Identification of Routing Determinants in the Cytosolic Domain of a Secretory Granule-associated Integral Membrane Protein*

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We have investigated the trafficking of integral membrane peptidylglycine α-amidating monooxygenase (PAM) in the neuroendocrine AtT-20 cell line. This bifunctional enzyme has two domains which together catalyze the COOH-terminal α-amidation of peptidylglycine substrates yielding amidated products stored in secretory granules. As soluble proteins, both catalytic domains were independently targeted to secretory granules. In contrast, membrane PAM was largely localized to the trans-Golgi network (TGN). Upon truncation of its cytoplasmic COOH-terminal domain, membrane PAM was less efficiently cleaved by secretory granule enzymes and accumulated on the plasma membrane. When transferred to the luminal domain of the interleukin 2 receptor α-chain (Tac protein), the cytoplasmic domain of PAM caused rerouting of Tac from the surface to the TGN and supported internalization of Tac antibody from the plasma membrane. To define sequences in the cytoplasmic domain of integral membrane PAM involved in its trafficking, we expressed PAM proteins containing truncations, deletions, or point mutations in the COOH-terminal cytoplasmic domain. PAM proteins were not retained in the TGN when half of the cytoplasmic domain was deleted; such proteins accumulated on the plasma membrane, were not efficiently internalized, and were cleaved to generate a bifunctional PAM protein that was not stored in secretory granules. A tyrosine-based internalization motif was identified, which was not required for efficient cleavage of full-length integral membrane PAM by secretory granule enzymes. Deletion of an 18-amino acid domain surrounding this Tyr residue both diminished cleavage of membrane PAM by secretory granule enzymes and eliminated internalization of PAM from the plasma membrane. The cytoplasmic domain is responsible for retaining membrane PAM in the TGN and for retrieving membrane PAM from the cell surface, while the luminal catalytic domains of PAM appear to be responsible for targeting the protein to secretory granules.

In neurons and endocrine cells, biologically active peptides are stored in secretory granules which undergo regulated release in response to external stimuli. Enroute through the secretory pathway, inactive propeptides are activated through the sequential action of specialized, neuroendocrine-specific peptide processing enzymes; these modifications include endoproteolysis, exoproteolysis, and for over half of all known peptides, COOH-terminal α-amidation (1–4). The peptide processing enzymes must be targeted together with their prohormone substrates to the regulated secretory pathway (5–8). Some enzymes are soluble proteins (PC1/3, PC2, and carboxypeptidase H), while others are integral membrane proteins (cytochrome b°60) (2, 9–11). Peptidylglycine α-amidating monooxygenase (PAM) occurs in both integral membrane and soluble forms (4).

While many studies have focused on the trafficking of soluble proteins within the regulated secretory pathway, little is known regarding signals that mediate the trafficking of integral membrane proteins (12–16). Since PAM functions late in the secretory pathway, we have used this protein as a model for studying the trafficking of soluble and integral membrane secretory granule-associated proteins. PAM is a bifunctional enzyme with monooxygenase (PHM) and lyase (PAL) domains; these two enzymes act in sequence to catalyze the production of α-amidated peptides (4). Amidated peptides are first detected in the TGN, indicating that PAM is functional in this subcellular compartment (17).

When PAM-1 is expressed in AtT-20 cells, endoproteolytic processing generates soluble PHM proteins; much of the PAL remains membrane-bound, although some soluble PAL is also generated (18). Soluble PAM proteins are stored in secretory granules and undergo regulated release, while membrane PAM accumulates in the TGN region of endocrine and nonneuroendocrine cells. Newly synthesized membrane PAM was retained within the cell, with little newly synthesized protein reaching the plasma membrane. When the storage of soluble and integral membrane PAM proteins was compared in metabolic labeling experiments, integral membrane PAM proteins were less efficiently stored in granules (18–20). Instead, membrane PAM proteins were enriched in distal elements of the TGN, partially overlapping the more proximal tubulovesicular structures that contain TGN-38 (21).

