PHM antiserum. As expected, newly synthesized PAM proteins were visualized in medium from cells chased for 3 h at 37°C but not in medium from cells chased at 20°C (not shown). In extracts from cells chased at 37°C all of the PAM proteins underwent endoproteolytic processing to generate the smaller proteins described above; for both soluble and integral membrane forms of PAM, this processing was virtually eliminated in cells chased at 20°C (Fig. 5). When cells chased at 20°C were warmed to 37°C, the endoproteolytic cleavage of PAM was again observed (not shown). These data indicate that endoproteolytic processing within exon A
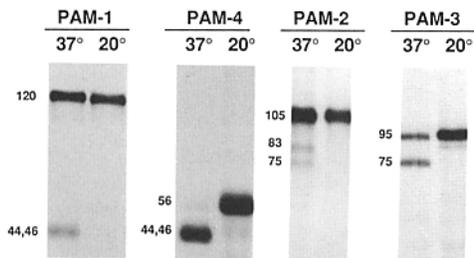
Figure 5. Effects of 20°C temperature blockade on the endoproteolytic processing of integral membrane and soluble PAM proteins. Cells expressing PAM-1, -2, -3, or -4 were incubated for 15 min with 400 μCi [³⁵S]methionine/cysteine and chased for 3 h in CSFM at 37°C or for 3 h in CSFM-AIR at 20°C. Immunoprecipitates were analyzed as described; apparent molecular masses are in kD. Similar results were obtained in three separate experiments with chase times ranging from 2 to 4 h.

Figure 6. Kinetics of secretion of newly synthesized PAM proteins. AtT-20 cells were labeled with 250–400 μCi [³⁵S]-methionine or [³⁵S]methionine/cysteine for 15 min and chased in CSFM for a total of 8 h. At the times indicated in the figure, medium was collected and replaced with fresh medium. Samples were prepared by immunoprecipitation using PHM antiserum and were analyzed by SDS-PAGE on 8 or 10% gels; apparent molecular masses are in kD. Similar results were obtained in four independent comparisons. *FE*, final extracts.

in PAM-1 and PAM-4 and after the lyase domain in PAM -2, and -3 occurs only after PAM proteins have exited the TGN.

Secretion of PAM Proteins

We next wanted to determine whether the soluble PAM proteins and PAM proteins which resulted from the endoproteolytic cleavage of integral membrane PAM proteins were secreted from AtT-20 cells with similar kinetics. Cells expressing integral membrane and soluble PAM were labeled with [³⁵S]methionine for 15 min and chased in CSFM for a total of 8 h. At intervals throughout the chase period, medium was collected and replaced with fresh medium; samples were analyzed by immunoprecipitation using PHM antiserum. In cells expressing soluble PAM-3 and -4, secretion of newly synthesized PAM was evident during the first hour of chase, peaked during the second hour of chase, and remained elevated during the 2–4-h chase period (Fig. 6). In addition to the 95-kD PAM-3 protein visualized during the first h of chase, a small amount of bifunctional 75-kD PAM-3 was visualized during later chase periods. Only the 56-kD full-length PAM-4 protein was secreted during the first h of chase while 44–46 kD PHM proteins were secreted during later times of chase. The basal secretion of newly synthesized PAM-3 and PAM-4 was greatly diminished after 4 h of chase. Thus, peak secretion of newly synthesized soluble PAM proteins occurred 1–2 h after a 15-min biosynthetic pulse. Additionally, the decrease in cell-associated PAM-3 and -4 from 0 to 8 h can be accounted for by basal secretion (not shown).

