Localization of integral membrane peptidylglycine α -amidating monooxygenase in neuroendocrine cells

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SUMMARY

Peptidylglycine α -amidating monooxygenase (PAM) catalyzes the amidation of glycine-extended peptides in neuroendocrine cells. At steady state, membrane PAM is accumulated in a perinuclear compartment. We examined the distribution of membrane PAM in stably transfected AtT-20 cells and compared its localization to markers for the *trans*-Golgi network (TGN), endosomes, and lysosomes. At the light microscopic level, the distribution of membrane PAM does not overlap extensively with lysosomal markers but does overlap with TGN38 and with SCAMP, a component of post-Golgi membranes involved in recycling pathways. By immunoelectron microscopy, membrane PAM is present in tubulovesicular structures which constitute the TGN; some of these PAM-containing tubulovesicular structures are more distal to the Golgi stacks and do

not contain TGN38. While some POMC-derived peptides are present in tubulovesicular structures like those that contain membrane PAM, the majority of the POMC-derived peptides are present in secretory granules. There is little overlap between the steady state distribution of membrane PAM and internalized FITC-transferrin in the early endosomes. Few of the perinuclear PAM-containing structures are labeled with HRP or WGA-HRP even following long incubations. Therefore, membrane PAM is localized to perinuclear tubulovesicular structures which are partially devoid of TGN38 and are not all endosomal in origin.

Key words: neuroendocrine, TGN, secretory granule

INTRODUCTION

Although substantial progress has been made in understanding the biogenesis of synaptic vesicles (reviewed by De Camilli and Jahn, 1990; Sudhof and Jahn, 1991; Bauerfeind and Huttner, 1993; Kelly and Grote, 1993; Bennett and Scheller, 1994), there are still many unanswered questions about the biogenesis of the large dense core granules in which neurons and endocrine cells store bioactive peptides. A number of studies have addressed the role of condensation and receptor-mediated processes in the routing of soluble content proteins to large dense core granules (Burgess and Kelly, 1987; Chanat and Huttner, 1991; Arvan and Castle, 1992; Kuliawat and Arvan, 1992, 1994; Chidgey, 1993; Cool and Loh, 1994; Colomer et al., 1996), but the routing of membrane proteins to these granules remains largely unexplored. We have used peptidylglycine α -amidating monooxygenase (PAM), a Type I integral membrane protein that catalyzes one of the last essential steps in the biogenesis of neuroendocrine peptides and a step common to many bioactive from species as diverse as Hydra and man (Fig. 1), as a tool to investigate the biogenesis of large dense core granules (Milgram et al., 1992, 1993, 1994, 1996; Eipper et al., 1993).

The two enzymes that comprise the luminal domain of the

bifunctional integral membrane PAM protein can be expressed independently as soluble proteins and both domains are efficiently targeted to large dense core granules when expressed in AtT-20 cells (Milgram et al., 1992). In contrast, light microscopic studies of stably transfected AtT-20 corticotrope tumor cells expressing integral membrane forms of PAM indicate that the majority of the PAM protein is localized in a perinuclear compartment overlapping the Golgi complex and *trans*-Golgi network (TGN) (Milgram et al., 1993, 1994). When expressed in non-neuroendocrine cells, membrane PAM is also retained in the perinuclear region overlapping markers of the Golgi complex/TGN (Tausk et al., 1992). In both cell types, localization of membrane PAM to this region of the cell is dependent on information in the 86 amino acid COOH-terminal cytosolic tail; truncated membrane PAM proteins lacking all but 9 amino acids of the cytosolic tail are largely localized to the plasma membrane (Tausk et al., 1992; Milgram et al., 1993). By appending the transmembrane and cytosolic domains of PAM to the luminal domain of the interleukin 2 receptor alpha chain (Tac protein), this plasma membrane protein is rerouted to the perinuclear region of the cell (Milgram et al., 1996).

Surface immunoprecipitation experiments indicate that very little newly synthesized integral membrane PAM reaches the

plasma membrane; in contrast, over half of the newly synthesized truncated PAM reaches the plasma membrane within 2–3 hours of synthesis (Milgram et al., 1993). Although little full-length membrane PAM is located on the cell surface at steady state, incubation of live cells with antibodies that recognize the luminal domain of membrane PAM results in the rapid internalization of the PAM/PAM antibody complex; truncated membrane PAM proteins fail to undergo internalization (Milgram et al., 1993). Mutation of a single Tyr residue in the COOH-terminal cytosolic domain compromises the ability of integral membrane PAM to undergo internalization but does not eliminate its ability to localize to the perinuclear region or undergo neuroendocrine cell-specific endoproteolytic cleavage (Milgram et al., 1996). In our current working model, membrane PAM retrieved from the compartment in which endoproteolytic cleavage of POMC and PAM begins is retained in the perinuclear region and recycled.

We sought to use confocal and electron microscopy to define better the major compartments in which integral membrane PAM is localized. To carry out these studies, we first generated monoclonal antibodies to PAM and polyclonal antibodies to the luminal domain of TGN38 (Ladinsky and Howell, 1992). We compared the distribution of PAM to that of TGN38 and POMC-derived peptides, soluble constituents of large dense core granules. In addition, we compared the distribution of PAM to that of internalized fluorescein-conjugated transferrin (FITC-Tf), a marker of early endosomes, and internalized horseradish peroxidase (HRP) and WGA-HRP, markers of the entire endocytic pathway (Orci et al., 1986; Schmid et al., 1988; Back et al., 1993; Watts, 1992; Thilo et al., 1995; Futter et al., 1996). In AtT-20 cells, integral membrane PAM displays a unique distribution; the majority of the membrane PAM is localized to tubulovesicular structures in a region of the TGN distal to the major site of TGN38 accumulation and clearly distinct from the endosomal compartment. The precise composition and function of this compartment remain to be determined.