The expression of integral membrane PAM proteins truncated 9 amino acids following the transmembrane domain resulted in an accumulation of PAM on the surface of AtT-20 cells; a smaller percentage of the truncated protein yielded monofunctional PHM proteins stored in secretory granules (19). While full-length integral membrane PAM proteins underwent efficient internalization from the cell surface, the truncation mutant did not (19). These data suggest that some of the signals mediating the trafficking of integral membrane PAM reside in the COOH-terminal cytoplasmic domain. The fact that soluble PHM and PAL proteins are independently targeted to secretory granules argues that the luminal domain also plays a role in the trafficking of membrane PAM, however,

The abbreviations used are: PAM, peptidylglycine α-amidating monooxygenase; TGN, trans-Golgi network; PHM, monooxygenase domain; PAL, lyase domain; PCR, polymerase chain reaction; Ab, antibody; FITC, fluorescein isothiocyanate; PMA, phorbol 12-myristate 13-acetate; CLV, constitutive-like vesicle.

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the luminal domains acting alone are unable to target membrane PAM proteins efficiently to the regulated secretory pathway.

In this paper we demonstrate that the cytoplasmic domain of PAM contains information sufficient to redirect the cell surface-associated protein Tac to the TGN. Additionally, we have used site-directed mutagenesis to identify an internalization signal in the PAM cytoplasmic domain. A comparison of the trafficking of the internalization mutant and other cytoplasmic domain mutants argues that additional signals governing routing in the TGN and immature granules are contained within the PAM cytoplasmic domain. Together with the luminal domains, these cytosolic signals play an important role in the trafficking of membrane PAM within the regulated secretory pathway.

MATERIALS AND METHODS

Construction of Expression Vectors—The construction of pBS.PAM-1 was described (18). To generate cDNAs encoding truncated PAM proteins, PCR was performed on the pBS.PAM-1 template using a unique primer in the lyase domain and a mutagenic antisense primer consisting of a stop codon and the appropriate gene-specific sequence. To generate point and deletion mutants, the double PCR method was used with mutagenic sense and antisense primers paired with the T7 primer and a sense PAM-specific primer, respectively (22). PCR products were inserted into pBS.PAM-1 using a unique BglII site. All inserts were ligated into pCIS.2CXXNH (18).

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Tissue Culture and Transfections—AtT-20 cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 medium containing 10% fetal bovine serum (HyClone, Logan, UT) and 10% Nu-Serum (Collaborative Research, Bedford, MA); transfected cell lines were grown in the presence of 0.5 mg/ml G418. Stable AtT-20 cell lines expressing various PAM proteins were established by co-transfecting the pCIS expression vector and pMT.neo or pMC.neo (Stratagene, La Jolla, CA) using Lipofectamine (Life Technologies, Inc.). G418-resistant cell lines were selected, expanded, and screened for expression of PAM protein by immunofluorescent staining using rabbit polyclonal antibodies to PHM or PAL (19). Cell lines expressing Tac and TaccPAM were screened by immunofluorescent staining using antibody 7G7 directed against the lumenal domain of Tac (19). The localization of the transfected proteins were compared using electron microscopy (Carl Zeiss Inc., Thornwood, NY) using FITC filters and cells were photographed using a Kodak T-Max 400 film (Eastman Kodak Co.). Rabbit polyclonal antisera directed against the lumenal domain of Tac was used at a final dilution of 1:1000, and spent medium from a mouse hybridoma line secreting monoclonal antibody to recombinant PAM COOH-terminal domain (6E6) was diluted 1:50.

RESULTS

PAM Cytoplasmic Domain Redirects Tac from the Cell Surface to an Intracellular Compartment—In previous experiments we demonstrated that truncation of the cytoplasmic domain of membrane PAM resulted in accumulation of the mutant protein on the surface of AtT-20 and hEK-293 cells, suggesting that the cytoplasmic domain was important for sorting within the secretory pathway of both cell types (19). To separate the effects of the transmembrane and cytoplasmic domains from effects of lumenal sorting signals, we constructed a fusion protein consisting of the lumenal domain of Tac and the TGN. Additionally, we have shown that Tac accumulates on the cell surface (24, 26, 27). AtT-20 cells stably expressing Tac were fixed and stained with an antibody directed against the lumenal domain of Tac (Fig. 2A). Intense surface staining in the absence of Triton X-100 (Fig. 2B) demonstrated that a significant amount of the expressed protein was present on the surface (Fig. 2A). The steady state localization of the Tac/PAM chimera differed significantly from the localization observed for full-length Tac. In the absence of permeabilization, almost no staining was observed in cells expressing Tac/PAM, in permeabilized cells staining was tightly concentrated in the perinuclear region (Fig. 2A, left). The localization of Tac/PAM closely resembled that of PAM-1, which was largely coincident with TGN-38, a marker defining the TGN (Fig. 2B). Little immunofluorescent staining was observed in the secretory granule-enriched peripheral processes of cells expressing Tac/PAM.

