Response of an Integral Granule Membrane Protein to Changes in pH*

L. Chastine Bell-Parikh‡, Betty A. Eipper§, and Richard E. Mains¶¶
From the Department of Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

A key feature of the regulated secretory pathway in neuroendocrine cells is lumenal pH, which decreases between trans-Golgi network and mature secretory granules. Because peptidylglycine α-amidating monoxygenase (PAM) is one of the few membrane-spanning proteins concentrated in secretory granules and is a known effector of regulated secretion, we examined its sensitivity to pH. Based on antibody binding experiments, the noncatalytic linker regions between the two enzymatic domains of PAM show pH-dependent conformational changes; these changes occur in the presence or absence of a transmembrane domain. Integral membrane PAM-1 solubilized from rat anterior pituitary or from transfected AtT-20 cells aggregates reversibly at pH 5.5 while retaining enzyme activity. Over 35% of the PAM-1 in anterior pituitary extracts aggregates at pH 5.5, whereas only about 5% aggregates at pH 7.5. PAM-1 recovered from secretory granules and endosomes is highly responsive to low pH-induced aggregation, whereas PAM-1 recovered from a light, intracellular recycling compartment is not. Mutagenesis studies indicate that a transmembrane domain is necessary but not sufficient for low pH-induced aggregation and reveal a short lumenal, juxtamembrane segment that also contributes to pH-dependent aggregation. Taken together, these results demonstrate that several properties of membrane PAM serve as indicators of granule pH in neuroendocrine cells.

Maturation of bioactive peptides in the nervous and endocrine systems involves a series of post-translational processing steps that occur as precursor proteins and their products traverse the secretory pathway (1–6). As proteins move through the endoplasmic reticulum and trans-Golgi network (TGN)

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‡ Present address: Center for Experimental Therapeutics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104.

¶ Present address: Dept. of Neuroscience, University of Connecticut Health Center, 263 Farmington Ave., Farmington, CT 06030-3401.

¶¶ To whom correspondence should be addressed: Dept. of Neuroscience, University of Connecticut Health Center, 263 Farmington Ave., Farmington, CT 06030-3401. Tel.: 860-679-8894; Fax: 860-679-1060; E-mail: mains@uchc.edu.

1 The abbreviations used are: TGN, trans-Golgi network; PHM, peptidylglycine α-amidating monooxygenase; PAL, peptidyl-α-hydroxyglycine α-amidating lyase; PALm, approximately 70-kDa membrane-bound PAL; PAM, peptidylglycine α-amidating monoxygenase; Tac, interleukin-2 receptor α chain; LDCV, large dense core vesicle; TMD, transmembrane domain; CD, COOH-terminal domain of PAM, rPAM-1(900–976); Ab, antibody; mAb, monoclonal antibody; IL-2Ra, interleukin-2 receptor α; CHAPS, 3-[3-cholamidopropyl]dimethylammonio-1-propanesulfonic acid; CSFM, complete serum-free medium; PIPES, 1,4-piperazinedithanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; MES, 4-morpholineethanesulfonic acid; TPP, Tac-PAM-Tac; PPT, PAM-PAM-Tac; PTT, PAM-Tac-Tac.
zation; these effects on regulated secretion and the cytoskeleton are not observed when soluble PAM constructs are expressed (29–31). The PAM cytosolic domain interacts with and affects the localization of several cytoskeletal-associated cytosolic proteins (30–33). Expression of PAM-1 in A1T-20 cells leads to rearrangement of both actin and intermediate filament components of the cytoskeleton and results in a significant decrease in regulated secretion (29).

Because regulated secretion is affected by molecules in and on the LDCV, it stands to reason that there is some level of communication between the lumen of the LDCV and the cytosol. Luminal proteins such as carboxypeptidase E and the chromogranins display pH-dependent protein-protein interactions, conformational changes, and membrane association (17, 18, 21, 22, 34). Although some of these proteins are membrane-associated, none actually spans the vesicle membrane. Because PAM was known to exhibit pH-sensitive changes in catalytic rate, we explored the possibility that PAM, like other proteins in the regulated secretory pathway, exhibited additional pH-sensitive properties.

In this study we explored the possibility that PAM could serve in a signal transduction system linking the lumenal environment to cytosolic factors that regulate the cytoskeleton or other cellular functions. We employed subcellular fractionation, sucrose gradient sedimentation, and immunoprecipitation to explore the sensitivity of PAM to changes in pH. Our results demonstrate that PAM is highly responsive to the pH changes that characterize granule maturation and support the possibility that PAM acts as a pH sensor within LDCVs.

