Targeting of Membrane Proteins to the Regulated Secretory Pathway in Anterior Pituitary Endocrine Cells*

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Unlike the neuroendocrine cell lines widely used to study trafficking of soluble and membrane proteins to secretory granules, the endocrine cells of the anterior pituitary are highly specialized for the production of mature secretory granules. Therefore, we investigated the trafficking of three membrane proteins in primary anterior pituitary endocrine cells. Peptidylglycine α-amidating monooxygenase (PAM), an integral membrane protein essential to the production of many bioactive peptides, is cleaved and enters the regulated secretory pathway when expressed at levels 40-fold higher than endogenous levels. Myc-TMD/CD, a membrane protein lacking the luminal, catalytic domains of PAM, is still stored in granules. Secretory granules are not the default pathway for all membrane proteins, because Tac accumulates on the surface of pituitary endocrine cells. Overexpression of PAM is accompanied by a diminution in its endoproteolytic cleavage and in its BaCl₂-stimulated release from mature granules. Because internalized PAM/PAM-antibody complexes are returned to secretory granules, the endocytic machinery of the pituitary endocrine cells is not saturated. As in corticotrope tumor cells, expression of PAM or Myc-TMD/CD alters the organization of the actin cytoskeleton. PAM-mediated alterations in the cytoskeleton may limit maturation of PAM and storage in mature granules.

Peptidylglycine α-amidating monooxygenase (PAM)† is a bifunctional enzyme involved in the posttranslational processing of many prohormones and neuropeptides. PAM catalyzes the formation of α-amidated peptides from peptide precursor molecules with a COOH-terminal glycine. In neurons and endocrine cells, biologically active peptides are stored in secretory granules that undergo regulated release in response to external stimuli. Localized in secretory granules of many neural and endocrine tissues, PAM is one of a small number of posttranslationally processing enzymes occurring naturally in soluble and membrane forms (1–4). For this reason, we have used PAM to investigate the trafficking of soluble and membrane proteins into secretory granules (5, 6).

Soluble and membrane PAM are targeted to the regulated secretory pathway in different ways (5, 6). By stably expressing wild type and mutant PAM in AT-20 corticotrope tumor cells, protein domains containing trafficking information were identified (7–9). The two catalytic domains of PAM can be expressed independently, and both are efficiently targeted to secretory granules. In contrast, membrane PAM is predominantly localized in the trans-Golgi network (TGN) at steady state. The small amount of membrane PAM on the cell surface is rapidly internalized and accumulates in the TGN region (7). The cytosolic domain of PAM contains information necessary for the trafficking of this membrane protein within the secretory pathway (7). Membrane proteins lacking the cytosolic domain are less extensively cleaved by secretory granule endoproteases, accumulate on the plasma membrane, and fail to undergo internalization. A marker protein lacking both catalytic domains of PAM, consisting only of the PAM signal sequence followed by its transmembrane/cytosolic domain, is highly localized to the TGN region of AT-20 cells (10); appending the cytosolic domain of PAM to Tac, a plasma membrane protein, rerouted Tac to the TGN (11). The cytosolic tail of PAM interacts with proteins that affect cytoskeletal organization and expression of membrane PAM alters the organization of the actin cytoskeleton, perhaps explaining the ability of membrane PAM to affect secretory granule biogenesis (12).

The endocrine cells of the anterior pituitary are highly specialized for the production of peptide hormones, their storage, and regulated release from mature secretory granules (4). Our initial studies of PAM trafficking in anterior pituitary endocrine cells identified similarities and clear differences in the trafficking of endogenous PAM in primary cells and exogenous PAM in AT-20 tumor cells. As in AT-20 cells, the endogenous PAM in pituitary cells is subjected to endoproteolytic cleavage, generating soluble PHM and PAL. In both cases, PAM processing products are subjected to regulated exocytosis. Unlike transfected AT-20 cells, immunostaining and subcellular fractionation identifies PAM mainly in pituitary secretory granules. Anterior pituitary cells rapidly internalize membrane PAM from the cell surface, but unlike AT-20 cells, massive accumulation of retrieved PAM in the TGN region is not observed in primary pituitary cells.

To elucidate the trafficking of membrane proteins in endocrine cells and understand the distinctly different steady state localization of endogenous PAM in primary pituitary cells and transfected PAM in AT-20 cell lines, we investigated the trafficking of three membrane proteins in primary anterior pituitary: membrane PAM (PAM-1); a truncated version of PAM...
with only a small luminal epitope (Myc-TMD/CD); and the interleukin-2 receptor α chain (Tac), normally a T-lymphocyte plasma membrane protein (13, 14). We used recombinant adenovirus to overexpress membrane PAM or Myc-TMD/CD in anterior pituitary cells; Tac was expressed in the same cells by transfection. Using immunofluorescent staining and subcellular fractionation, we show that the transmembrane and cytosolic domains of PAM are sufficient to target the protein to the secretory granules. In parallel, the capacity of pituitary endocrine cells to cleave and store greatly increased amounts of PAM has been assessed. Although overexpression of PAM alters the organization of the actin cytoskeleton, neither the regulated secretion or the endocytic machinery is affected in anterior pituitary cells.