Since PAM proteins are efficiently internalized from the cell surface, while Tac proteins are not, antibody internalization assays were performed on AtT-20 cells expressing Tac and Tac/PAM (Fig. 2A, right). Even after 30 min of chase, significant amounts of Tac Ab remained on the surface of cells expressing Tac and little internalization of the Tac/PAM complex was observed. In contrast, the TaccPAM-Tac Ab complex was internalized from the surface of the transfected cell lines and was collected in the perinuclear region (Fig. 2A, right). Thus, the cytoplasmic domain of PAM contains positive sorting information, which both causes the TaccPAM chimera to be localized largely to the TGN and causes internalization and efficient clearance of the TaccPAM chimera from the surface of cells.

Analysis of PAM-1 Truncation Mutants: Steady State Localization and Internalization—We next examined the role of the cytoplasmic domain routing determinants in the presence of the wild type PAM lumenal domain. All of the mutant PAM-1 proteins were active in vitro PHM and PAL enzyme assays, indicating that mutations in the cytoplasmic domain did not lead to misfolding of the expressed proteins. The levels of expression of the mutant proteins were compared using en-
The steady state localization of PAM-1 to the TGN could arise by retention signals as well as by retrieval of the protein from the plasma membrane. In order to compare the relative importance of these two types of signals, the access of newly synthesized PAM proteins to the cell surface was determined by surface immunoprecipitation following metabolic labeling (Fig. 4). Although very little newly synthesized PAM-1 protein is accessible to externally applied antibody within 2 h of synthesis, almost all of the newly synthesized PAM-1/936 protein is accessible to antibody within the same time period. In deleting the final 40 amino acids of PAM-1, a signal localizing PAM-1 to the TGN region of AT-20 cells is lost. In order to determine whether this signal was only recognized in neuroendocrine cells, similar experiments were carried out on non-neuroendocrine human embryonic kidney cells (hEK-293) expressing PAM-1 (Fig. 4) (32). Again, very little newly synthesized PAM-1 protein was accessible to externally applied antibody within 2 h of synthesis. Unless membrane PAM proteins reach the plasma membrane but remain inaccessible to antibody, retention signals play a key role in the routing of membrane PAM. Furthermore, these experiments indicate that the routing determinants in the cytoplasmic domain of PAM-1 are recognized in cells that have a regulated pathway and in cells that lack such a specialized pathway.

Analysis of Truncation Mutants: Endoproteolytic Processing, Storage, and Secretion—We previously demonstrated that PAM-1 is cleaved by a secretory granule-specific protease to generate soluble 45-kDa PHM and integral membrane PAL. Since this cleavage is limited to neuroendocrine cells and occurs distal to the site of 20°C temperature block (30), its occurrence is taken as an indication of entry into immature secretory granules. Therefore, we performed pulse-chase biosynthesis experiments to compare the proteolytic processing of PAM-1 and PAM-1/936. The pattern of proteolytic processing provides a biochemical assessment of the trafficking of membrane PAM through the regulated pathway. Cells expressing PAM-1 or PAM-1/936 were labeled for 15 min and chased 0 or...
4 h in basal medium; equal aliquots of cell extracts and media were immunoprecipitated with PHM antiserum (Fig. 5). After the 15-min pulse, all of the newly synthesized PAM-1 protein was full-length, while after the 4-h chase, most of the PAM-1 had been endoproteolytically processed and all of the full-length form (120 kDa) was fully glycosylated (Fig. 5). AtT-20 cells have a significant rate of constitutive-like (or basal) secretion (31) and 50% of the soluble PHM generated through the cleavage of PAM-1 is secreted from cells, while the remainder is stored. After the 4-h chase, monofunctional 45-kDa PHM was present in approximately equal amounts in the cell extract and medium of cells expressing wild type PAM-1.