MATERIALS AND METHODS

Reagents—A1T-20 cells expressing PAM-1, PAM-1/899, PAM-2, PAM-3, TEP (35), and myc-TMD-CD (previously called kp-myc-CD) (36) were described previously. HEK293 cells expressing PAM-1 were described (37). Antisera to PAM were described previously: mouse monoclonal antibody specific for the COOH-terminal domain (6E6; rPAM-1/898–976); CD mAb (38); rabbit polyclonal antiserum raised against PHM (Abs 1761 and 1764; rPAM-1/37–382), PAL (Ab 471), Exon 16 (also called Exon A) (Ab 629), and the COOH-terminal domain (Ab 571; CD polyclonal Ab) (39, 40). Although Ab 475 was generated to rPAM-1/37–382, it fails to detect rPAM-1/37–382 and will be referred to as an Exon 15 (rPAM-1/389–392) antibody (41). Antibody against the COOH terminus of Tac (IL-2Rα, C-20) was from Santa Cruz Biotechnology. The expression vectors pCIS.PPT, pCIS.PPT, and pCD-M.Tac were generously provided by Dr. Sharon Milgrom (University of North Carolina, Chapel Hill, NC). Transfected cell lines were plated onto gelatinized or poly-lysine and NuSerum and kept in growth medium (Dulbecco’s modified Eagle’s medium/ Ham’s F-12 medium containing 10% fetal calf serum (HyClone, Logan, UT), 10% NuSerum (Collaborative Research, Bedford, MA), and antibiotics) containing 0.5 mg/ml G418. Triton X-100, CHAPS, Sarkosyl, and octyl-β-glucopyranoside were from Calbiochem. Complete serum-free medium (CSFM) is Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium containing insulin and transferrin. CSFM-Air has HEPES buffer substituted for the bicarbonate (42).

Generation of Stable Cell Lines—To express soluble, cytosolic CD, PBS.KrPAM-1 was used to amplify the appropriate fragment with 5′ SalI and 3′ BamHI restriction sites (43). This fragment was inserted into SuI-BamHI cut pRKS (33) to produce myc-Gly3'-Ser-Thr-PAM (891–976). The construct was verified by sequencing. For expression of myc-CD (pRk.myc-CD), PAM/Tac chimera (pCIS.PPT or pCIS.PPT), and Tac (pCDM.Tac), A1T-20 cells were co-transfected with expression vector (20 μg) and pCl.neo (5 μg) using Lipofectin (Life Technologies, Inc.). Stably transfected lines were selected and maintained in growth medium plus 0.5 mg/ml G418. For each cell line, two clones with similar expression levels were analyzed (35).

Extraction of PAM—Stably transfected A1T-20 or HEK293 cells were kept for 2 days in growth medium. The cells of one confluent well of a 2.0-ml linear 5% sucrose gradient with a 50% sucrose interface were recovered in the supernatant and used to perform most of the experiments described. Detergent-insoluble PAM (I) was recovered by suspending the pellet in 250 μl of co-immunoprecipitation buffer, pH 7.5, with protease inhibitors and 1% Triton X-100 using a motor-driven Potter-Evveljheim homogenizer with a Teflon pestle at 4 °C. Homogenate was centrifuged for 20 min at 250,000 × g at 4 °C, and the supernatant (100 μl) gradient was used for sucrose gradient sedimentation studies. Detergent-soluble PAM (II) was recovered in 500 μl of co-immunoprecipitation buffer adjusted to pH 5.5 or to 7.5 and containing 10 μl of the specified polyclonal antibody. Antibody binding proceeded for 2 h at 4 °C. Protein A-agarose beads (Sigma) were preblocked with 2.0 mg/ml bovine serum albumin in phosphate-buffered saline and then equilibrated with pH 5.5 or 7.5 co-immunoprecipitation buffer. Following antibody binding, the samples were centrifuged for 20 min at 5000 rpm at 4 °C in a tabletop centrifuge, and the supernatant (200 μl for each pH) was incubated with pretreated Protein A beads (60 μl of a 33% slurry) for 1 h at 4 °C. The beads were pelleted and washed twice with co-immunoprecipitation buffer of the same pH. Proteins eluted by boiling for 5 min in Laemmli sample buffer (1% SDS/wt., 8% urea, 5% 2-mercaptoethanol (v/v), 50 mg Tris-HCl, pH 6.8) were fractionated by SDS-PAGE. Western blots were visualized using PAM CD mA b 66E at the PHM Ab. Antibody Binding to PVDV—Multiple aliquots of an A1T-20 PAM-1 cell extract prepared as described above were fractionated by SDS-PAGE. Following transfer, the PVDF membrane was cut into strips of two lanes each for antibody binding so that each antibody and pH was tested in duplicate. All membranes were initially blocked for 45 min with 5% milk in 50 ml Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20 for 2 h at 4 °C. Membrane strips were incubated for 2 h with Exon 16 Ab, Exon 15 Ab, or PAM CD mA b diluted into either TTBS, pH 7.5 or 50 ml Na-MES, 0.1% Tween 20, pH 5.5. Subsequent rinses and secondary antibody binding used TTBS, pH 7.5. Protein was visualized and quantified as described above.