MATERIALS AND METHODS

Primary Anterior Pituitary Cell Cultures—Cultures were prepared as described (4). Anterior pituitaries were dissected from the neurointermediate lobes of 5–20 adult male Harlan Sprague-Dawley rats (175–200 g; Charles River, Wilmington, MA) and dissociated with collagenase (Type I, Worthington Biochemical Corporation, Lakewood, NJ), hyaluronidase (Sigma), and benzozan (EM Science, Darmstadt, Germany), followed by trypsin (Sigma). This procedure consistently produced 1.5 × 10^6 cells/anterior pituitary. The dissociated cells were plated on protamine-coated culture wells in Dulbecco’s modified Eagle’s medium with Ham’s F-12 medium supplemented with 10% fetal clone III bovine serum (Hyclone, Logan, UT) and 10% Nu-Serum IV (Collaborative Research, Bedford, MA) for the first 24 h and then maintained in the same medium containing 10 μM cytosine arabinoside (Sigma).

Recombinant Adenovirus Constructs—Where indicated, anterior pituitary cells were infected with 2 × 10^6 plaque-forming units/ml of recombinant adenoviruses (V) 72 h after plating. Recombinant adenoviruses have been described previously (15): PAM-IV encodes full length rat PAM-1 (nucleotides 293–3245); POMC encodes mouse POMC (nucleotides 1–39); HEK293-CRES cells were then cotransfected with the shuttle vector and purified V5 adenoviral DNA to produce recombinant adenovirus. The next day, the virus-containing cell culture medium was replaced with serum-containing medium; for all experiments the cells were used 48 h after infection, but cells were viable for at least 2 weeks after infection. Expression vectors were kindly provided by Drs. B. Dor spray (Cell Biology and Metabolism Branch, National Institutes of Health), was transiently transfected in anterior pituitary cells using GenePorter (Gene Therapy Systems) following the manufacturer’s protocols. Mouse AtT-20 corticotropin tumor cells expressing PAM-1 (AtT-20 PAM-1) were grown in Dulbecco’s modified Eagle’s medium with Ham’s F-12 medium supplemented with 10% fetal serum, 10% Nu-Serum IV, and G418 as described previously (6).

Secretion Experiments and Analysis—For each secretion experiment, pituitary cell cultures were initially rinsed for three 30-min periods in complete serum-free medium/BSA: Dulbecco’s modified Eagle’s medium with Ham’s F-12 medium supplemented with 10% fetal clone III bovine serum, 10% Nu-Serum IV, and G418 as described previously (6).

PhM and PAL Assays—PHM and PAL activity were measured in cell extracts or media as described previously using α-N-acetyl-Tyr-Val-Gly and α-N-acetyl-Tyr-Val-OH-GLY substrates, respectively (17). Samples were assayed in duplicate, and reactions were carried out for 1.5 h.

PHM and PAL specific activity is expressed as picomoles of product formed per hour (units) per microgram of protein or as a percentage of the corresponding total enzyme activity in the cell extract.

ACTH Radioimmunoassay—Anterior pituitary cells were extracted in 5% acetic acid containing protease inhibitors; the soluble material was precipitated, dissolved in homogenization buffer, centrifuged to remove insoluble material, and stored frozen. Assays were performed on media and cell extracts using antibody Kathy (1:20,000) and [125I]ACTH (1–39) (PerkinElmer Life Sciences). Antiserum Kathy only recognizes POMC products in which the COOH-terminal end of ACTH (1–39) is exposed (18).

Subcellular Fractionation—Cultures prepared from anterior pituitary cells were harvested at 4 °C in 10 volumes of homogenization buffer containing 0.32 M sucrose, 10 mM Tris-HCl, pH 7.4, and protease inhibitors, passed six times through a 26-gauge needle, and then 12 times through a ball-bearing homogenizer (H & Y Enterprises, Redwood City, CA) (8). Cell debris (P1) was removed by centrifugation at 800 × g for 5 min. The resulting supernatant (S1) was separated into a P2 pellet and soluble fraction (S2) by centrifugation at 20,000 × g for 30 min (2). The P2 pellet largely enriched in secretory granules was resuspended in homogenization buffer and fractionated further on a discontinuous sucrose gradient. Resuspended P2 was layered onto a density gradient consisting of 200 μl each of 0.4, 0.6, 0.8, and 1.0% sucrose, 350 μl each of 1.2, 1.4, and 1.6% sucrose, and 200 μl of 2.0% sucrose; this gradient was designed to keep the densest secretory granules from pelleting. Gradients were centrifuged for 2 h at 120,000 × g. 150-μl fractions were collected from the top of the gradient, and proteins in an equal fraction of each sample were analyzed. Finally, a P3 pellet and a cytosolic fraction (S3) were obtained following centrifugation of S2 at 350,000 × g (2, 4).