When cells expressing PAM-1/936 were analyzed, we observed a different processing and secretion pattern (Fig. 5). After 4 h of chase, a significant amount of the PAM-1/936 protein had undergone endoproteolytic processing to generate a bifunctional 107-kDa PAM protein recovered from medium, but not from cell extracts; the 107-kDa bifunctional protein accounted for 60–80% of the PHM protein secreted from cells expressing PAM-1/936. In contrast, 107-kDa bifunctional PAM accounted for less than 3% of the PAM protein secreted by cells expressing PAM-1. A similar 107-kDa bifunctional PAM protein was released by hEK-293 cells transfected with PAM-1/899, indicating that this processing site is not neuroendocrine-specific (32). A significant amount (20–40%) of monofunctional 45-kDa PHM was generated from PAM-1/936. Only 30% of the
conjugated goat anti-rabbit IgG to visualize the PAM antibody. Cells were fixed, permeabilized, and incubated with FITC-body for 10 min at 37°C and chased for 15 min in the absence of monovalent Fab fragments replaced whole antiserum (19). Internalization of PAM antibody by AtT-20 PAM-1 cells occurred when selected cells.

Secretion of the 107-kDa bifunctional PAM protein generated from PAM-1 was stored for 4 h, while 50% of monofunctional 45-kDa PHM generated from PAM-1 was stored for >4 h (Fig. 5).

We next used responsiveness to secretagogue to determine which PAM proteins were stored in secretory granules. After a 15-min labeling period and a 3-h chase in basal medium, cells expressing full-length or truncated PAM-1/936 were incubated with or without PMA for 1 h. Phorbol ester treatment stimulated the secretion of monofunctional PHM 2–3 fold from cells expressing either PAM-1 or PAM-1/936 (Fig. 5). By contrast, secretion of the 107-kDa bifunctional PAM protein generated from proteolytic processing of PAM-1/936 was not increased by exposure of cells to phorbol esters (Fig. 5). Taken together, the immunofluorescent staining and biosynthetic labeling experiments indicate that the signals responsible for the more efficient storage of PAM-1-derived proteins in secretory granules, and the formation of 45-kDa PHM versus 107-kDa bifunctional PAM, involve information distal to Tyr936.

Nested Deletions: Steady State Localization, Internalization, Endoproteolytic Processing, and Secretion—To identify the signals responsible for the wild type pattern of PAM processing and secretion, we evaluated the steady state distribution, internalization, processing, and secretion of a set of intermediate truncation mutants. Cells expressing PAM proteins terminating at amino acids 971, 961, 957 (data not shown), and 953 have steady state distributions more similar to that observed in cells expressing full-length PAM-1 than PAM-1/936 (Fig. 6). There was intense staining in the TGN region of transfected cells, and staining was also evident at the tips of processes (Fig. 6, arrows). In the absence of permeabilization with TX-100, very little staining was observed (data not shown), indicating that these truncated PAM-1 proteins did not accumulate on the cell surface. PAM proteins truncated at residue 971, 961, or 953 were efficiently internalized from the cell surface (Fig. 6, lower panels). However, in cells expressing PAM-1/953, the distribution of the PAM-PAB complex differed from the pattern observed in cells expressing the longer truncation mutants or full-length PAM-1 (Figs. 3 and 6, lower panels). The internalized PAM-1/953-PAB complex was in larger structures not as heavily concentrated in the perinuclear region and resembling lysosomes in AtT-20 cells.

Cells expressing PAM-1 proteins terminating at residues 971, 961, and 957 displayed the wild type endoproteolytic processing and secretion pattern with little production of the 107-kDa PAM protein seen in cells expressing PAM-1/936 (Fig. 7, upper panels). For these three mutants, approximately 50% of the recovered monofunctional PHM was cell-associated after a 4-h chase. Incubation of cells expressing these truncation mutations with phorbol esters significantly increased secretion of monofunctional PHM, indicating that the protein was stored in secretory granules (Fig. 7, lower panels). The variable magnitude of secretagogue-stimulated 45-kDa PHM release reflects variability among individual clonal cell lines (18).

Although not yielding a 107-kDa PAM protein, cells expressing PAM-1/953 did not exhibit a wild type processing pattern (Fig. 7). Not all of the newly synthesized PAM-1/953 protein could be recovered in the final extract or medium after the 4-h
chase (Fig. 7, upper panels). The disappearance of PAM protein during the chase may be related to the altered pattern observed for internalized PAM-1/953 PAM Ab complex. However, release of the 45-kDa PHM protein stored in PAM-1/953 cells was sensitive to stimulation by PMA.