Antibody Binding to A1T-20 Cells—A1T-20 cells expressing PAM-1/ 899 were plated onto poly-lysine treated 4-well glass slides and grown for 2 days in growth medium beneath. Molecular mass markers (50, 20, 10, 7, 5, 2, and 1 kDa) in co-immunoprecipitation buffer adjusted to pH 5.5 or pH 7.5 (42, 45) were used for internal or external standards. Samples (up to 200 μl) loaded on top of the sucrose gradients were centrifuged for 5 h at 4 °C, 50,000 rpm (214,000 × g) in a Ti-55 swinging bucket rotor in a TL100 centrifuge (Beckman). Fractions (169 μl) from each gradient were collected from the top down. Particulate matter that was not resuspended in the bottom of each tube was not measured. The following were the buffer conditions: PBS sample buffer (169 μl) and heating for 10 min at 37 °C or by adding immunoprecipitation buffer (169 μl) pH 7.5, with detergent (as specified). This final fraction was collected and labeled "Particulate." Gradient fractions were analyzed on 10% polyacrylamide, 0.25% N,N'-methylene-bisacrylamide/SDS gels. Following transfer to PVDVF membranes (Millipore Corp.), PAM proteins were visualized with Exon A antibody.
Alternative splicing generates isoforms of PAM lacking the transmembrane domain and/or Exon 16 (Fig. 2A). To delineate the contribution of these domains to the observed pH sensitivity, natural isoforms lacking these domains were exposed to antibody at pH 5.5 and at pH 7.5 (Fig. 2B). The ability of the Exon 15 antibody to recognize native PAM-1 at pH 5.5 reflects a pH-dependent change in PAM-1 rather than a pH sensitivity of the antibody. Similarly, the Exon 15 antibody detected PAM-1 equally well at pH 7.5 and at pH 5.5 on Western blots (not shown).

Membrane PAM Exhibits pH-dependent Conformational Changes—Immunoprecipitation of membrane PAM requires its solubilization. We carried out additional antibody binding experiments with live cells to determine whether PAM embedded in the lipid bilayer still exhibited pH-sensitive epitope masking. AtT-20 cells expressing PAM-1/899, a membrane protein with a truncated cytosolic domain but possessing the transmembrane domain and/or Exon 16 (which lacks Exon 15 and Exon 16) was greatly reduced at pH 5.5. In contrast, the ability of the CD polyclonal antibody to immunoprecipitate PAM-3 was not pH-dependent (Fig. 2B). From these results, we conclude that sequences contained within Exons 15 and 16 exhibit pH-dependent changes in conformation. Additionally, based on the response of PAM-3, it appears that the transmembrane domain is not required for this pH-responsive change in antibody reactivity.
tified using immunofluorescent staining with a fluorescein isothiocyanate-conjugated secondary antibody (Fig. 3). As expected, cells visualized with PHM antibody showed binding of antibody that was independent of pH (Fig. 3A). The Exon 15 antibody showed robust surface staining when the incubation was carried out at pH 7.3 but failed to produce any signal above background when the incubation was carried out at pH 5.7 (Fig. 3B). These data support the hypothesis that membrane PAM undergoes physiologically relevant pH-dependent changes in conformation.