MATERIALS AND METHODS

Growth and manipulation of AtT-20 cell lines

The generation and characterization of AtT-20 cells stably expressing rat PAM-1 were described (Milgram et al., 1992). Cells were grown in DMEM-F12 containing 10% Fetal Clone and 10% Nu-Serum containing 500 µg/ml G418.

To compare the distribution of early endosomes and membrane PAM, PAM-1 AtT-20 cells were rinsed with pre-warmed Dulbecco's modified Eagle's medium containing 2 mg/ml bovine serum albumin (DMEM/BSA) and incubated with 30 µg/ml fluorescein-conjugated transferrin (Molecular Probes, Inc.) for 30 minutes. Cells were rinsed 3 times with DMEM/BSA, fixed with 4% paraformaldehyde for 60 minutes, and processed for immunofluorescence microscopy. To visualize the endosomal pathway using HRP as a marker, PAM-1 cells were incubated in DMEM for 1 hour at 37°C prior to incubation with 1 mg/ml HRP (Sigma Type II) in DMEM for 3 hours at 37°C. To visualize the endosomal pathway using WGA-HRP (Sigma Type IV), AtT-20 PAM-1 cells were preincubated at 4°C in DMEM for 1 hour followed by incubation with WGA-HRP (30 µg/ml) for 1 hour at 4°C in DMEM previously equilibrated to 4°C. The cells were then washed, warmed to 37°C and incubated for 1 hour at 37°C to allow internalization of the WGA-HRP bound to proteins present on the cell surface. Cells were fixed and processed for electron microscopy.

Generation of PAM antisera

Rabbit polyclonal antibodies generated against PHM (Ab 475), PAL (Ab 471), and cytosolic domain (CD, Ab 571) were described (Yun et al., 1993). The antigen used to generate monoclonal antibody 18E5, which recognizes an epitope in the monooxygenase domain, was purified PAM-3 (Husten et al., 1993). Monoclonal antibody 6E6 was generated using recombinant CD [rPAM-1(898-976)] (Yun et al., 1993).

Primary inoculations of mice were performed with ~20 µg of antigen emulsified in Complete Freund's adjuvant. C57-B16 and Balb-C mice were used to generate the PHM and CD antibodies, respectively. Mice which showed a primary immune response were boosted several times using 5 µg of antigen emulsified in incomplete Freund's adjuvant; an additional 3 boosts of 5 µg each were performed on alternate days immediately preceding the fusion. Fusions of isolated splenocytes and P3.653 myeloma cells were performed using polyethylene glycol. Hybridomas were cloned by limiting dilution in 96 well plates and grown in DMEM-F12 supplemented with 20% fetal calf serum, 10% NCTC (Sigma), insulin (0.2 µU/ml), sodium pyruvate (0.05 mg/ml), oxalacetic acid (0.15 mg/ml), hypoxanthine (100 µM), aminopterin (0.4 µM), and thymidine (16 µM). In the initial fusions and subclonings, a feeder layer of peritoneal macrophages was used to sustain low density growth of cells; later the addition of 10% Hybridoma cloning factor (Origen) to the medium negated the need for macrophages. Hybridomas were selected based on their ability to recognize PAM-1 stably expressed in AtT-20 cells either by immunofluorescence staining of fixed cells or by western blotting of membrane proteins. Positive hybridomas were subcloned through at least 3 rounds of limiting dilution.

Generation of TGN-38 antisera

We generated rabbit polyclonal antisera directed against a region of the rat TGN38 luminal domain (residues 155-249) purified following overexpression in *Escherichia coli*. The plasmid was generated by reverse transcription PCR of rat anterior pituitary mRNA using TGN38-specific sense (nt 474-490) and antisense (nt 758-741) primers containing *NdeI* and *EspI* restriction sites, respectively. The PCR fragment was subcloned into complementary restriction sites in the pET.11C plasmid and expressed in BL21 *E. coli* as described (Yun et al., 1993). The TGN38 fragment was solubilized from a washed bacterial pellet by homogenization in 30% acetic acid and centrifugation at 10,000 *g* for 20 minutes at 4°C. The supernatant was lyophilized, resuspended in 50% acetic acid containing 5% β-mercaptoethanol, and fractionated on a Sephadex G-75 gel filtration column equilibrated and eluted with 10% formic acid. The purified TGN38 fragment was subjected to amino acid analysis and found to have a single Met residue preceding the expected N-terminal sequence (D¹⁵⁵-K-T-S-G-G).

Antisera were generated by injecting rabbits with 150 µg of recombinant TGN38 fragment linked to keyhole limpet hemocyanin using glutaraldehyde and 150 µg of recombinant TGN38 which was not linked to carrier protein. Rabbits were boosted with 75 µg of each antigen and plasma was collected biweekly. Antisera 1479 and 1481 showed high levels of immunoreactivity toward endogenous TGN38 in mouse anterior pituitary AtT-20 cells and toward the recombinant TGN38 fragment on solid phase assay.