The kinetics of secretion of soluble proteins derived from integral membrane PAM-1 and -2 differed from the kinetics observed for secretion of soluble PAM proteins. During the first hour of chase, little secretion of soluble monofunctional PHM or bifunctional PAM was observed in cells expressing PAM-1 and PAM-2, respectively (Fig. 6). In cells expressing PAM-1, secretion of newly synthesized monofunctional 44–46 kD PHM was highest during the 2–4-h chase period and continued at a high rate during the 4–6-h period. The secretion of soluble PAM proteins derived from newly synthesized PAM-2 followed similar kinetics. Secretion of 83- and 75-kD bifunctional proteins was first observed during the second hour of chase and the peak of secretion of newly synthesized PAM-2 was during the 2–4-h chase period. Thus
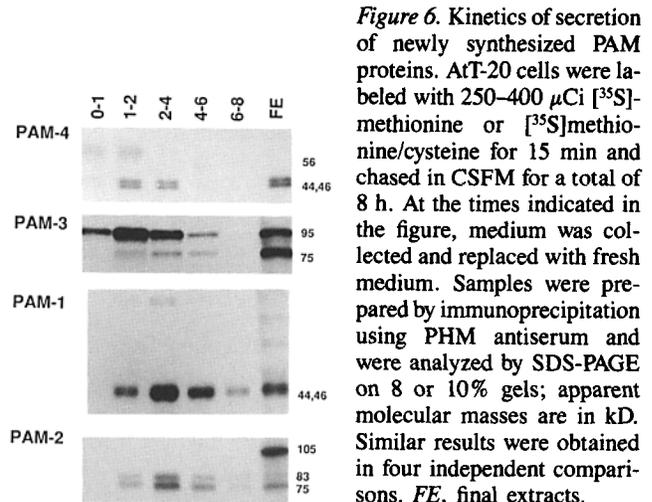
secretion of soluble proteins generated through endoproteolysis of membrane-associated PAM proteins occurs at a slower rate than the secretion of soluble PAM proteins.

Kinetics of Packaging into the Regulated Secretory Pathway

We previously demonstrated that cAMP analogues and phorbol esters stimulated the secretion of soluble PAM-3 and -4 as well as the soluble proteins which resulted from the endoproteolytic processing of integral membrane PAM-1 and -2 (Milgram et al., 1992, 1993). To determine when soluble and integral membrane PAM proteins first reach a stimulatable compartment, AtT-20 cells expressing PAM-3 and PAM-2 were incubated with [³⁵S]methionine for 15 min and chased at 37°C for 1–5 h. During the 1–2, 2–3, or 4–5-h chase period, cells were incubated with control medium or medium containing 15 nM phorbol, 12-myristate-13 acetate. One hour after the biosynthetic pulse, when ~50% of the newly synthesized PAM has reached the Golgi complex (Fig. 2), an approximately twofold stimulation of secretion of 95-kD PAM-3 was observed (Fig. 7). PAM-3 accumulated in secretory granules as the length of the chase in basal medium increased and the extent of stimulation of PAM-3 increased to a maximum of approximately eightfold during the 4–5-h chase interval (Fig. 7). At later times of chase, increased secretion of both full length and processed PAM-3 was observed in response to secretagogue. Similarly, soluble proteins derived from the endoproteolytic processing of PAM-2
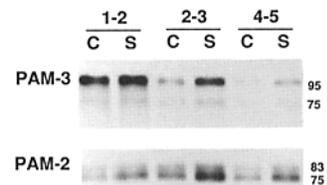

Figure 7. Kinetics of stimulated secretion. Cells expressing PAM-2 or PAM-3 were incubated with 300 μCi [³⁵S]-methionine and chased in CSFM for 1–5 h. At the times indicated the CSFM was removed and replaced with either CSFM for control samples or with medium containing 15 nM phorbol, 12-myristate-13 acetate. Equal amounts of control and challenge media were immunoprecipitated with PHM antiserum and immunoprecipitates were analyzed after fractionation on 10% SDS-PAGE gels. Similar results were obtained in another identical experiment.

were secreted in response to stimulation. At all times examined, phorbol esters stimulated the secretion of both the 83- and 75-kD bifunctional PAM-2–derived proteins; however, there was a greater stimulation of secretion of the 75-kD form. Thus integral membrane and soluble PAM proteins reach the regulated secretory pathway with similar kinetics.