Internalization but Not Access to Secretory Granules Is Dependent on Tyr936—Our analyses of the truncation mutants raised the possibility that the same signal that mediated trafficking within the regulated secretory pathway was also responsible for internalization from the cell surface. Most commonly, internalization signals consist of tyrosine residues localized within a specific conformation or di-leucine motifs (33). We sought to determine whether either of the two tyrosine residues in the cytoplasmic domain of PAM were involved in PAM trafficking. Tyr936 and Tyr965 were individually mutated to alanine, and stable AtT-20 cells were generated.

The steady state distribution of the mutant PAM proteins was determined following immunofluorescent staining of permeabilized cells. In cells expressing PAM-1/Y965A, PAM was concentrated in the TGN and staining was detectable at the tips of the processes (Fig. 8, upper panels, white arrows). The steady state distribution of PAM-1/Y965A resembled that of PAM-1 (Fig. 3, upper panels). In contrast, the steady state localization of PAM-1/Y936A differed from both that of PAM-1 and PAM-1/936. Unlike PAM-1/Y936, the PAM-1/Y936A point mutant was not localized primarily on the plasma membrane. Some of the PAM-1/Y936A protein was detected at the tips of processes; staining in the TGN region was still apparent, but was more compact than the pattern observed in AtT-20 cells expressing PAM-1/Y965A or wild type PAM-1.

Mutating Tyr936 to Ala altered the internalization of PAM proteins from the surface of cells. Internalization assays were performed by incubating duplicate wells of cells with antibody at 37 °C for 10 min, followed by 10 min of chase in medium...
lacking antibody; cells were visualized with or without permeabilization (Fig. 8, lower panels). Mutation of Tyr936 significantly altered the collection of PAM proteins in perinuclear vesicles and the majority of the protein remained on or near the cell surface following 10 min of chase (Fig. 8, lower panels). In marked contrast, the PAM-1/Y965A protein was concentrated in the perinuclear region and was completely removed from the cell surface following 10 min of chase (Fig. 8, lower panels). The PAM-PAM Ab complex remaining of the surface of PAM-1/Y936A cells showed a patchy pattern unlike the uniform distribution of PAM-PAM Ab complex remaining on the surface of PAM-1/Y936 cells (Fig. 3, lower panels).

We next examined the proteolytic processing and secretion of PAM-1/Y936A (Fig. 9, upper panels). Cells expressing PAM-1/Y936A displayed a pattern similar to the wild type endoproteolytic processing and secretion pattern (Fig. 9, upper panels). Approximately 60% of the recovered monofunctional PHM was cell-associated after a 4-h chase. The predominant PHM protein present in conditioned medium was monofunctional 45-kDa PHM and no 107-kDa bifunctional PAM was apparent in the chase media. Furthermore, phorbol esters significantly increased the secretion of monofunctional PHM from cells expressing PAM-1/Y936A (Fig. 7, upper panels), indicating that the 45-kDa protein was stored in secretory granules.

While the proteolytic processing of PAM-1/Y936A was indistinguishable from wild type PAM-1 (see Fig. 5), we considered the possibility that disrupting the internalization of PAM by mutation of Tyr936 might change the kinetics of processing and secretion. Previously, we demonstrated that the constitutively secreted form of monofunctional PHM from cells expressing wild type PAM-1 peaked 2–4 h after a 15-min biosynthetic pulse. To compare nonstimulated secretion from cells expressing PAM-1/Y936A and PAM-1/Y965A, cells were pulse-labeled with [35S]methionine for 15 min and chased for a total of 8 h in basal medium. At intervals throughout the chase, the secretion of PHM proteins was determined following immunoprecipitation. Although the efficiency with which PAM-1/Y936A and PAM-1/Y965A were internalized from the cell surface differed greatly, the processing and secretion of the two proteins were very similar (Fig. 9, lower panels). Secretion of monofunctional 45-kDa PHM was first apparent during the 1–2-h chase time, and the peak of secretion occurred during the 2–4-h chase. This processing and secretion pattern is indistinguishable from the pattern for cells expressing wild type PAM-1 (20) and very different from cells expressing PAM-1/Y936. The fact that virtual elimination of the internalization of PAM-1 from the plasma membrane by mutation of Tyr936 to Ala fails to greatly affect cleavage of PAM-1 by secretory granule-associated endoproteases supports the conclusion that most PAM-1 enters secretory granules without first reaching the plasma membrane. Newly synthesized PAM-1/Y936A was selectively immunoprecipitated from the cell surface as described in Fig. 4; after 1 h of chase, only 3% of the newly synthesized PAM-1/Y936A protein was accessible to antibody. In comparison, 1% of the newly synthesized PAM-1 and 27% of the newly synthesized PAM-1/936 were accessible to antibody. At longer chase times, the percentage of PAM-1 at the surface did not increase, while the fraction of PAM-1/Y936A increased slowly and the fraction of PAM-1/936 rose dramatically (Fig. 4). Since PAM-1/Y936A cannot be efficiently internalized, these data support the conclusion that membrane PAM is retained within the cell, with only a small fraction of the newly synthesized protein reaching its final destination by first traveling to the plasma membrane.