Immunofluorescence staining

AtT-20 PAM-1 cells were grown on Lab-Tek chamber slides coated with 0.1 mg/ml poly-L-lysine 48–72 hour prior to immunostaining. Cells were rinsed with phosphate buffered saline (PBS) and fixed in either 4% paraformaldehyde in PBS for 20 minutes at 22°C or in ice-cold methanol for 1 minutes. Cells fixed with paraformaldehyde were permeabilized for 20 minutes at 22°C with 0.075% Triton X-100 in PBS. Cells were blocked and stained as described (Tausk et al., 1992). SCAMP antiserum was kindly provided by Dr David Castle, University of Virginia and used at a final concentration of 1:500. Rat mon-

oclonal antibody 1D4B, kindly provided by Dr Tom August through the Developmental Studies Hybridoma Bank, was diluted 1:50. All other primary antibodies were generated in our lab and used at the following dilutions: PHM (Ab475), 1:500; PHM (Ab18E5, ascites), 1:100; PAM cytosolic domain (mouse monoclonal 6E6, ascites or conditioned medium), 1:1,000 or 1:50; TGN38, 1:500; and β -endorphin (JH2), 1:3,000. Secondary antisera were diluted 1:200–1:400 in PBS containing 2 mg/ml BSA.

Confocal microscopy

Specimens were imaged using either a Bio-Rad MRC600 or a Zeiss LSM410 confocal microscope. The Gain and Black Level controls of the instrument were set such that the signal from the specimens utilized the entire available range of 256 gray levels. Specimens double-labeled with FITC and Texas Red were imaged by simultaneous collection of the two fluorescent signals using the microscope's dual channel filter blocks. The optical section thickness was approximately 1.5 μ m for the Bio-Rad images or 0.9 μ m for the Zeiss images. At each area of interest, 8 images from each channel were averaged, and the resulting averaged images were merged and pseudo-colored to produce a co-localization image. Controls for fluorescent bleed-through in the co-localization images were performed by imaging the appropriate single-labeled specimens that had been labeled in parallel with the double labeled specimens. Only when no image was generated in the 'wrong' channel for each of the single-labeled specimens was the double-labeled specimen imaged.

Electron microscopy

LRGold sections

Cells were rinsed once in a modified Hanks' buffer, then fixed on the culture dish for 1 hour at 22°C with 3% formaldehyde and 0.05% glutaraldehyde in the same buffer (Roth et al., 1985). The cells were then scraped from the dish and pelleted in fixative for 10 minutes at 14,000 rpm prior to processing for LRGold embedment and immunolabeling according to the method of Berryman et al. (1992). For labeling of sequential sections with different antibodies, a single section was placed onto each grid and the order of the individual sections was maintained.

Ultrathin cryosections

Small pieces of fixed cell pellets prepared as described above were rinsed briefly in modified Hanks' buffer and infiltrated for 2 hours at 22°C in polyvinylpyrrolidone/sucrose (Tokuyasu, 1989). After freezing in liquid nitrogen, specimens were sectioned and labeled as described (Tokuyasu, 1989). After labeling, sections were stained for 20 minutes with 2% OsO₄, rinsed 2 minutes with dH₂O, and further stained with 2% uranyl acetate for 45 minutes. Final embedding and contrasting of the sections was accomplished by incubating the sections in 2.8% polyvinyl alcohol (PVA, *M_r* 10,000) for 2 minutes followed by 3 minutes in 2.8% PVA containing 0.0015–0.002% lead citrate.

Visualization of the endocytic pathway

Cells incubated with HRP or WGA-HRP were fixed and processed for ultrathin cryosections as described, with the exception that the fixation was carried out on ice. Double labeling of the internalized HRP and PAL antibody was performed using the same protocol described for single labeling of ultrathin cryosections. The primary (polyclonal mouse anti-HRP and rabbit anti-PAL) and secondary antibodies (donkey anti-mouse 6 nm colloidal gold and donkey anti-rabbit 12 nm colloidal gold) were applied simultaneously. Control experiments in which either of the single primary antibodies was applied followed by the mixed secondary antibodies resulted in binding of only the appropriate size gold-conjugate.

Immunoreagents for electron microscopy

All colloidal gold-conjugated secondary antisera and affinity-purified

polyclonal mouse anti-HRP were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Affinity-purified rabbit anti-PAL (Ab471) was used at 1:1,000, rabbit anti-ACTH antibody (Ab Kathy) was used at 1:10,000, rabbit anti-PAM recombinant CD (Ab571) was used at 1:1,000, mouse anti-HRP was used at 20 μ g/ml and affinity-purified rabbit anti-TGN38 was used at 1:50. Secondary antibodies were diluted to an *A*₅₂₀ of 0.05, except for the labeling of sequential sections for TGN38 and PAL, in which case the dilution was to an *A*₅₂₀ of 0.1. All immunoreagents used for labeling LRGold sections were diluted in 50 mM Tris-HCl, 150 mM NaCl, pH 7.4, containing 5% normal goat serum; those used for ultrathin cryosections were diluted in PBS containing 10% fetal calf serum.