**Detergent Extraction of PAM-1 Is pH-dependent**—During the preparation of extracts for the antibody binding experiments, it became apparent that pH had a remarkable effect on the ability of detergent to extract PAM-1 from cell membranes. When PAM-1 AtT-20 cells were incubated in buffer containing 1% Triton X-100 at pH 7.5, >80% of the 120-kDa PAM-1 and 70-kDa PALm appeared in the soluble fraction (Fig. 4A). At pH 5.5, less than 45% of the total PAM-1 was solubilized by Triton X-100; PAM-1 was not degraded at pH 5.5, it was recovered in the insoluble fraction. Soluble 45-kDa PHM was recovered in the soluble fraction at both pH 7.5 and pH 5.5.

To further address the possibility that pH might affect protein stability, other detergents were utilized to test extraction of PAM at low pH (Fig. 4B). Octyl-β-glucopyranoside (1%) and CHAPS (1%) showed properties very similar to those of Triton X-100, with most of the PAM-1 remaining in the insoluble fraction at pH 5.5. Both sarkosyl (1%) and SDS (1%) were effective at solubilizing essentially all of the PAM-1 from AtT-20 membranes at pH 5.5. The sarkosyl- and SDS-solubilized samples were analyzed for retention of PHM enzymatic activity (Fig. 4C). As expected with a strong denaturant, the SDS-solubilized PAM lost nearly all enzymatic activity. The sarkosyl-solubilized preparation was catalytically active, although its specific activity was lower than that of Triton X-100-solubilized PAM, demonstrating that the structural integrity of PAM is not drastically compromised by lowering the pH to 5.5.

**PAM-1 Undergoes pH-dependent Aggregation**—We next sought a method that would let us determine whether these pH-dependent changes in the conformation of PAM might affect its ability to interact with itself or with other proteins. Detergent extracts of PAM-1 AtT-20 cells were fractionated on linear sucrose gradients at pH 5.5 or pH 7.5 (Fig. 5). The gradients were centrifuged for 5 h at 214,000 × g, and fractions were collected from the top down. Any material that accumulated as a pellet at the bottom of the gradient was recovered using SDS sample buffer. Based on fractionation of marker proteins of known Svedberg (S) value and native molecular mass (45), the full-length PAM-1 monomer is expected to localize to gradient fractions 5–7 (Fig. 5).

Because of the pH dependence of PAM-1 extraction, equal volumes of pH 7.5 cell extracts were used as inputs for both the pH 5.5 and 7.5 sucrose gradients. At pH 7.5 (Fig. 5A), PAM-1 (120 kDa) and its proteolytic cleavage products, PALm (70 kDa) and PHM (45 kDa), fractionated as predicted by their monomeric molecular masses, e.g. the peak of PAM-1 occurred in fractions 5–7. PAM-1 and PALm were more broadly distributed...
than PHM, but only ~6% of the PAM-1 reached the bottom of the gradient at pH 7.5. By contrast, at pH 5.5, 17% of the PAM-1 sedimented to the bottom of the gradient (Fig. 5 B). The behavior of PALm was less sensitive to pH, with 4% of the PALm recovered in the pellet fraction at pH 5.5 and 2% recovered at pH 7.5. The behavior of soluble PHM, which localized to gradient fractions 2–4, was identical at pH 5.5 and pH 7.5.

We were concerned that the pH-dependent aggregation of PAM-1 and PALm might reflect an irreversible, nonspecific process. Control experiments included preparation of cell extracts at pH 5.5 followed by analysis on separate sucrose gradients at pH 5.5 and 7.5; the same pH-dependent aggregation of PAM-1 and PALm was apparent (data not shown). Additionally, when the particulate fraction from a pH 5.5 sucrose gradient was solubilized at pH 7.5 and tested for PHM activity, it was clear that PAM-1 recovered from the pellet retained full activity. In a separate set of control experiments, 0.2% Triton X-100 was included in the sucrose gradients; the presence of detergent throughout the gradient did not diminish the aggregation of PAM-1 at pH 5.5 (data not shown). To further address concerns about specificity, we visualized total gradient protein. A small subset of the proteins applied to a pH 7.5 gradient was recovered from the pellet; at pH 5.5, the pellet still contained only a small fraction of the total protein, but additional proteins were apparent.