Immunoblot Analysis—Samples were fractionated by 10 or 12% SDS-polyacrylamide gel electrophoresis and subjected to Western blot analysis using rabbit polyclonal antisera to PHM (JH1761, 1:1000), exon A (JH629, 1:1000), and PAL (JH471, 1:1000) or mouse monoclonal antibodies to the cytosolic domain of PAM (6E6, 1:50; Refs. 4, 15, and 19), Myc epitope (9E10 hybridoma, 1:50; Ref. 10), and synaptobrevin-2 (VAMP-2, 1: 5000; Synaptic Systems; Ref. 20). Proteins were visualized using the ECL kit (Amer sham Pharmacia Biotech) (21).

Immunofluorescent Staining—PAM, Myc-TMD/CD, POMC, TGN38, and VAMP-2 were detected in anterior pituitary cells using indirect immunofluorescence. Cells (200,000 cells/well of a 4-well slide) were fixed with 4% paraformaldehyde in phosphate-buffered saline (50 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) for 30 min, permeabilized with 0.075% Triton-X 100, and blocked with 2 μg/ml BSA in phosphate-buffered saline for 1 h at room temperature. Cells were incubated overnight at 4 °C with rabbit polyclonal antibodies against PAM (exon A (JH629), PAL (JH471), or TGN38 (JH481) (19), and monoclonal antibodies to Myc (10), PAM-CD (6E6), and ACTH (Novostra Labs; Ref. 15), synaptobrevin-2 (VAMP-2; Synaptic Systems; Ref. 20), γ-adaptin (AP-1; Transduction Laboratories; Ref. 22), and α-adaptin (AP-2) (Transduction Laboratories) (25). Permeabilized or nonpermeabilized anterior pituitary cells expressing rat Tac protein were stained using a monoclonal antibody 7G7 (AMAC, Westbrook, ME) directed against the laminar domain of Tac. The antigen-antibody complexes were visualized using FITC-conjugated goat anti-rabbit IgG (Caltag, San Francisco, CA) or Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). To detect filamentous actin, cells were stained with FITC-phalloidin (0.1 μg/ml; Sigma). Cells were viewed with a Zeiss Axioskop microscope (Carl Zeiss, Thornwood, NY) and photographed with a Micromax CCD camera (Princeton Instruments, Princeton, NJ) or a Spot RT camera (Diagnostic Instruments, Sterling Heights, MI).

Antibody Internalization Experiments—Primary pituitary cells were incubated in complete serum-free medium/BSA containing a 1500 dilution of rabbit polyclonal antisera to PAM exon A (JH629) for 20 min at 37 °C and either prepared immediately for immunofluorescence staining or chased in antibody-free medium for 1 or 2 h at 37 °C (4, 9). To determine whether the endocytosis of PAM were affected by the binding of bivalent PAM antibody, control experiments were performed using monoclonal Fab fragments. We generated Fab fragments by digestion of IgG preparations containing PAM exon A antibody with immobilized papain. The Fab fragments were then purified by affinity chromatography on semicarbazide-derivatized IgG. Fab fragments were detected in nondigested IgG and Fc fragments using immobilized protein A-Sepharose (Sigma). The purity of the Fab fragments was verified by SDS-polyacrylamide gel electrophoresis. Primary pituitary cells were incubated with exon A antibody or Fab fragments prepared from the same serum for 20 min at 37 °C and then harvested or chased in the absence of antibody for additional time periods, as described above.
**RESULTS**

**Adenovirus-mediated Increase in PAM Expression in Anterior Pituitary Cells**—To evaluate the level of protein expression achieved following infection of primary cells with adenovirus, PHM and PAL specific activities were compared in PAM-1V-infected anterior pituitary (AP), or stably transfected AtT-20 PAM-1 cells (Fig. 1, top panel). PHM and PAL specific activities increase 40–50-fold after PAM-1V infection, reaching levels substantially higher than in adult rat anterior pituitary or stably transfected AtT-20 PAM-1 cells (Fig. 1, top panel). Specific activities in PAM-1V-infected anterior pituitary cells are about 2-fold higher than in adult rat atrium, the richest source of PAM in essentially all of the primary pituitary cells (Fig. 1, D and E).