RESULTS

Different domains of PAM are concentrated in different compartments of AtT-20 cells

In previous studies we used immunofluorescence microscopy to localize integral membrane PAM to the perinuclear region and secretory granules in AtT-20 cells (Milgram et al., 1992, 1993). PAM proteins expressed in AtT-20 cells are subject to endoproteolytic cleavage in a compartment beyond the site of the 20°C temperature block (Milgram et al., 1994; Milgram and Mains, 1994); some of the products generated are soluble (e.g. 44–46 kDa PHM) while others remain membrane associated (e.g. 70 kDa PAL and all products containing the COOH-terminal domain; Fig. 1). Several new reagents were generated for the experiments described below. Monoclonal antisera to PHM and to the PAM COOH-terminal domains (Fig. 1) allowed us to perform double-labeling experiments and compare the distribution of membrane PAM to marker proteins for various cellular compartments. In addition, because the perinuclear accumulation of membrane PAM suggested overlap with the TGN, we generated a rabbit polyclonal antibody using as immunogen residues [155–249] of the TGN38 luminal domain.

On western blots monoclonal antibody 6E6 recognized full-length membrane PAM-1 as well as the 70 kDa membrane-associated monofunctional PAL observed in the crude particulate fraction of AtT-20 PAM-1 cells; no cross-reactive proteins were observed in the soluble fraction (Fig. 2). In contrast, the monoclonal antibody directed against the PHM domain (mAb 18E5) recognized full length PAM-1 and soluble PHM (44–46 kDa); this 44–46 kDa PHM is the same protein recognized by

Fig. 1. The PAM protein and antibodies used. The major protein products generated from PAM-1 stably expressed in AtT-20 cells are shown. The specificities of the antisera used in these studies are indicated below the figure.

Fig. 2. Characterization of antisera. The specificities of the newly generated antisera were evaluated by western blot. Protein (30 µg) from a whole homogenate (H) or crude particulate (P) or soluble (S) fraction prepared from AtT-20 PAM-1 cells was electrophoresed on 8 or 10% SDS-PAGE gels and transferred to Immobilon-P. Primary antisera were used at the following concentrations: spent medium from monoclonal antibody 6E6 (mAb6E6), 1:50; ascites from mAb18E5, 1:100; Ab 1479, 1:1,000. Goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP were used at a final concentration of 1:10,000 and immune complexes were visualized using chemiluminescent detection (Amersham).

our polyclonal antisera against the PHM domain (Ab475). When the specificity of the polyclonal antibodies to TGN38 was assessed on western blots, we observed an ~70 kDa protein in the particulate fraction of AtT-20 cells (Fig. 2). Proteins of similar mobility were observed in the particulate fractions of protein extracts prepared from rat liver and anterior pituitary (not shown).

Since the trafficking of soluble and integral membrane proteins derived from PAM-1 are clearly not identical (Milgram et al., 1994), the staining patterns observed with antisera to the COOH-terminal cytosolic domain of PAM and the luminal PHM domain of PAM were compared to each other and to the staining observed for POMC (Fig. 3). The staining patterns observed were overlapping. The COOH-terminal

cytosolic domain of PAM which remains membrane-associated, was localized primarily to the perinuclear region (Fig. 3, middle); the tips of the cellular processes were stained, but much less intensely. The PHM domain, which is cleaved from the membrane, was also localized to the perinuclear region (Fig. 3, left). In addition, the PHM domain was visualized in punctate structures throughout the cytosol and at the tips of the cellular processes (Fig. 3, left). The POMC-derived peptide β-endorphin, was also visualized in the perinuclear region and in punctate structures throughout the cytosol (Fig. 3, right); as for PHM, β-endorphin-containing structures were easily visualized at the tips of the processes.

Localization of PAM at the EM level

PAM proteins in AtT-20 cells were visualized at the EM level with antisera to the PAL domain (Ab471), and to the COOH-terminal cytosolic domain (Ab571). Since very little PAL is cleaved from the transmembrane domain of PAM-1 in AtT-20 cells, these antisera should detect the same major proteins, 70 kDa membrane-associated PAL and 120 kDa PAM-1 (Figs 1 and 2). With both PAM antisera, staining was most prevalent in the vicinity of the TGN (Fig. 4) and tubulovesicular structures similar to those described by other investigators were visualized (Luzio et al., 1990; Tooze and Hollinshead, 1992; Connolly et al., 1994); the tubuloreticular structure of the TGN was more apparent in fresh frozen sections (Fig. 4C to F), and PAM protein was sometimes identified along the entire extent of an individual tubule (Fig. 4F). The average diameter of these tubular structures was 49±15 nm (n=118). In some sections PAM staining was apparent in structures that contain condensing secretory protein (Fig. 4A). PAM staining was also commonly observed in what appear to be accumulations of vesicular structures (Fig. 4C,E,F). Although membrane PAM protein was not prevalent in mature secretory granules, PAM was visualized in immature secretory granules (Fig. 4A).

To visualize the regulated secretory pathway in AtT-20 cells, PAM-1 AtT-20 cells were also stained with a POMC antiserum. This antiserum recognizes the COOH-terminus of ACTH and only detects POMC products after endoproteolytic cleavage has occurred at this site (Schnabel et al., 1989). POMC-derived cleavage products were detectable in the TGN and were especially prevalent within mature secretory granules (Fig. 4G-I).

Fig. 3. Immunofluorescence localization of PAM and POMC in AtT-20 PAM-1 cells. PAM-1 AtT-20 cells were visualized with mouse monoclonal antibody 6E6 to the COOH-terminal domain of PAM-1 (B), mouse monoclonal antibody 18E5 to the PHM domain (A), or rabbit polyclonal antiserum JH2 to β-endorphin (C). *, tips of cell processes where secretory granules accumulate; arrows, Golgi/TGN region of cell.