**Endogenous Pituitary PAM Undergoes pH-dependent Aggregation**—Although AtT-20 cells have served as an excellent model for corticotrope function and allowed examination of site-directed mutant forms of PAM, the endocrine cells of the anterior pituitary are more efficient at assembling large dense core vesicles. If the pH-dependent aggregation of PAM plays a role in granule biogenesis, it should also occur in pituitary extracts. To test this hypothesis, detergent extracts of rat pituitary were subjected to sucrose gradient fractionation at pH 7.5 (Fig. 6A) and at pH 5.5 (Fig. 6B). The endogenous PAM-1 in pituitary extracts exhibited an even more robust pH-dependent
aggregation than did exogenous PAM-1 expressed in AtT-20 cells. Nearly 40% of the total pituitary PAM-1 pelleted through the gradient at pH 5.5, whereas less than 5% of the endogenous PAM-1 pelleted at pH 7.5. Thus the pH-dependent aggregation of PAM-1 is not simply the result of overexpression of PAM-1 or expression in tumor cells. The striking increase in pH-dependent aggregation of membrane PAM in anterior pituitary extracts, compared with AtT-20 cells, suggests that this aggregation is not purely a biophysical property of membrane PAM.

We wanted to know whether the pH-dependent aggregation of PAM was limited to neuroendocrine cells. To address this question, HEK 293 cells expressing PAM-1 were utilized. When analyzed in the same manner, PAM-1 expressed in HEK 293 cells displayed pH-dependent sedimentation (data not shown). Although HEK 293 cells do not produce large dense core vesicles, their ability to release soluble PAM from membrane PAM is responsive to phorbol ester stimulation (49, 50).

**PAM in Different Subcellular Fractions Is Differentially Sensitive to pH**—To investigate the role of pH-dependent aggregation in the trafficking of PAM in more detail, we compared the ability of PAM localized to different subcellular organelles to undergo this process. From our previous studies we knew that anterior pituitary PAM was largely recovered in three subcellular fractions (46). Membrane PAM recovered from the membrane-enriched pellet (P2) resides in a light vesicle compartment involved in the post-TGN intracellular recycling of PAM; these gradient fractions were pooled (Fig. 7, light vesicles). Much of the PAM recovered from the secretory granule-enriched pellet (P3) co-localized with secretory granules following gradient fractionation. PAM recovered from the high speed pellet (P4) co-localized with endocytic markers following gradient centrifugation, as expected.

PAM-1 from the secretory granule-enriched P3 fraction was highly susceptible to pH-dependent aggregation (Fig. 7); fully 30% of the secretory granule PAM-1 aggregated at pH 5.5, whereas only 8% aggregated at pH 7.5. PAM-1 in the endosome-enriched P4 fraction also exhibited robust pH-dependent aggregation; at pH 5.5, 39% of the PAM-1 in this fraction aggregated, whereas 18% aggregated at pH 7.5. In contrast, PAM-1 from the P2-derived light vesicle recycling compartment exhibited negligible pH-dependent aggregation (20% at pH 5.5 versus 18% at pH 7.5). Although not pH-dependent, a significant amount of the PAM-1 in the endosomal compartment was aggregated. Importantly, these results demonstrate that the tendency of PAM-1 to aggregate at low pH depends on its original subcellular localization before homogenization.

**The Region of PAM Essential for pH-dependent Aggregation**—To determine whether specific regions of PAM were essential for pH-dependent aggregation, isoforms and truncated variants of PAM were evaluated (Fig. 8). We first investigated the role of the COOH-terminal domain of PAM, which is known to interact directly with at least three cytosolic proteins (32). To do so, we fractionated extracts of AtT-20 cells expressing PAM-1/899, which lacks most of the COOH-terminal domain (Fig. 8A). Robust pH-dependent aggregation was observed, with 18% of the PAM-1/899 pelleted at pH 5.5 and only <5% pelleted at pH 7.5.

We next evaluated the role of the luminal, catalytic domains of PAM. A myc-tagged construct with a leader sequence but lacking both catalytic domains of PAM (myc-TMD-CD) (Fig. 8B) also underwent pH-dependent aggregation; 26% of the myc-TMD-CD pelleted at pH 5.5, whereas only ~5% pelleted at pH 7.5. PAM-1/899 and myc-TMD-CD share only the transmembrane domain and 8–10 juxtamembrane residues to both sides of the transmembrane domain.

The fact that these two proteins aggregate to a similar extent suggests that the pH-responsive region(s) of PAM may be contained within the common sequence. To test this prediction, two PAM proteins lacking the transmembrane domain were examined; PAM-3 is a natural isoform (Fig. 5C), and myc-CD was constructed by deleting the leader sequence and transmembrane domain from myc-TMD-CD (Fig. 8D). Neither PAM-3 nor myc-TMD-CD exhibited any significant aggregation at pH 5.5 or at pH 7.5 (pH 7.5 not shown).