To assess the fraction of cells expressing virally encoded PAM, we used indirect immunofluorescence (Fig. 1, bottom panels). Virally encoded PAM-1 is expressed at varying levels in essentially all of the primary pituitary cells (Fig. 1, A and B). Consistent with the 50-fold increase in PHM and PAL specific activities, endogenous PAM is not visualized under the same conditions (Fig. 1, D and E). Because these cultures contain very few nonendocrine cells (4), almost all of the virally encoded PAM is expressed in cells proficient at producing secretory granules. Punctate staining for virally encoded PAM is observed throughout the cytosol of most of the endocrine cells (Fig. 1C). The vesicular staining pattern observed for virally encoded PAM mimics the pattern observed previously for endogenous PAM in pituitary cells (4).

**Subcellular Localization of Virally Encoded PAM in Anterior Pituitary Cell Culture**—To better evaluate the subcellular localization of PAM, PAM-1V-infected pituitary cultures were simultaneously visualized for PAM (Fig. 2A) and TGN38 (Fig. 2B), a trans-Golgi network marker (25). PAM (in red) is localized to vesicular structures distributed throughout the cell, whereas TGN38 (in green) is highly localized to a compact, reticular structure in the perinuclear region of each cell (Fig. 2B, arrows). Very little PAM is identified in the TGN region (Fig. 2C). In contrast, double immunofluorescent staining of PAM-1V-infected pituitary cells for PAM (Fig. 2D) and VAMP-2 (Fig. 2E), a secretory granule marker (20), reveals a largely overlapping distribution. As with endogenous anterior pituitary PAM (4), overlaying the PAM and VAMP-2 images (Fig. 2F, PAM in green and VAMP-2 in red) reveals a range of colors, with some structures enriched in PAM and others in VAMP-2. Despite massive adenovirus-mediated overexpression, membrane PAM is largely localized to vesicular structures with no accumulation on the cell surface or in the TGN area.

**Localization of Myc-TMD/CD in Anterior Pituitary Cells**—The routing of integral membrane PAM involves contributions from the two luminal, catalytic domains and from the COOH-terminal cytoplasmic domain (11). To evaluate the importance of the transmembrane and cytoplasmic domains of PAM in trafficking in anterior pituitary endocrine cells, we generated a recombinant adenovirus encoding Myc-TMD/CD (Fig. 3). In this chimeric protein (Fig. 3A), the PAM signal and NH2-terminal regions allow the Myc epitope tag, which precedes the catalytic regions. Specific activities in PAM-1V-infected pituitary cells are about 2-fold higher than in adult rat atrium, the richest source of PAM (4). The vesicular staining pattern observed for virally encoded PAM mimicks the pattern observed previously for endogenous anterior pituitary PAM (4). Western blot analysis of extracts of cultures infected with the Myc-TMD/CD virus identified a single 23-kDa protein that is recognized by antibodies to Myc and to the cytosolic domain of intact PAM-1, is largely localized to secretory granules (Fig. 3F).

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protein synthesis to production of secretory granules (26, 27). Therefore, we considered the possibility that secretory granule localization might represent the default trafficking pathway in these cells. To address this issue, we transfected anterior pituitary cultures with Tac, the interleukin-2 receptor a chain, a membrane protein that normally resides on the surface of T-lymphocytes (13, 14). Tac was previously shown to accumulate on the surface of AtT-20 cells (11). Immunofluorescent staining of nonpermeabilized pituitary cells, using an antibody against the luminal domain of Tac, demonstrates that a significant amount of the expressed protein is present on the cell surface (Fig. 4D). The localization of Tac is distinctly different from that of PAM-1 or Myc-TMD/CD, which are not visualized without permeabilization (11). Thus, the transmembrane/cytosolic domain of PAM is sufficient to direct the protein to secretory granules in pituitary endocrine cells. The ability of Myc-TMD/CD to direct trafficking to secretory granules is not apparent in AtT-20 cells because both Myc-TMD/CD and membrane PAM are largely localized to the TGN area of AtT-20 cells at steady state.

Processing of Overexpressed PAM-1 in Anterior Pituitary Cells—Despite the high levels of expression achieved using adenovirus, anterior pituitary endocrine cells appear to be able to accommodate the exogenous PAM in vesicular structures (4). To investigate the ability of pituitary cells to cleave the increased amounts of PAM, we used Western blot analysis (Fig. 5). Consistent with the dramatic increase in enzyme activity, analysis of the same amount of protein from infected versus uninfected cells fails to visualize endogenous PAM when the exposure times are restricted to yield a nonsaturated signal for the infected cells; therefore, longer exposures are shown for uninfected cultures. In PAM-1V-infected pituitary cells, intact PAM-1 (125 kDa) is processed into membrane PAL (70 kDa), soluble PAL (50 kDa), and soluble PHM (45 kDa) (Fig. 5A). Production of these proteins requires cleavage within exon A and between PAL and the transmembrane domain. As described previously (4), PAM-1-derived proteins of similar mass are identified in uninfected cell extracts (Fig. 5A).