Fig. 4. Electron microscope localization of PAM and POMC in AtT-20 PAM-1 cells. Ultrathin LRGold or cryosections were labeled with antibodies directed against PAM (PAL antibody or the COOH-terminal domain, CD antibody), or POMC (ACTH) as described in Material and Methods. Regions containing well-preserved Golgi complexes or mature secretory granules were selected for analysis. Tubuloreticular structures (C-F,H, long filled arrows), immature secretory granules (A,G, short filled arrows), mature secretory granules (I, short open arrows), the *trans*-Golgi network (F, large arrowhead), and clathrin-coated vesicles (A,B, small arrowheads) are indicated. G, Golgi.

When detected in the TGN-region, POMC-derived products were often concentrated in a single or a few tubuloreticular structures (Fig. 4H). In contrast to integral membrane PAM, POMC-derived peptides were heavily concentrated in mature secretory granules (Fig. 4I).

Localization of PAM compared to organelle marker proteins

With the goal of identifying the nature of the perinuclear tubuloreticular compartment enriched in membrane PAM, confocal microscopy was used to compare the distribution of PAM and marker proteins concentrated in particular subcellular compartments. SCAMP37, one of a set of secretory carrier membrane proteins described by Brand and Castle (1993), serves as a marker for membranes that function in cell surface

recycling; SCAMP37 is found in endosomes, synaptic vesicles, secretory granules and transport vesicles (Brand and Castle, 1993). AtT-20 cells visualized simultaneously with antisera to the COOH-terminal cytosolic domain of PAM and SCAMP showed significant co-localization of the two antigens (Fig. 5A). Lysosome-associated membrane protein-1 (LAMP-1) is an integral membrane protein residing primarily in lysosomes (Fukuda, 1991). The large punctate structures visualized with the LAMP antibody were widely distributed throughout the cytosol and were not concentrated in the perinuclear region (Fig. 5B). Although presumably targeted to lysosomes for degradation, very little PAM is resident in lysosomes at steady state.

The distribution of membrane PAM strongly suggested a Golgi/TGN localization; therefore we compared the distribu-

Fig. 5. Confocal microscopy to compare the steady state localization of PAM and marker proteins for organelles of the secretory pathway. The steady state distribution of membrane PAM was compared to localization of the secretory carrier membrane proteins (SCAMPS) (A) which are broadly distributed in the secretory and endocytic pathways and to the distribution of markers for lysosomes (LAMP-1) (B) and TGN (TGN-38) (C). Cells were fixed and simultaneously incubated with monoclonal antibody to the PAM COOH-terminal domain (mAb6E6) and rabbit anti-SCAMP, rat anti-LAMP or rabbit anti-TGN-38. PAM proteins were visualized with Texas Red conjugated-donkey anti-mouse IgG and organelle makers with FITC-conjugated donkey anti-rabbit IgG (TGN38; SCAMP) or anti-rat IgG (LAMP-1). To compare the distributions of membrane PAM and early endosomes, AtT-20 PAM-1 cells were incubated for 30 minutes with 30 µg/ml FITC-conjugated transferrin. After the incubation, cells were fixed, permeabilized and stained with monoclonal antibody to the PAM COOH-terminal domain (mAb6E6) and Texas Red-conjugated goat anti-mouse serum to visualize the steady state distribution of membrane PAM. All samples were analyzed by confocal microscopy using 1.5 µm optical sections. Differential interference contrast (right) and fluorescence images (left) are shown. Bar, 20 µm.

tion of PAM to the distribution of TGN38. TGN38 was identified by screening a cDNA expression library with a polyclonal antiserum generated to detergent extracted rat liver Golgi membranes (Luzio et al., 1990). Although its function is still not clear, TGN38 is thought to play a role in vesicle budding from the TGN (Luzio et al., 1990; Stanley and Howell, 1993). AtT-20 cells visualized simultaneously with antibodies to the COOH-terminal domain of PAM and TGN38 showed a very similar pattern of staining. Both antibodies visualized a complex tubular network located to one side of the nucleus (Fig. 5C). Although staining for PAM was clearly detected in the tips of the cells (red stain), no staining for TGN38 was detected in this region. Furthermore, at the level of resolution of the confocal microscope, the distributions of TGN38 and PAM in the perinuclear region were not identical.

Although most of the membrane PAM protein seems to be localized in the vicinity of the TGN, our earlier antibody internalization experiments clearly demonstrated that the small amount of membrane PAM that reached the plasma membrane was efficiently internalized (Milgram et al., 1993). We compared the steady state distribution of membrane PAM to the distribution of internalized FITC-conjugated transferrin to determine whether a significant amount of membrane PAM protein was in an early endosomal compartment. Cells stably expressing PAM-1 were incubated with FITC-transferrin at 37°C for 30 minutes. Following fixation, the localization of membrane PAM was determined using antisera to the COOH-terminal domain of PAM and TRITC-conjugated secondary antiserum; the distributions of FITC-transferrin and membrane PAM were compared using confocal microscopy (Fig. 5D). There was minimal overlap between the internalized transferrin and the steady state distribution of membrane PAM, demonstrating that very little of the PAM protein resides in early endosomes at steady state.