Analysis of Internal Deletion Mutant Identifies a Region Required for Routing in the Regulated Pathway—Alternative splicing generates an integral membrane PAM protein lacking 18 amino acids in the cytoplasmic domain (Exon 26; amino acids 900–917) (34). When this naturally occurring protein (PAM-1/990–917) was expressed in fibroblasts, its pattern of expression and secretion was identical to full-length PAM-1 (32). When AtT-20 cells expressing PAM-1/900–917 were analyzed, we also observed that the steady state distribution, processing, and secretion patterns were identical to full-length PAM-1 (data not shown). Thus amino acids 900–917 do not contain routing information, and more distal determinants can be moved 18 amino acids closer to the transmembrane domain without altering the trafficking of PAM.

To define more precisely the trafficking signals important for routing in the regulated secretory pathway, we generated a deletion mutant lacking 18 amino acids including the Tyr, which is critical for internalization of PAM-1 (Tyr936). The steady state distribution of PAM-1/933–950 differed from the distribution of full-length PAM-1 but was not identical to that of PAM-1/936 (Fig. 10, upper panels). In the absence of permeabilization, significant staining was observed on the surface of cells expressing PAM-1/933–950 (Fig. 10, lower panels). Consistent with our other results indicating that Tyr936 plays a critical role in the internalization of PAM-1, PAM-1/933–950 proteins were not internalized from the surface of transfected cells (Fig. 10, upper panels).

We next examined the endoproteolytic processing and secretion of PAM-1/933–950. After a 15-min pulse, all of the newly synthesized protein was the full-length unprocessed form. Dur-
The routing of integral membrane PAM in AtT-20 cells clearly involves contributions from the two luminal catalytic domains and the COOH-terminal cytoplasmic domain. Monofunctional soluble PHM and PAL proteins are efficiently targeted to regulated secretory granules when expressed in AtT-20 cells; thus, each enzyme contains the information necessary to reach secretory granules (18, 20). Integral membrane PAM is cleaved to yield soluble PHM that is stored in regulated secretory granules, but a large amount of the membrane PAM remains localized in the vicinity of the TGN (21). To examine the possibility that the cytoplasmic domain of integral membrane PAM might function as a targeting signal, we transferred the transmembrane and cytoplasmic domains of PAM to Tac; the chimeric protein was rerouted from the cell surface to the TGN and internalized from the plasma membrane, but was not concentrated in secretory granules. These data directly demonstrate that the cytoplasmic domain/transmembrane domain of PAM contains protein trafficking information. Additional experiments will be needed to determine whether a small fraction of the Tac-PAM chimera enters secretory granules. The fact that membrane PAM proteins terminating at Gly\textsuperscript{999} are not as efficiently routed within the regulated pathway as PAM-1 argues that cytoplasmic signals are important for the trafficking of membrane PAM within the regulated secretory pathway.

We proceeded to define more precisely trafficking signals contained within the PAM cytoplasmic domain by performing site-directed mutagenesis using the PAM-1 protein, since our previous data demonstrated an important role for the lumenal domains in PAM trafficking (18–20). Since the routing and processing of PAM-1 proteins as short as PAM-1/957 was indistinguishable from the routing and processing of PAM-1, the 20 amino acids distal to amino acid 957 are not required for the entry or retention of integral membrane PAM within the regulated secretory pathway. These data rule out the highly acidic PEST sequence, which includes residues 954–970 (35; Fig. 1) as an important mediator of PAM trafficking. The instability of the PAM-1/953 protein in our pulse-chase studies and the different pattern of the internalized PAM-PAM Ab complex in these cells suggest that sequences immediately distal to 953 positively affect the stability of membrane PAM proteins. Deletion of 18 amino acids proximal to the transmembrane domain (PAM-1/990–917) is also without effect on routing and processing of membrane PAM. Routing information in the cytoplasmic domain is completely eliminated upon truncation at residue 936 (PAM-1/936), and both internalization and processing by secretory granule-associated enzymes are impaired upon deletion of residues 928–945 or 933–950.