To clarify the identity of the cleavage products, antisera to PHM, PAL, and CD were also used (Fig. 5B). Again, longer exposures of blots from uninfected cells are shown to facilitate comparison of the products of endogenous PAM processing (primarily PAM-2 and PAM-3, with small amounts of PAM-1) and virally expressed PAM-1 processing. The PHM antibody recognizes PAM-1 and PHM, and the PAL antibody recognizes PAM-1, mPAL, and sPAL. The CD antibody identifies PAM-1 along with mPAL and a 27-kDa protein containing the transmembrane and cytosolic domains of PAM (Fig. 5B). Despite the high level of expression, substantial amounts of virally encoded PAM-1 are cleaved into products resembling the endogenous products (Ref. 4 and Fig. 5B). The increased fraction of protein recovered as intact PAM-1 or membrane PAL in virally infected cells suggests that the cleavage capacity of the cells becomes limiting.

Basal and Stimulated Secretion of PAM Proteins from PAM-1V-infected Pituitary Cells—To evaluate the ability of pituitary endocrine cells to store increased amounts of PAM in the regulated secretory pathway, we compared the basal and stimulated secretion of PHM activity from PAM-1V-infected and uninfected cultures (Fig. 6A). PHM secretion was examined during two sequential basal collection periods before stimulation with BaCl2 or PMA (4). Data are expressed in units (Fig. 6A, left panel) and as percentages of cell content of PHM activity (Fig. 6A, right panel). Basal secretion of PHM activity increases about 10-fold when cell content of enzyme rose 40-fold in response to the PAM-1V infection. Overexpression of PAM-1 did not result in increased basal secretion of PHM activity when expressed as a percentage of cell content/h (0.55 ± 0.1% versus 1 ± 0.1% of cell content/h, infected versus uninfected, respectively; Fig. 6A, right panel). Overexpression of membrane PAM does not lead to increased basal secretion of enzyme.

Secretion was stimulated with Ba2+, which mimics the effect of Ca2+ on regulated exocytosis (28), or with PMA, which acts via protein kinase C to stimulate a cascade of protein phosphorylations and induce Ca2+-independent release (29). BaCl2 stimulates secretion from mature granules, whereas PMA stimulates Ca2+-independent exocytosis from both immature and mature pools of vesicles (30). BaCl2 is more effective at stimulating secretion of PHM activity from uninfected cells (3.5 ± 0.5-fold; 4.5 ± 0.5% of cell content/h) than from infected cells (2.5 ± 0.5-fold over basal; 1.3 ± 0.2% of cell content/h). In contrast, PMA is at least as effective on infected cells (6 ± 0.4% of cell content/h) as on uninfected cells (5 ± 0.5% of cell content/h). PMA stimulates PHM secretion from infected cells 10-fold, whereas secretion from uninfected cells is only stimulated 4-fold. The ability to stimulate secretion demonstrates the presence of a significant amount of virally encoded PAM in the...
regulated secretory pathway.

The PAM proteins secreted during basal and challenge periods were subjected to Western blot analysis using antisera to PHM (Fig. 6B). Under basal conditions, soluble, bifunctional PAM proteins of 110 and 105 kDa are secreted along with 45-kDa PHM (Fig. 6B). Addition of BaCl₂ or PMA stimulated secretion of all three PAM proteins. As expected from the enzyme assays, PMA was a more potent secretagogue than BaCl₂. Secretion of the 105- and 110-kDa PAM proteins was more responsive to PMA than to BaCl₂. This result suggests that both bi- and mono-functional PAM proteins derived from PAM-1 processing are stored in the secretory granules of PAM-1V-infected pituitary cells.

Effect of Exogenous PAM-1 Expression on Pituitary Hormone Secretion—Overexpression of PAM-1 in AtT-20 cells impairs the regulated secretion of the endogenous peptide hormone, ACTH (16). Given the extremely high levels of PAM expression achieved by infection of primary pituitary cells, we wanted to determine whether secretion of endogenous hormone were affected. Secretion of GH from somatotropes and ACTH from corticotropes was evaluated under basal and stimulated conditions (Fig. 7, A and B). Based on Western blot analysis, BaCl₂ is as effective at stimulating infected and uninfected somatotropes (Fig. 7A). PMA is a more effective GH secretagogue than BaCl₂, with no differences observed between infected and uninfected cells. PMA is more effective than BaCl₂ (10-fold versus 2-fold) at stimulating ACTH secretion, with no differences observed between infected and uninfected cells (Fig. 7B). Unlike the situation in AtT-20 corticotrope tumor cells, overexpression of PAM-1 does not impair regulated secretion from primary somatotropes or corticotropes.