Kinetics of the Appearance of Integral Membrane PAM at the Cell Surface

We previously demonstrated that integral membrane PAM-1 and -2 reached the surface of AtT-20 cells intact and were subsequently internalized but not immediately degraded in lysosomes (Milgram et al., 1993). Therefore, when considering the trafficking of integral membrane PAM proteins, it is necessary to determine not only the kinetics of secretion of the processed proteins, but also the kinetics of appearance of the full length protein at the cell surface. AtT-20 cells expressing PAM-1, PAM-2, or PAM-3 were incubated for 30 min with [^{35}S]methionine and chased at 37°C for 0, 1, 2, or 3 h. At the end of the chase period intact cells were placed at 4°C and PHM antiserum was added to bind to PAM proteins accessible on the cell surface; detergent was added to lyse cell membranes and cell surface-associated PAM proteins were immunoprecipitated. Then, one sixth of the supernatant from the cell-surface immunoprecipitation was immunoprecipitated with PHM antiserum to determine the total amount of cell-associated integral membrane PAM protein. For comparison, the same experiments were performed using AtT-20 cells expressing a truncated PAM-1 protein terminating nine amino acids following the transmembrane domain at Gly[899] (PAM-1/899). We previously demonstrated that large amounts of this protein were on the cell surface when expressed in AtT-20 cells and did not undergo rapid internalization (Milgram et al., 1993).

Very little newly synthesized integral membrane PAM-1 or PAM-2 was accessible to the PHM antiserum in the extracellular space (Fig. 8). Longer exposures of the autoradiograms demonstrated that less than 2–5% of the PAM-1 or PAM-2 full length protein was accessible to PHM antibody after 1, 2, or 3 h of chase. In contrast, ∼20% of the newly synthesized PAM-1/899 was present at the cell surface and was accessible to antibody after 1 h of chase. By 3 h, between 50 and 70% of the PAM-1/899 protein had reached the plasma membrane. Longer exposures of the autoradiogram demonstrated that most of the PAM-1/899 not located at the cell surface was monofunctional 44–46 kD PHM, although some full length PAM-1/899 was also observed at all times of chase.

Discussion

Maturation and Endoproteolytic Processing

Soluble and integral membrane PAM proteins exit the ER and reach the medial Golgi apparatus with similar kinetics, acquiring resistance to endoglycosidase H with a $t_{1/2}$ of ∼60 min (Fig. 2). Within 1–2 h after a short biosynthetic pulse, newly synthesized PAM proteins begin to undergo endoproteolytic cleavage and their secretion becomes responsive to secretagogues (Figs. 3, 4, and 7). Incubation of cells at 20°C virtually abolished the processing of all forms of PAM (Fig. 5), indicating that for both soluble and integral
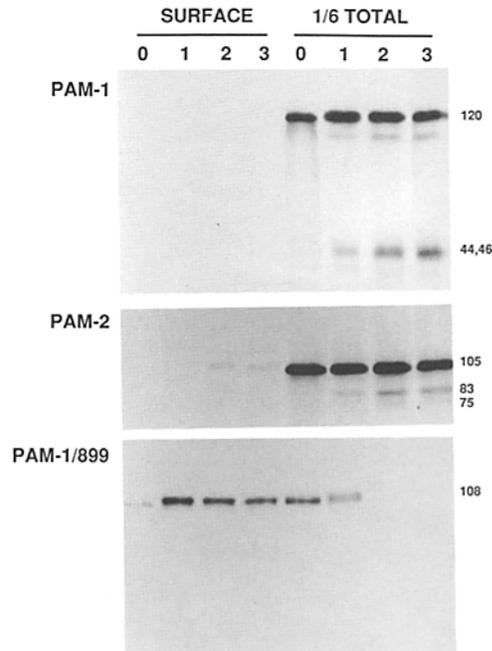

Figure 8. Kinetics of appearance of full length and truncated integral membrane PAM at the cell surface. Biosynthetic labeling was performed as described for 30 min and cells expressing PAM-1, -2, or PAM-1/899 were chased for 0–3 h. Cell surface-associated PAM proteins were immunoprecipitated after incubation of intact cells with PHM antiserum at 4°C as described. One sixth of the whole cell extract was then immunoprecipitated to determine the amount of PAM not present at the cell surface. Immunoprecipitates were analyzed on 8% SDS-PAGE gels; apparent molecular masses are in kD.

membrane PAM proteins, these endoproteolytic cleavages must occur in a post-TGN compartment (Griffiths et al., 1985). Taken together with our observation that expression of PAM proteins in the hEK-293 fibroblast cell line yields no 45-kD PHM from PAM-1 or PAM-4 and no 75-kD PAM from PAM-2 or PAM-3 (Tausk et al., 1992), these data argue that in AtT-20 cells, the endoproteolytic cleavage of integral membrane and soluble PAM occurs in the regulated secretory pathway. The times at which processing is first observed suggest that integral membrane and soluble PAM proteins exit the TGN and first become accessible to the relevant endoproteases at similar rates (Fig. 3).