This region of the cytoplasmic domain contains a Tyr-based internalization motif that plays a critical role in the internalization of PAM-1 from the plasma membrane. The GY\textsuperscript{936}SRK sequence in PAM shares some similarity with the SYTRF sequence in the transferrin receptor, the DYQRL sequence in TGN38 and the KYSKV sequence in the cation-independent mannose 6-phosphate receptor (33, 36). Elimination of this Tyr-based internalization motif does not compromise the access of membrane PAM to secretory granule enzymes as dramatically as elimination of cytoplasmic domain routing information or deletion of 18 amino acids surrounding Tyr\textsuperscript{936}. The bifunctional 107-kDa PAM protein produced when all routing information is deleted from the cytoplasmic domain of PAM is not generated in AtT-20 cells expressing PAM-1/Y936A, indicating that internalization is not an essential step in the formation of this product.

Integral membrane PAM proteins, which were not efficiently internalized due to the Y936A mutation, were nonetheless efficiently processed to generate 45-kDa PHM, which was stored in secretory granules. Monofunctional PHM was secreted with similar kinetics from cells expressing PAM-1/Y936A and PAM-1, ruling out a significant, or obligatory, pathway from plasma membrane to secretory granules, the site where 45-kDa PHM is generated (20). Thus the signal that mediates the internalization of PAM does not play a critical role in the trafficking of PAM within the regulated secretory pathway. Distinct internalization and TGN localization signals are found in the cytoplasmic domains of furin and TGN-38 (26, 27, 37, 38). These results are consistent with our observation that far less than 10% of the full-length membrane PAM reaches the cell surface following a short biosynthetic pulse (20). Thus, the cytoplasmic domain contains an internalization signal as well as additional information responsible (together with the luminal domains) for the trafficking of PAM within the regulated secretory pathway.

A working model consistent with these data is outlined in Fig. 11. Both integral membrane and soluble PAM proteins...
Experiments are currently in progress to identify proteins that differ in cells that lack a regulated secretory pathway.

Integral membrane PAM (and the soluble proteins that are generated through endoproteolytic cleavage) also partition between mature secretory granules and CLVs during the granule maturation process. In the absence of cytoplasmic signals, integral membrane PAM proteins reach the cell surface in CLVs (dashed line). The major cytoplasmic signal (heavy arrow) removes integral membrane PAM from the CLV pathway (perhaps following fusion of the CLVs with an endosomal compartment), while a minor signal mediated pathway retrieves any integral membrane PAM that reaches the cell surface. Shaded arrows represent pathways that may be inferred from the data but have not been directly demonstrated. PM, plasma membrane; ENDO, endosomal system; L, lysosomes.

The signals that mediate the trafficking of PAM via specific protein-protein interactions.

**Fig. 11. Signal-mediated trafficking of integral membrane PAM proteins.** Packaging into immature secretory granules (ISG) involves lumenal interactions for both soluble and integral membrane PAM proteins. Soluble PAM proteins may be retained in mature secretory granules (MSG) and undergo regulated secretion, or be diverted to constitutive-like vesicles (CLV). Integral membrane PAM (and the soluble proteins that are generated through endoproteolytic cleavage) also partition between mature secretory granules and CLVs during the granule maturation process. In the absence of cytoplasmic signals, integral membrane PAM proteins reach the cell surface in CLVs (dashed line). The major cytoplasmic signal (heavy arrow) removes integral membrane PAM from the CLV pathway (perhaps following fusion of the CLVs with an endosomal compartment), while a minor signal mediated pathway retrieves any integral membrane PAM that reaches the cell surface. Shaded arrows represent pathways that may be inferred from the data but have not been directly demonstrated. PM, plasma membrane; ENDO, endosomal system; L, lysosomes.

**Signal Targeting PAM**

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