Internalization of Exogenous PAM-1 in Anterior Pituitary Cells—The steady state localization of PAM reflects a balance of fluxes between different subcellular compartments. Internalization of membrane PAM from the cell surface is an important component of PAM trafficking and can be monitored by incubating live cells with antiserum to a lumenal domain of PAM. In PAM-1 transfected AtT-20 cells, PAM internalized from the surface of primary pituitary cells is distributed to vesicular structures dispersed throughout the cell (4). To determine whether overexpression of PAM in anterior pituitary cells overloads the sorting machinery and alters trafficking of membrane PAM in the endocytic pathway, we studied PAM internalization in PAM-1V-infected pituitary cells.

**Fig. 3. Myc-TMD/CD localization in anterior pituitary cells.** A, diagram showing Myc-TMD/CD relative to PAM-1. B–I, primary pituitary cells infected with the Myc-TMD/CD virus were simultaneously visualized with two antisera. B and C, Myc-TMD/CD protein was localized using antisera to PAM-CD (FITC) and to Myc (Cy3). Myc-TMD/CD (D, arrowheads) was visualized simultaneously with TGN38 (E, arrows); the superimposed images are shown in F. Myc-TMD/CD (G) was visualized simultaneously with VAMP-2 (H). The superimposed images (I) demonstrate PAM/VAMP colocalization (yellow, arrows). The scale bar for B–H is shown in B.
Pituitary cells expressing virally encoded PAM were incubated with exon A antisera for 20 min, washed and either harvested or chased for 1 or 2 h. The exon A antisera binds to PAM-1 on the cell surface, and the PAM/PAM antibody complex can then undergo endocytosis. The internalized PAM/PAM-antibody complex is visualized using a fluorescent secondary antibody. Localization of the internalized PAM/PAM-antibody complexes was then compared with the steady state distribution of various organelle markers. Substantial amounts of PAM/PAM-antibody complex are internalized from the surface of PAM-1V-infected pituitary cells (Fig. 8). The signal is much more robust than that observed from uninfected cells. The intensity of the signal obtained from the internalized PAM/PAM-antibody complex is internalized from the surface of pituitary cells was not caused by the binding of bivalent antibodies.

After the pulse incubation with the exon A antibody, PAM/PAM antibody complexes are present in small vesicles of uniform size that are distributed throughout the cytosol (Fig. 8A). These PAM/PAM-antibody complexes are often colocalized with AP-2, an early endosomal marker (Refs. 23 and 31 and Fig. 8B). After 1 h of chase, internalized PAM/PAM-antibody complexes are in a more heterogeneous collection of vesicles predominantly in the TGN area (Fig. 8E). These vesicles colocalize with γ-adaptin AP-1, part of the adaptor complex required for the assembly of clathrin-coated buds from the Golgi (Refs. 31 and 32 and Fig. 8F). Although internalized PAM/PAM-antibody complexes are observed in the TGN area, no accumulation is observed. After a 2-h chase, the PAM/PAM-antibody containing vesicular structures are coincident with secretory granules visualized by antisera to VAMP-2 (20) (Fig. 8, G and H). Overexpression of PAM in anterior pituitary cells does not appear to overload the endocytic machinery; internalization of PAM-1 is similar in infected and uninfected cells (4).

Effect of Membrane Protein Overexpression on Filamentous Actin Organization in Anterior Pituitary Cells—The cytoskeleton, along with many cytosolic proteins, governs secretory granule formation, maturation, translocation, and exocytosis (33–35). PAM-1 interacts, via its cytosolic domain, with proteins that play a powerful role in cytoskeletal organization (12, 36). In AtT-20 cells, expression of PAM-1 alters the organization of the actin cytoskeleton (12, 16). Using FITC-phalloidin, we studied the distribution of filamentous actin in pituitary endocrine cells expressing PAM-1 or Myc-TMD/CD (Fig. 9). For comparison, filamentous actin staining was evaluated in uninfected cells and in POMCV-infected pituitary cultures. Uninfected (Fig. 9, A and B) or POMCV-infected (Fig. 9, C and D) cells show organized patches of filamentous actin throughout the cytosol, with more intense signal at the margins of the cell immediately beneath the plasma membrane (Fig. 9, B and D).

DISCUSSION

The single most striking difference between the behavior of endogenous PAM in primary pituitary cells and exogenous PAM in AtT-20 cells is the steady state localization of the protein (4). In pituitary cells, as in hypothalamic neurons and cultured atrial myocytes, PAM is concentrated in secretory granules (3, 37). When integral membrane PAM proteins are expressed in the neuroendocrine AtT-20 cell line, much of the protein is found in the vicinity of the Golgi apparatus (4, 19). At the immunoelectron microscopic level, a significant amount of overlap is observed between transfected PAM and endogenous TGN38, with PAM expression enriched in the more distal compartments of the TGN (19).