**Analysis of PAM Co-immunoprecipitates**—Proteins recovered from the particulate fraction of these sucrose gradients must have passed through a 50% sucrose pad. Their density suggests the involvement of protein-protein interactions; in contrast, proteins that reside in lipid rafts float up out of dense sucrose solutions to equilibrate at 10–15% sucrose (51, 52). We used a co-immunoprecipitation paradigm to explore the possibility that membrane PAM proteins might interact with each other in a pH-dependent manner. AtT-20 cells stably expressing PAM-2 (Fig. 9A) or PAM-1/899 (Fig. 9B) were infected with an adenovirus encoding PAM-1. AtT-20 cells expressing only PAM-2, PAM-1/899, or virally encoded PAM-1 were analyzed as controls. We then asked whether selective immunoprecipitation of PAM-1 at low pH resulted in co-precipitation of PAM-2 or PAM-1/899. Although binding of the Exxon 16 antibody to PAM-1 is pH-sensitive, the addition of an excess of this polyclonal antiserum allowed precipitation of PAM-1 at pH 5.5; PAM-2 is not recognized by this antiserum. When extracts of
cells co-expressing PAM-1 and PAM-2 were analyzed, PAM-1 appeared in the pellet, but PAM-2 did not (Fig. 9A). Antibody to the cytosolic domain of PAM was used to immunoprecipitate PAM-1 from cells co-expressing PAM-1/899 (Fig. 9B); this antiserum does not recognize PAM-1/899. PAM-1/899 was not co-precipitated with PAM-1 (Fig. 9B). Neither the lumenal nor the cytosolic domain of membrane PAM supports a pH-dependent interaction among membrane PAM proteins. Because similar proteins (e.g. insulin and proinsulin) can co-crystallize (24), our data suggest that the presence of integral membrane PAM in low pH aggregates involves the interaction of PAM with other proteins.

Transfer of the Ability to Aggregate: Analysis of Tac/PAM Chimeras—To further delineate the features essential for pH-dependent aggregation, we utilized Tac (IL-2Rα), a transmembrane protein that resides on the plasma membrane when expressed in most cells, including AtT-20 cells (35). When extracts of AtT-20 cells expressing full-length Tac were resolved on sucrose gradients, less than 5% of the Tac exhibited pH-dependent aggregation (Fig. 10). Thus pH-dependent aggregation is not an intrinsic property of membrane proteins. Because PAM and Tac are topologically similar, PAM/Tac chimeras were constructed so that the contributions of the lumenal, transmembrane and cytosolic domains to aggregation could be assessed.

In the PPT chimera, the nine cytosolic juxtamembrane residues present in PAM-1/899 were replaced by the cytosolic domain of Tac. Like PAM-1/899, PPT exhibited pH-dependent aggregation. In the PTT chimera, the PAM transmembrane domain and six preceding lumenal juxtamembrane residues...
were also replaced by the corresponding Tac domains. PTT exhibited pH-dependent aggregation. Although a transmembrane domain is required for aggregation, the PAM transmembrane domain can be replaced by the Tac transmembrane domain. The PAM TMD is not unique in its ability to facilitate aggregation. The TPP chimera includes lumenal PAM residues 861–866 along with the PAM transmembrane and cytosolic domains. Unlike PAM, the TPP chimera did not aggregate at low pH (Fig. 10). The myc-TMD/CD protein, which includes only three additional PAM lumenal residues (Lys858-Leu859-Ser860), exhibited robust pH-dependent aggregation (Fig. 10B). The only lumenal membrane-proximal sequence common to the proteins exhibiting pH-dependent aggregation is Lys858-Leu859-Ser860. The pH-dependent aggregation of these chimeras requires a TMD, is affected by the lumenal sequence, and is independent of the cytosolic domain.

**DISCUSSION**

The production of bioactive peptides in neuroendocrine and endocrine cells involves a complex series of steps that occur in a characteristic order as proteins traverse multiple subcellular compartments (1–6). One of the key features distinguishing early and late compartments of the secretory pathway is luminal pH. As pituitary proteins move from the TGN into immature and mature secretory granules, luminal pH becomes progressively more acidic. In the anterior pituitary, the lumen of the LDCV is approximately pH 5.0–5.5. The acidic pH of the mature granule is thought to limit additional proteolytic processing, by removing peptides from free solution and possibly decreasing prohormone convertase activity, allowing vesicles to be stored for long periods of time and released only upon appropriate stimulation (8, 11, 12, 53).