It is not until exiting the TGN that differences in the trafficking of soluble and integral membrane PAM proteins appear. Although both PAM-1 and PAM-4 are processed within exon A to generate 44–46-kD monofunctional PHM proteins, soluble PAM-4 is processed much more rapidly (Figs. 3 and 4). While processing of both PAM-2 and PAM-3 begins at the same time following a biosynthetic pulse, the processing of the soluble PAM-3 protein is more extensive (Figs. 3 and 4). The less efficient processing of integral membrane PAM compared to soluble PAM suggests that the integral membrane forms have access to the endoproteases responsible for their cleavage for shorter lengths of time. Alternatively, it is possible that the endoproteases responsible for processing PAM cleave integral membrane proteins less efficiently than soluble ones.

To explore directly the hypothesis that the less efficient

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cleavage of integral membrane PAM proteins was accompanied by their less efficient storage in secretory granules, we followed the fate of newly synthesized PAM proteins for up to 16 h after a short biosynthetic pulse. There was considerably less integral membrane PAM (70-kD PAL and intact PAM-2) remaining cell-associated following a 16 h chase compared with soluble PAM proteins, indicating that integral membrane PAM proteins are not efficiently stored in secretory granules (Fig. 4).

The Trafficking of Soluble PAM Proteins

When expressed in AtT-20 cells, soluble monofunctional and bifunctional PAM proteins are targeted to the regulated secretory pathway and accumulate in secretory granules (Fig. 7 and Milgram et al., 1992). Each catalytic domain contains the routing information needed to enter the regulated pathway (Milgram et al., 1992). However, AtT-20 cells also display a significant amount of nonstimulated secretion of these soluble PAM proteins (Fig. 6). The nonstimulated secretion of soluble PAM proteins exhibited kinetics similar to the nonstimulated secretion of granule-associated proteins from the parotid gland and pancreatic islets and was highest 1–2 h after a 15-min biosynthetic pulse (Fig. 6) (von Zastrow and Castle, 1987; Kuliawat and Arvan, 1992).

In neuroendocrine cells, secretory granule biogenesis proceeds through the formation of immature granules which are less dense than mature storage granules (Tooze et al., 1991; Grimes and Kelly, 1992). The budding of vesicles from immature granules and/or the fusion of several immature granules with subsequent loss of excess membrane could account for the change in size during granule maturation; the vesicles which bud from immature granules should contain soluble proteins excluded from the granule core as well as membrane. Indeed, other investigators have demonstrated that vesicular budding from immature granules accounts for much of the nonregulated release of secretory granule-associated proteins; this secretion is referred to as constitutive-like secretion (von Zastrow and Castle, 1987; Kuliawat and Arvan, 1992; Arvan and Castle, 1992). In the primary tissues studied, constitutive-like secretion represents <5% of the newly synthesized secretory protein; in AtT-20 cells the early phase secretion of soluble PAM proteins was ~10-fold greater, accounting for ~50% of the newly synthesized soluble PAM protein. This is consistent with observations from many laboratories that transformed cells display considerably higher rates of nonstimulated secretion of granule-associated proteins than the corresponding primary tissue (discussed in Matsuuchi and Kelly, 1991). Nonetheless, the kinetics of secretion of soluble PAM-3 and -4 would suggest that much of the secretion in AtT-20 cells was constitutive-like in nature and was not unregulated release from mature secretory granules. The overlap in the kinetics of constitutive and constitutive-like secretion complicates the analysis of these two types of secretion (Arvan et al., 1991) and some of the soluble PAM protein secreted in the absence of stimulation could reach the cell surface in constitutive vesicles. The fact that >90% of the PAM-4 undergoes an endoproteolytic cleavage which is limited to the regulated pathway suggests that constitutive secretion is not a major pathway.