To explore trafficking to secretory granules in cells equipped to assemble a large number of secretory granules, we expressed three membrane proteins in primary anterior pituitary cells. Using the adenovirus system, PAM-1 is expressed at varying levels in essentially all of the primary pituitary cells. Following infection, PAM enzyme activity, both PHM and PAL, is 40–50-fold higher in adult rat anterior pituitary or stably transfected AtT-20 PAM-1 cells. Adenovirally mediated PAM expression in pituitary is high, but only double the physiological level of PAM in adult rat atrium (24). The overexpressed membrane PAM is localized primarily to vesicular structures, with very little PAM in the TGN region and no accumulation of PAM on the cell surface. This distribution stands in sharp contrast to the TGN accumulation observed in AtT-20 PAM-1 cells (19). The largely overlapping PAM and VAMP-2 localization, with variable ratio of PAM and VAMP-2, is consistent with current
models of vesicle biogenesis and with continuous removal of membrane proteins and lumenal content via clathrin coated vesicles (38, 39).

Our earlier studies in AtT-20 cells clearly identified routing determinants in both the luminal and cytosolic domains of membrane PAM (8, 9, 16, 19). Expressed independently, both luminal domains are efficiently stored in secretory granules (4, 40). Studies in AtT-20 cells indicated that the cytosolic domain of membrane PAM contains endocytic trafficking information, but a role for cytosolic signals in granule entry was not apparent. PAM mutants truncated so that they lack most of the cytosolic domain are less extensively cleaved by secretory granule membrane endoproteases, are localized on the plasma membrane, and fail to undergo internalization (19). Like intact PAM, Myc-TMD/CD, lacking both catalytic (lumenal) domains, is highly localized to the TGN region of AtT-20 cells (10), and appending the cytosolic domain of PAM to Tac reroutes Tac to the TGN (11). In contrast, Myc-TMD/CD is enriched in the secretory granules of anterior pituitary endocrine cells. This result suggests the presence of granule targeting signals in the transmembrane and/or cytosolic domain of PAM.

AtT-20 cells contain far fewer secretory granules than pituitary endocrine cells (18, 41). In many systems, luminal proteins enter vesicular traffic derived from the TGN by default (42). Sorting of regulated secretory proteins is then accomplished passively by removal from immature granules of a subset of protein components. Therefore, we considered the possibility that secretory granules are a default pathway for membrane proteins in pituitary cells. However, Tac, which accumulates on the cell surface when expressed in a variety of endocrine and nonendocrine cells (11, 13), also localizes to the plasma membrane of anterior pituitary endocrine cells. Therefore, pituitary secretory granules do not represent a default pathway, and the granule localization of Myc-TMD/CD indicates that it contains a secretory granule routing signal.

P-selectin is a platelet and endothelial cell granule membrane protein. In AtT-20 cells, exogenous P-selectin is targeted to secretory granules by signals in its cytoplasmic tail and transmembrane domain (43–45). Recently, Cutler and co-workers (46) demonstrated that the same cytoplasmic signal is required for the appearance of P-selectin in immature and mature dense core vesicles as well as synaptic-like microvesicles in PC12 cells.

Anterior pituitary endocrine cells show an immense storage capacity for exogenous PAM. Remarkably, overexpression of PAM-1 in pituitary cells actually decreases the basal secretion of PHM activity as a percentage of cell content. Although high levels of expression do not lead to PAM accumulation on the cell surface, there is clearly some limitation to PAM proteolytic processing, with intact membrane PAM accounting for a higher percentage of the PAM protein in infected cells than in uninfected cells. Secretion of PHM is stimulated by both BaCl2 and PMA. Secretion of PHM from the BaCl2 responsive compartment is limited in pituitary cells overexpressing PAM (1.3% of cell content/h versus 4.5% from uninfected cells). Assuming that BaCl2 is stimulating Ca2+-dependent exocytosis from mature granules (28, 47), these data suggest that access to mature granules is limited upon overexpression of membrane PAM. BaCl2-stimulated release of GH and ACTH occurs normally following overexpression of PAM, indicating that mature granules form normally. Access of PAM to mature granules seems to be impaired.

PMA stimulated release of a comparable percentage of enzyme content/h from uninfected and PAM-1-infected pituitary cultures (5 ± 0.5% and 6 ± 0.4% respectively). PMA stimulates protein kinase C, inducing Ca2+-independent exocytosis via phospholipase D, which is known to activate secretory vesicle budding from the TGN (29, 48). Thus PMA is able to affect secretion at earlier steps of the secretory pathway, including immature as well as mature granules (diagramed in Fig. 10).
PAM-1 overexpression in pituitary endocrine cells results in a dramatic expansion of the PAM content of the immature and mature pools of granules (Fig. 10); basal secretion from infected cells is stimulated 10-fold by PMA, whereas basal secretion from uninfected cells is stimulated only 4-fold. The limited endoproteolytic cleavage of virally expressed PAM also suggests accumulation in immature granules. PMA stimulated GH and ACTH secretion normally following overexpression of PAM-1.