Immunoelectron microscopy to compare the distributions of PAM and TGN38

We next used electron microscopy to address more directly the question of whether TGN38 and membrane PAM were co-localized within overlapping compartments in AtT-20 cells. At the EM level, TGN38 was localized within tubular elements at the *trans*-side of the Golgi stacks; relatively little TGN38 was present in the more reticular area somewhat removed from the *trans*-most cisterna of the Golgi (Fig. 6A). Visualization of PAM and TGN38 on serial sections confirmed the presence of PAM in regions coincident with TGN38 as well as in more distally located regions enriched in tubulovesicular structures (Fig. 6B). To compare the patterns observed with the PAM and TGN38 antibodies, we counted the number of gold particles falling within 0.2 and 0.6 μ m of the distal-most Golgi cisternae. For the TGN38 antibody, most of the gold particles fell within 0.2 μ m of the last Golgi cisternae and few were between 0.2 and 0.6 μ m. In contrast, for the PAM antibody, only half of the gold particles were within 0.2 μ m of the distal-most Golgi cisternae and half were between 0.2 and 0.6 μ m. Therefore we conclude that a significant amount of the membrane PAM that is located in the perinuclear region is contained in structures that are not as enriched in TGN38 as the more proximal tubular elements of the *trans*-Golgi network.

Fig. 6. Comparison of the distribution of TGN38 and PAM antibodies near the Golgi apparatus. Adjacent LRGold sections of the same Golgi-region labeled for TGN38 (A) or PAM (PAL antibody, B). Arrows indicate PAM labeling in the vicinity of the Golgi stack, predominantly more distal from the *trans*-side of the stack than the TGN38 label (arrowheads). To confirm our qualitative impression, we counted gold particles within 0.2 μ m of the distal-most cisternae and up to 0.6 μ m beyond; 86% of the particles for the TGN38 antibody ($n=90$) fell within 0.2 μ m of the distal-most cisternae, while only 48% of the particles for the PAM antibody ($n=297$) fell into this region.

Identification of PAM in the endocytic pathway

Internalized HRP has been used to define the endocytic pathway in AtT-20 cells (Tooze and Hollinshead, 1992; Back et al., 1993). In order to determine whether a significant amount of PAM were associated with endocytic structures at steady state, we visualized HRP internalized by AtT-20 cells with an anti-HRP antibody and 6 nm colloidal gold-labeled secondary antibody and simultaneously visualized PAM with an anti-PAL antibody and a secondary antibody coupled to 12 nm colloidal gold (Fig. 7A,B,D,E,H). Tubulovesicular structures enriched in PAM contained very little HRP while those regions enriched in HRP contained very little PAM (Fig. 7A,B,D,E). Structures resembling multivesicular bodies (Tooze and Hollinshead, 1992) often contained both HRP and PAM (Fig. 7H); proteins in these structures are thought to be targeted to lysosomes. In the multivesicular bodies, PAM was always associated with internal membranes.

Fig. 7. A comparison of the distribution of membrane PAM and internalized HRP and the steady state localization of membrane PAM in endosomal and lysosomal structures. AtT-20 PAM-1 cells were incubated for 3 hours at 37°C with 1 mg/ml Sigma type II HRP, fixed and processed for ultrathin cryosections. (A,B,D,E,H) Sections were doubly labeled to visualize internalized HRP (mouse anti-HRP and donkey anti-mouse 6 nm colloidal, indicated by arrows in A,B,E) and PAL (rabbit anti-PAL and donkey anti-rabbit 12 nm gold). (C,F,G) Micrographs of ultrathin cryosections stained with PAL or COOH-terminal (CD) domain antibodies as described in Fig. 4; HRP was not present in these samples. G, Golgi.

In AtT-20 cells stained with PAM antisera without prior internalization of HRP, PAM was also visualized in structures comprising the endocytic pathway (Fig. 7C,F,G). Cup-shaped structures similar to those characterized as early endosomes by HRP-internalization experiments and resembling structures defined as early endosomes by other investigators (Tooze and Hollinshead, 1992) were visualized with antisera to both PAL and the COOH-terminal domain (Fig. 7C,F,G); structures resembling late endosomes and multivesicular bodies were also observed to contain membrane PAM (not shown).

It is clear that internalized membrane proteins do not always follow the path taken by internalized soluble marker proteins like HRP (Back et al., 1993). Therefore, we performed similar experiments using WGA-HRP, which binds to glycoproteins on the plasma membrane of cells prior to internalization

(Krijnse-Locker et al., 1995). AtT-20 PAM-1 cells were first incubated at 4°C with 30 µg/ml WGA-HRP for 60 minutes to allow the WGA-HRP to adhere to the cell surface. The cells were extensively washed and returned to 37°C for one hour. After fixation, the distribution of WGA-HRP and PAM were visualized using the same primary and secondary antibodies as for the soluble HRP and PAM co-localization studies (Fig. 8).

WGA-HRP was observed at the plasma membrane (Fig. 8B), in endosomes (Fig. 8B,C), multivesicular bodies (Fig. 8D) and lysosomes (Fig. 8A). The tubulovesicular structures enriched in membrane PAM did not contain large amounts of WGA-HRP (Fig. 8A,E); although WGA-HRP was visualized in this compartment following one hour of chase, the amount of WGA-HRP in compartments containing integral membrane PAM was relatively small. These results indicate that the post-TGN tubu-

Fig. 8. A comparison of the distribution of membrane PAM and internalized WGA-HRP. AtT-20 PAM-1 cells were incubated at 4°C with 30 μg/ml WGA-HRP (Sigma, Type IV) for 1 hour and then chased at 37°C for 1 hour. Cells were fixed and processed for ultrathin cryosections as described. Sections were doubly labeled to visualize internalized WGA-HRP (mouse anti-HRP and donkey anti-mouse 6 nm colloidal gold) and PAL (rabbit anti-PAL and donkey anti-rabbit 12 nm gold). G, Golgi complex; open arrowheads, plasma membrane; arrows, organelles enriched in WGA-HRP.

loreticular compartment enriched in membrane PAM is distinct from the major compartments of the endosomal pathway but is accessible at least to a limited extent via endocytosis.