Trafficking of Integral Membrane PAM

If the trafficking of integral membrane and soluble PAM pro-

teins were the same, then the kinetics of appearance of integral membrane PAM proteins at the cell surface would be the same as for soluble PAM proteins. Since integral membrane PAM proteins can reach the cell surface intact or undergo endoproteolysis and secretion (Milgram et al., 1993), cell surface expression of intact, integral membrane PAM as well as secretion of the described cleavage products must be considered. The kinetics of secretion of monofunctional and bifunctional PAM proteins generated through cleavage of integral membrane PAM-1 and -2 differed from the kinetics of secretion observed for soluble PAM proteins (Fig. 6), and biosynthetic labeling followed by immunoprecipitation of cell surface–associated integral membrane PAM demonstrated that newly synthesized full length PAM-1 and PAM-2 did not reach the cell surface in appreciable amounts during a 3-h chase period (Fig. 8). Therefore, the kinetics of the appearance of integral membrane PAM at the cell surface differs from the kinetics of secretion of soluble PAM proteins. Taken together these data strongly argue that upon exiting the TGN, the trafficking of integral membrane and soluble secretory granule-associated proteins differ.

We previously demonstrated that integral membrane PAM proteins which reached the cell surface were rapidly internalized (Milgram et al., 1993). Therefore, we were concerned that the newly synthesized PAM-1 and -2 reached the cell surface in significant amounts during the first 3 h of chase but was not detected in our cell surface immunoprecipitation experiments due to their rapid internalization. However, when cells were labeled with [³⁵S]methionine and chased at 37°C in the presence of PHM antiserum, no significant increase in the amount of integral membrane PAM which had reached the cell surface was observed (not shown). These data strongly suggest that, unlike the synaptic vesicle protein synaptophysin, integral membrane PAM proteins are not all transported to the cell surface before packaging into secretory granules (Regnier-Vigouroux et al., 1991). However, it is possible that some of PAM proteins bound to the antibody at the cell surface did not remain bound throughout the chase period at 37°C and that our experiments under-represented the percentage of integral membrane PAM which reached the cell surface in constitutive or constitutive-like vesicles.

Immunoprecipitations of cell surface–associated PAM-1/899 clearly demonstrates that a signal in the cytoplasmic domain of integral membrane PAM is responsible for the differences in the trafficking of integral membrane and soluble PAM proteins. By comparison to the full length integral membrane PAM, significant amounts of this truncation mutant reached the cell surface within one hour of a biosynthetic pulse with kinetics similar to those observed for the nonstimulated secretion of soluble PAM-3 and -4 (Fig. 8). In previous experiments, the secretion of a novel bifunctional PAM protein from cells expressing PAM-1/899 was highest during the 1–2- and 2–4-h chase periods (Milgram et al., 1993). Together these data suggest that the PAM-1/899 protein reached the cell surface primarily in constitutive-like vesicles. In contrast, full length integral membrane PAM proteins may be diverted from these constitutive-like vesicles and would therefore not be accessible to PHM antibodies in our cell surface immunoprecipitation experiments (Fig. 8). All of the PAM-1 and 30–40% of the PAM-1/899 protein was processed in exon A to generate monofunctional PHM. Since this cleavage is restricted to the regulated secre-

tory pathway, these integral membrane PAM proteins must initially reside in immature secretory granules.

A Model for PAM Trafficking

The data are consistent with a model whereby integral membrane and soluble PAM proteins are packaged into immature granules by passive sorting (Arvan and Castle, 1992). Both soluble and integral membrane PAM proteins are retained in maturing granules; however, integral membrane PAM, perhaps due to its inability to aggregate well in the granule lumen or due to active removal mediated by interactions with the cytoplasmic domain, is less efficiently retained during the granule maturation process. Soluble PAM proteins not retained in the storage granule during the maturation process would be secreted from constitutive-like vesicles at early times after a biosynthetic pulse (Fig. 9), while integral membrane PAM would reach the cell surface intact. Our inability to detect large amounts of integral membrane PAM on the surface of cells at early times after a biosynthetic pulse (Fig. 8) raises the possibility that full length integral membrane PAM proteins are retrieved from constitutive-like (or constitutive) vesicles before these vesicles reach the plasma membrane (Fig. 9). Additionally, we previously demonstrated that integral PAM proteins could be internalized from the cell surface after exocytosis (Fig. 9). The retrieval of integral membrane PAM, either from the cell surface or from intracellular vesicles, with subsequent return to the TGN could account for our observation that at steady state much of the integral membrane PAM was concentrated in perinuclear vesicles and endosomes (Milgram et al., 1992, 1993). Our inability to account for all of the newly synthesized integral membrane PAM in cell extracts or medium after long chase incubations, suggests that a portion of the newly synthesized integral membrane PAM may undergo intracellular degradation. However, the addition of lysosomotropic amines to chase media for 16 h after a short biosynthetic pulse did not affect the fate of the newly synthesized integral membrane PAM in transfected AtT-20 cells (not shown). Interestingly, similar results on the stability of integral membrane PAM were obtained in primary cultures of cardiac myocytes, which also contain secretory granules and express high levels of endogenous integral membrane PAM (Maltese and Eipper, 1993).