PAM catalyzes one of the final steps in peptide biosynthesis and is present in mature LDCVs. It is one of the few peptide biosynthetic enzymes that spans the LDCV lipid bilayer. The COOH-terminal domain of membrane PAM is exposed to cytosolic pH, whereas the luminal domains are exposed to the increasingly acidic environment in the lumenal compartment. Overexpression of PAM-1 alters cytoskeletal organization in anterior pituitary endocrine cells and in corticotrope tumor cells (29–31, 54). Mutagenesis studies indicate that the COOH-terminal cytosolic domain is essential to the ability of PAM to affect cytoskeletal organization and regulated secretion (31). Because PAM functions in both the luminal and cytosolic compartments, we hypothesized a role for PAM in signaling from the secretory granule lumen to the cytosol. Because mature vesicles are distinguished by their acidic pH, we searched for effects of pH on PAM.

**PAM Is Sensitive to pH**—Based on several criteria, PAM exhibits different properties at neutral pH and at pH 5.5, the pH measured in the lumen of mature LDCVs (8, 10). Importantly, PAM exhibits optimal catalytic activity at pH 5 to 5.5 (2, 43). At pH 5.5, Triton X-100, CHAPS, and octyl-β-glucopyranoside fail to solubilize the majority of the PAM-1 from AtT-20 cells; in contrast, most of the PALm and all of the 45-kDa PHM are solubilized under the same conditions. All three detergents solubilize PAM-1 very effectively at pH 7.5. It is not clear whether pH-dependent interactions of PAM with cytosolic proteins or luminal proteins are responsible for its differential solubility.

The linker region (Exons 15 and 16) separating PHM and PAL is subject to epitope masking at pH 5.5. Antibodies to exon 15 and exon 16 bound denatured PAM-1 at pH 5.5 but failed to immunoprecipitate native PAM at pH 5.5. At pH 7.5 both antibodies effectively immunoprecipitated PAM-1. Epitope masking of this type suggests the occurrence of a pH-dependent conformational change in the luminal domain of PAM. Because PAM-1/899 accumulates on the surface of AtT-20 cells, we could demonstrate that pH-dependent epitope masking occurs when live cells are exposed to medium titrated to pH 5.5. This surface configuration of PAM-1/899 conveniently mimics the vesicle lumen-to-cytosol signaling configuration and demonstrates that pH-dependent epitope masking occurs while PAM is an integral membrane protein.

Based on sedimentation through 5–20% linear sucrose gradients, PAM-1 undergoes reversible, pH-dependent aggregation as the pH is lowered from 7.5 to 5.5. Because pH-dependent aggregation of PAM occurs in anterior pituitary as well as in transfected AtT-20 cells, it is not an artifact of PAM-1 overexpression or expression in tumor cells. The domains of PAM-1 involved in pH-dependent epitope masking are not required for pH-dependent aggregation. Because integral membrane forms of PAM do not co-immunoprecipitate and membrane PAM in anterior pituitary aggregates more effectively than membrane PAM in AtT-20 extracts, the involvement of additional proteins in the aggregation process is strongly suggested.

**Features Affecting the pH-dependent Aggregation of PAM**—The ability of anterior pituitary PAM-1 to aggregate in response to low pH varies dramatically with its subcellular localization. PAM-1 solubilized from a post-TGN compartment that contains recycling and constitutive-like secretory vesicles does not show increased aggregation in response to low pH. In contrast, aggregation of PAM-1 solubilized from secretory granules or endosomes was greatly increased at pH 5.5. These observations do not allow us to distinguish between two extreme possibilities. First, the PAM protein itself may carry a modification that facilitates aggregation. Alternatively, other proteins present in the different subcellular fractions may be responsible for the differences observed.