The cytosolic tail of PAM interacts with proteins involved in cytoskeletal organization and expression of membrane PAM or Myc-TMD/CD alters regulated secretion in AtT-20 cells, perhaps by affecting the actin cytoskeleton (12, 16). Similarly, virally overexpressed PAM and Myc-TMD/CD affect filamentous actin organization in anterior pituitary cells (Fig. 10). In contrast, overexpression of the soluble POMC precursor is without effect on the actin cytoskeleton, ruling out nonspecific effects of adenovirus-mediated expression. PAM-1 and Myc-TMD/CD-infected cells exhibit an absence of punctate actin aggregates, instead exhibiting diffuse staining for filamentous actin and some concentration of filamentous actin in the TGN region (Fig. 10). The accumulation of filamentous actin in the TGN area may be involved in the accumulation of membrane PAM in immature granules following overexpression.

As observed for endogenous anterior pituitary PAM and for PAM-1 transfected AtT-20 cells, robust internalization of PAM/PAM-antibody complexes from the plasma membrane occurs under basal conditions. In both cell systems, internalized PAM/PAM-antibody complexes are initially present in small uniform vesicles distributed all over the cell. Later a more heterogeneous collection of intracellular vesicular structures is observed. PAM retrieved from the surface of anterior pituitary cells passes through an early endosomal compartment recognized by antisera to the plasma membrane adaptor, AP-2 (23, 31) (Fig. 6).

**Fig. 6.** Basal and stimulated secretion of PHM activity and PAM protein from PAM-IV-infected pituitary cells. Infected and uninfected cultures were incubated in basal medium for two sequential 1-h periods and then exposed to either 1 mM BaCl2 or 1 μM PMA for 1 h. Cultures were extracted in 20 mM Na-TES, 10 mM mannitol, 1% Triton X-100, pH 7.4. A, PHM activity measured in duplicate samples of medium was expressed as units of product formed per microgram of protein (left) and as percentage of cell content of enzyme activity (right). Data are the means ± S.D. for four cultures for each secretagogue; where error estimates are small, error bars are not visible. Similar results were obtained in three additional independent experiments. B, equal amounts of basal and stimulated media from PAM-IV-infected cells were analyzed by Western blot and probed with antiserum to PHM. Ab, antibody.

**Fig. 7.** Overexpression of PAM-1 does not impair regulated secretion. The secretion experiments described in the legend to Fig. 6 were subjected to additional analysis. A, GH secretion was evaluated by Western blot analysis of medium samples. B, ACTH secretion was determined by radioimmunoassay. Similar results were obtained in three additional complete experiments. Ab, antibody.
At later times, the recycled PAM/PAM-antibody complex is more localized with AP-1 (31, 32). By 2 h, internalized PAM largely colocalizes with VAMP-2 in secretory granules (20). As for endogenous PAM, the enzyme internalized from the surface of pituitary endocrine cells was never collected in the TGN region (4). Therefore, overexpression of membrane PAM in anterior pituitary endocrine cells does not overload the endocytic sorting machinery, and membrane PAM retrieved from the pituitary cell surface has rapid access to secretory granules. In the same context, it has been demonstrated that VAMP-2-stained synaptic vesicles in neuroendocrine PC12 cells form by budding from tubular extensions of sorting endosomes; granule proteins together with the transferrin receptor are delivered to the early endosomes, where they are sorted into synaptic-like microvesicles and recycling vesicles (49). In the current study we propose targeting of recycled membrane PAM from the pituitary cell surface to the secretory granules via the endosomal compartment.

In summary, we propose a model of PAM trafficking in primary pituitary cells where the transmembrane and/or cytosolic domains of PAM is sufficient to target the protein to secretory granules (Fig. 10). PAM proteins leaving immature secretory granules largely go to mature granules, with a smaller amount of PHM and the bifunctional enzyme undergoing constitutive-like secretion (4). Little membrane PAM reaches the cell surface, and the amount that does is rapidly internalized via the endosomal compartment for recycling toward the TGN or toward basal secretion (Fig. 10A). Upon overexpression, PAM proteins accumulate in immature secretory granules (Fig. 10B). The expression of membrane PAM affects cytoskeletal organization, probably via interactor proteins that recognize the CD of PAM, resulting in a delay of secretory granule maturation and an alteration of PAM proc-
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A Endogenous PAM

B Over-expressed PAM

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