If integral membrane PAM were returned to the TGN and repackaged into immature granules, then some of the integral membrane PAM would be retained in the maturing granule, some would reach the cell surface in constitutive or constitutive-like vesicles, while some might be returned to the TGN without traversing the cell surface (Fig. 9). The integral membrane PAM which was retained in granules during maturation would undergo endoproteolysis and exocytosis in a regulated manner (Figs. 4 and 7). Once integral membrane PAM was cleaved in immature granules, the soluble PAM-derived proteins would undergo the same fate as soluble PAM proteins. Some of the soluble proteins derived through cleavage of PAM-1 and PAM-2 would be stored in secretory granules while some would be released in the absence of stimulation (Fig. 9). A model for the trafficking of integral membrane PAM, which includes cycles of packaging into immature granules with subsequent cleavage, is consistent with our observation that the nonstimulated secretion of proteins derived from PAM-1 and -2 occurs over a longer
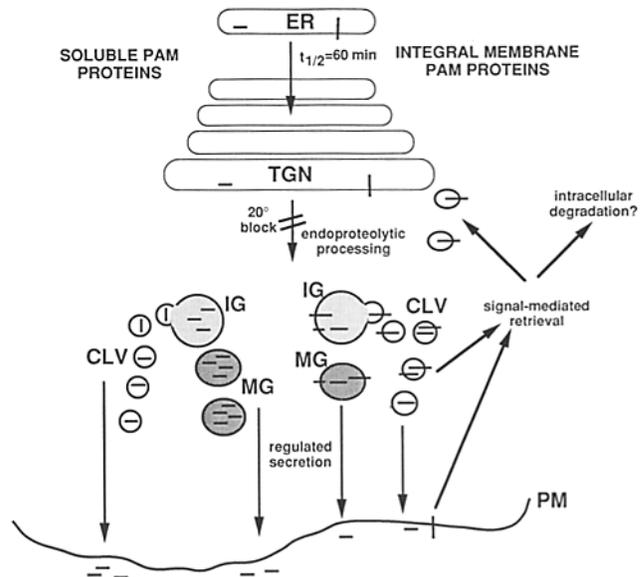

Figure 9. A model for trafficking of integal membrane and soluble PAM proteins. Integral membrane and soluble PAM proteins exit the ER and reach the Golgi complex with similar kinetics. Both integral membrane and soluble PAM proteins are packaged into immature granules (*IG*) and undergo endoproteolytic processing upon exiting the TGN. Some of the soluble PAM proteins are diverted from immature granules and are released in constitutive-like vesicles (*CLV*) while some of the soluble PAM proteins are stored in mature granules (*MG*) and undergo stimulus-induced secretion. Integral membrane PAM is also targeted to IGs, may undergo endoproteolytic processing and can be diverted from IGs. Integral membrane PAM is retrieved from IGs and the plasma membrane (*PM*) through a signal-mediated process. Integral membrane PAM which undergoes endoproteolytic cleavage yields proteins stored in mature granules or secreted under nonstimulated conditions in CLVs. Integral membrane PAM which does not undergo processing to generate soluble proteins is poorly stored in MGs and some may be degraded in the cell.

time period than was observed for the secretion of PAM-3 and -4 (Fig. 6).

Our data on the trafficking of integral membrane PAM in AtT-20 cells would suggest that an active, signal-mediated sorting event occurs to retrieve integral membrane PAM from intracellular vesicles or the cell surface. This retrieval process for membrane proteins apparently can overpower the sorting events which would otherwise propel the lumenal portion of the molecules into secretory granules. Mutagenesis studies are currently underway to identify the nature of this retrieval signal.

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