Support for the first possibility derives from the fact that the
The cytosolic domain of PAM is phosphorylated at multiple sites (50). Mutagenesis studies suggest that phosphorylation of Ser949 facilitates the entry/retention of PAM in LDCVs.2 P-CIP2, identified as a protein that interacts with the COOH-terminal domain of PAM, is a protein kinase that phosphorylates Ser949 in the cytosolic domain of PAM (55). Mutagenesis studies suggest that cytosolic proteins with the binding specificity of P-CIP2 mediate the effects of PAM on regulated secretion (31). A reasonable extension of the latter possibility is that, at low pH, PAM is recruited to interact with a lumenal binding protein that facilitates protein aggregation. For example, chromogranins A and B interact with the secretory granule membrane, specifically, with the inositol 1,4,5-trisphosphate receptor, at pH 5.5 and are released from the membrane at pH 7.5 (17).

In neuroendocrine cells, maturation of secretory granules involves the removal of membrane proteins via clathrin-coated vesicles containing the adapter protein AP-1 (56, 57). When analyzing anterior pituitary homogenates, we found clathrin, as well as γ-adaptin (104 kDa) and α-adaptin (112 kDa), in the particulate fraction following centrifugation at pH 5.5 but not at pH 7.5 (data not shown). The mass of the clathrin triskelion alone (>650 kDa) predicts migration to the bottom of the sucrose gradient. Localization of PAM to the bottom of the sucrose gradients may be due in part to interactions between PAM and adaptor-clathrin complexes.

**Features of PAM-1 Essential to pH-dependent Aggregation**—

Even when expressed in the same cell, PAM-1 was unable to co-immunoprecipitate PAM-2 or PAM-1/899 at low pH. Nevertheless, both PAM-2 and PAM-1/899 exhibit pH-dependent aggregation. The failure of PAM-2 and PAM-1/899 to co-immunoprecipitate with PAM-1 suggests that aggregation is selective and involves the interaction of PAM with other proteins.

Neither the catalytic domains nor the COOH-terminal domain of PAM was essential for pH-dependent aggregation. PAM-1/899 and myc-TMD-CD both exhibited pH-dependent aggregation, and the two proteins have in common only residues Lys858 through Gly859 of PAM; the transmembrane domain of PAM extends from Val867 to Ile890. Consistent with an essential role for the transmembrane region, PAM-3, which lacks residues 833–917, does not aggregate at low pH. However, replacement of the PAM transmembrane domain with the Tac transmembrane domain (PTT chimera) also supported pH-dependent aggregation. In the PTT chimera, the luminal domain of PAM terminates at Ser860 and is followed immediately by the Tac transmembrane domain. Unlike myc-TMD-CD, the TPT chimera did not undergo pH-dependent aggregation. Although the Lys858-Leu859-Ser860 sequence that is present in myc-TMD-CD and missing from the TPT chimera may be critical, it is possible that the presence of the Tac luminal domain prevents aggregation. The KLS sequence must be broadened to KL(SIV) to include known mammalian PAMs; data base searching in which KL(SIV) is forced to remain a fixed number of residues from a hydrophobic stretch (TMD), reveals only PAM when search criteria are very stringent and includes many integral membrane proteins as search criteria are relaxed.

Membrane association plays a critical role in the aggregation of other secretory proteins. For example, carboxypeptidase E is present within secretory granules in both a soluble form and a form capable of membrane association. Association of the latter form with the granule membrane occurs only at low pH; the soluble form is unaffected by pH (21). Similarly, full-length prohormone convertase 1 associates with membranes through an amphipathic helix near its COOH terminus (1, 58) and is recovered from the bottom of our sucrose gradients only at pH 5.5 (not shown). Mature, 65-kDa prohormone convertase 1, which lacks this amphipathic helix, does not undergo pH-dependent aggregation. Coomassie staining of the proteins recovered from the pellet fractions of the pH 7.5 and pH 5.5 gradients identifies a subset of proteins whose appearance in the pellet is pH-dependent (data not shown).

It has been hypothesized that pH-dependent aggregation facilitates the sorting of secretory proteins to the regulated pathway of neuroendocrine cells (59). In the case of PAM, we propose that aggregation serves a different function. PAM catalyzes one of the final steps in the biosynthesis of peptides, making it an ideal candidate for signaling completion of the process. The ability of PAM to interact with cytosolic proteins like P-CIP2 and Kalirin could then be modulated by lumenal pH. Others have proposed the presence of pH sensors in the regulated secretory pathway and in the endocytic pathway (60, 61), and a few proteins that serve as pH sensors throughout the cell have been identified (62, 63). Further experiments are required to achieve a better understanding of this phenomenon and its significance in the context of neuroendocrine cells.

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