DISCUSSION

Alpha amidation is an essential post-translational modification required to generate many bioactive peptides in neurons and endocrine cells. Since PAM functions only following proteolytic processing of peptide precursors, which occurs largely in the immature secretory granule compartment, we expected PAM to be targeted to immature granules along with its pro-

hormone substrates. The single PAM gene encodes multiple mRNAs which arise as a result of alternative mRNA splicing. Some of these mRNAs encode soluble PAM proteins while others encode type I integral membrane proteins. Our biochemical analyses of endogenous and transfected PAM proteins in several different neuroendocrine tissues and in AtT-20 cell lines clearly demonstrate that soluble forms of PAM are targeted to secretory granules (Milgram et al., 1994). However, the distribution of membrane PAM proteins is distinctly different. At the light microscope level, AtT-20 cells transfected with cDNAs encoding membrane PAM have significant amounts of membrane PAM localized in perinuclear vesicles, while the soluble enzyme generated by proteolytic processing

of the membrane bound precursor is also readily visualized in the distal processes of cells.

The most distal compartment of the Golgi complex consists of tubulovesicular networks which lose register with the Golgi stacks in three-dimensional images (Rambourg and Clermont, 1990; Ladinsky and Howell, 1992; Ladinsky et al., 1994). These tubulovesicular extensions of the *trans*-Golgi comprise the TGN and are thought to represent a site in the secretory pathway where protein sorting occurs (Griffiths et al., 1985; Orci et al., 1987; Klausner, 1989). In specialized secretory cells which contain regulated and constitutive secretory pathways, sorting of proteins destined for each of these pathways is also thought to occur, at least in part, in the TGN (Griffiths et al., 1985; Tooze et al., 1987; Sossin et al., 1990). In cells which lack a regulated secretory pathway, TGN38 is known to cycle between the cell surface and TGN; at steady state the majority of the protein is present in the TGN and the accumulation of this protein in the most distal aspects of the Golgi has provided an operational definition of the TGN for the last several years (Luzio et al., 1990; Ladinsky and Howell, 1992; Stanley and Howell, 1993). A recent examination of the TGN in cells which contain large numbers of secretory granules suggested that TGN morphology is cell-type specific (Clermont et al., 1995). In AtT-20 cells, which have a regulated secretory pathway but store relatively few secretory granules compared to primary corticotropes, tubulovesicular structures are easily observed in the vicinity of the *trans*-Golgi and TGN38 is accumulated in these structures. Thus we used TGN38 as a marker of the TGN in AtT-20 secretory cells.

It is clear from the immunoelectron microscopy that some of the membrane PAM is localized in the same compartment as TGN38, however, the PAM staining extends a greater distance from the Golgi complex than does TGN38 staining (Fig. 6). While we cannot rule out the possibility that in the more distal compartments TGN38 is in a complex making it inaccessible to antibody, the simplest interpretation of our results is that membrane PAM is localized in a tubulovesicular compartment which overlaps but is not identical to the TGN38 containing region of the TGN (Figs 4-6). Data from other laboratories indicate that the TGN is not a homogenous structure and that some proteins are not homogeneously distributed throughout the reticulum (Geuze et al., 1987, 1988; Berger et al., 1995). Therefore it is possible that in AtT-20 cells, tubulovesicular structures enriched in membrane PAM represent a distal subcompartment of the TGN. This is a complex region of the cell, and genetic studies in yeast have defined a compartment between the TGN and vacuoles (Cereghino et al., 1995; Horazdovsky et al., 1995).

Tubulovesicular processes extending from existing *trans*-Golgi elements have been observed in hippocampal astrocytes using NBD-ceramide to label the Golgi apparatus in living cells (Cooper et al., 1990). These processes may function to connect cisternae of the *trans*-Golgi and TGN into a single compartment where proteins can freely diffuse; sorting may occur as proteins move through this continuum. In three-dimensional reconstructions of the *trans*-Golgi and TGN using high voltage electron microscopic tomography, Ladinsky et al. (1994) demonstrated that vesicles which budded from TGN tubules contained one of two distinct coats; each TGN tubule produced vesicles containing a single coat, implying that protein sorting occurred within the TGN tubules. Entry of

proteins into tubules is also thought to be involved in sorting in the apical endosomal compartment of Caco-2 cells (Knight et al., 1995). While the structural elements of the TGN have been studied mainly in cells which lack the regulated secretory pathway, it is reasonable to infer the existence of similar structures in cells which contain both regulated and constitutive secretory pathways. The distinct distributions of PAM and TGN38 suggest that these proteins have been sorted in preparation for packaging into regulated and constitutive secretory vesicles, respectively. Since PAM and POMC must be packaged into the same secretory compartment (i.e. the immature secretory granule), we would expect membrane PAM and POMC to segregate into the same TGN ribbon. Electron micrographs of AtT-20 cells stained with an antiserum to ACTH demonstrate that some of the protein is found in the TGN and in the more distal tubulovesicular extensions, while the majority is present in immature and mature secretory granules; the subset of tubulovesicular extensions that stain for ACTH are generally heavily labeled (Fig. 4). Since the antiserum used recognizes cleavage products of POMC but not the POMC precursor, endoproteolytic cleavages must have occurred before this point.

In PC12 cells, secretory granule biogenesis proceeds through the formation of an intermediate, the immature granule, which is less dense than the mature granule (Tooze et al., 1991; Grimes and Kelly, 1992). The budding of vesicles from the immature granule and/or the fusion of several immature granules with subsequent loss of excess membrane could account for the change in size of the secretory granule during its maturation. Budding of membrane from the immature secretory granule is thought to account for the loss of proteins like the C-peptide of insulin, lysosomal-associated proteins and membrane-associated PAM proteins from this compartment (Kuliawat and Arvan, 1992, 1994; Milgram et al., 1994). Thus the tubulovesicular structures enriched in membrane PAM but lacking detectable amounts of TGN38 might represent a recycling compartment derived from the immature secretory granule; it is also possible that these structures represent vesicles of the constitutive-like secretory pathway, but surface immunoprecipitation indicates that very little newly synthesized PAM protein reaches the plasma membrane with the kinetics expected for this pathway (Milgram et al., 1994). A biochemical characterization of these structures and serial reconstructions of cells expressing PAM and TGN38 must be performed to distinguish these possibilities. Another integral membrane secretory granule-associated protein whose distribution in AtT-20 cells has been characterized is P-selectin. P-selectin is a cell adhesion molecule with a single transmembrane domain which 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 to secretory granules as a result of a signal in the cytoplasmic domain (Green et al., 1994; Koedam et al., 1992; Disdier et al., 1992). By immunoelectron microscopy, P-selectin was localized in mature secretory granules and little immunoreactivity was observed in the Golgi complex, suggesting that the distributions of PAM and P-selectin differ (Koedam et al., 1992). The differential localizations of PAM and P-selectin may be related to the fact that PAM can function in immature secretory granules while P-selectin normally functions at the cell surface of endothelial cells.

In light of our observations that membrane PAM could be internalized from the surface of transfected AtT-20 cells, transfected hEK-293 cells, primary pituitary cells and primary atrial myocytes, we expected that some of the membrane PAM would be localized in the endosomal system (Tausk et al., 1992; Milgram et al., 1993; Maltese and Eipper, 1993). Two types of experiments using transferrin to label early endosomes and relatively long incubations with HRP to label the entire endosomal system were used to identify membrane PAM in endosomes. AtT-20 cells contain a dense network of tubular endosomes which contain both transferrin receptor and synaptophysin (Tooze and Hollinshead, 1992). When we incubated AtT-20 cells with FITC-conjugated transferrin and then visualized the steady state distribution of membrane PAM using an antiserum specific for the cytosolic domain of PAM, we observed little overlap between the early endosomes and membrane PAM (Fig. 5D). These data indicate that membrane PAM does not accumulate in early endosomes to any significant degree. Furthermore, visualization of the entire endosomal system with HRP revealed structures similar to those described by Tooze and Hollinshead (1992); however, little of the membrane PAM was localized to this compartment (Figs 7, 8). Based on these data we do not think that membrane PAM is concentrated in the tubular, sorting endosomes at steady state. Based on the lack of overlap between soluble HRP internalized for 3 hours and the steady state distribution of membrane PAM, we also ruled out the possibility that membrane PAM resides primarily in late endosomes. However, labeling of the endosomal pathway in AtT-20 cells with a membrane-associated marker (WGA-HRP) for 1 hour and simultaneous visualization of membrane PAM demonstrated some overlap between the internalized WGA-HRP and membrane PAM (Fig. 8). These data are consistent with the fact that internalized proteins can reach the Golgi stacks and be packaged into exocytotic vesicles (Herzog and Farquhar, 1977; Green and Kelly, 1990; Back et al., 1993). Longer incubations might reveal a slightly higher degree of overlap.

It will be extremely important to extend our findings to primary tissues expressing endogenous membrane PAM. Biochemical studies using primary cultures of rat anterior pituitary demonstrated that a significant fraction of the membrane PAM (~30%) resided in a nongranule fraction distal to the site of the 20°C temperature block (Oyarce and Eipper, 1995). This compartment might be the same compartment that we observed in AtT-20 cells. The mechanisms responsible for sorting secretory granule-associated proteins from those destined for constitutive secretion remain poorly understood. The morphological characterization of the compartments involved using both soluble and membrane-associated marker proteins should facilitate our understanding of this process.

We thank Dr David Castle, University of Virginia for providing SCAMP antisera. Rat monoclonal LAMP antibody 1D4B was obtained from Dr Thomas August and the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA. We thank Dr Henry Keutmann for sequencing the recombinant TGN38 fragment, Richard Johnson for help with the expression and purification of TGN38, and Andrew Quon, Carla Berard, and Marie Bell for general laboratory assistance. We thank Kevin Trotter (UNC, CH) for maintaining cell lines and per-

forming immunofluorescence staining experiments. Supported by DK-32948 (R.E.M.), GM-15293 (S.L.M.) and NSF IBN9513671 (S.L.M.).

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(Received 30 September 1996 – Accepted 8 January 1997)