Cell, Vol. 94, 205–216, July 24, 1998, Copyright ©1998 by Cell Press

PACS-1 Defines a Novel Gene Family of Cytosolic Sorting Proteins Required for *trans*-Golgi Network Localization

Lei Wan, Sean S. Molloy, Laurel Thomas, Gseping Liu, Yang Xiang, Sheree Lynn Rybak, and Gary Thomas*
Vollum Institute
The Oregon Health Sciences University
Portland, Oregon 97201

Summary

We report the role of one member of a novel gene family, PACS-1, in the localization of *trans*-Golgi network (TGN) membrane proteins. PACS-1 directs the TGN localization of furin by binding to the protease's phosphorylated cytosolic domain. Antisense studies show TGN localization of furin and mannose-6-phosphate receptor, but not TGN46, is strictly dependent on PACS-1. Analyses in vitro and in vivo show PACS-1 has properties of a coat protein and connects furin to components of the clathrin-sorting machinery. Cell-free assays indicate TGN localization of furin is directed by a PACS-1-mediated retrieval step. Together, these findings explain a mechanism by which membrane proteins in mammalian cells are localized to the TGN.

Introduction

The regulation of membrane and protein traffic between secretory pathway compartments requires the orchestrated interaction of a large number of components, including various small molecules, lipid, cytosolic, and membrane proteins, and cytoskeletal elements (Cole and Lippincott-Schwartz, 1995; Schekman and Orci, 1996; Schmid, 1997). The secretory pathway compartments can be subdivided into two central membrane populations, the endoplasmic reticulum (ER)/Golgi system and the *trans*-Golgi network (TGN)/endosomal system. The ER/Golgi system performs the folding, oligomerization, and initial co- and posttranslational modifications of proteins transiting the secretory pathway. The TGN/endosomal system is central to the sorting, export, and recovery of numerous soluble and membrane-associated secretory pathway proteins. In addition to housing several biochemical reactions, the TGN orchestrates the routing of proteins to lysosomes, to regulated and constitutive exocytic pathways, and, in polarized cells, to apical and basolateral membranes.

For the ER/Golgi and TGN/endosomal systems to fulfill their manifold roles, resident proteins must be distinguished from the population of migrant molecules that transit through them. For example, the ER maintains populations of both soluble and membrane-anchored resident proteins that are localized by retrieval-based mechanisms. Soluble resident proteins (e.g., GRP78/Bip) contain a C-terminal -KDEL motif (Munro and Pelham, 1987) that binds to the KDEL receptor to direct retrieval from the Golgi, whereas resident membrane proteins contain a basic amino acid motif (e.g., KKXX) within their cytosolic domains that directs retrieval by binding to COPI coatomer (Letourneur et al., 1994). By contrast, resident Golgi cisternae glycosyltransferases are localized primarily by a retention mechanism, based in part on the length of their transmembrane domains (Munro, 1995). However, despite the central role of the TGN to the organization of the secretory pathway, little is known about the cellular machinery that directs the localization and distribution of membrane proteins within this dynamic compartment.

The endoprotease furin represents an excellent model with which to identify the factors that direct protein sorting within the TGN. Localization of furin to clathrin-coated regions of the TGN requires a motif within its cytosolic domain (cd) consisting of an acidic cluster (AC) of amino acids (..EECPS$_{773}$DS$_{775}$EEDE..) and containing a pair of casein kinase II (CKII) phosphorylatable serines (Molloy et al., 1994; Schäfer et al., 1995; Takahashi et al., 1995). Phosphorylation of the furin-cd AC is required for TGN localization of the endoprotease and enhances its association with the clathrin adaptor AP-1 (Dittié et al., 1997). These findings suggest that TGN localization of furin is mediated by an AP-1/clathrin-sorting step. Similarly, TGN localization of varicella-zoster virus gE (VZV-gE) also requires phosphorylation of an AC within its cd by CKII (Alconada et al., 1996; see also Figure 7A). By contrast, TGN localization of TGN38 requires a tyrosine-based motif (Wong and Hong, 1993), suggesting distinct sorting machinery is used to localize different membrane proteins to the TGN.

Like the plasma membrane-associated adaptor AP-2, AP-1 binds directly to many tyrosine-based and/or dileucine-like internalization/endosomal targeting signals (Ohno et al., 1996). However, direct binding of AP-1 to the structurally disparate furin TGN localization signal has not been demonstrated. Indeed, some membrane proteins contain sorting motifs that do not bind adaptors directly. Rather, additional cytosolic proteins are required to connect them to the clathrin-sorting machinery. For example, the HIV-1 *nef* gene product directs the internalization of CD4 receptor by connecting the cell surface protein to AP-2 (Foti et al., 1997). Similarly, β-arrestin directs the internalization of β-adrenergic receptor (β-AR) by connecting it directly to clathrin (Lin et al., 1997). These findings raise the possibility that localization of TGN proteins may similarly require the participation of connector proteins.

Here, we report the identification of one member of a novel gene family of cytosolic connector proteins, PACS (*p*hosphofurin *a*cidic *c*luster *s*orting proteins), that directs the localization of furin to the TGN. We show that PACS-1 binds directly to the TGN localization signal on the furin-cd and connects the endoprotease to the clathrin-sorting machinery. We use cell-free assays to show that the furin-cd AC is not necessary for TGN budding or retention but apparently functions in a PACS-1-mediated retrieval step. We also show that PACS-1 is required for the correct localization of the cation-independent mannose-6-phosphate receptor (CI-MPR),

*To whom correspondence should be addressed.

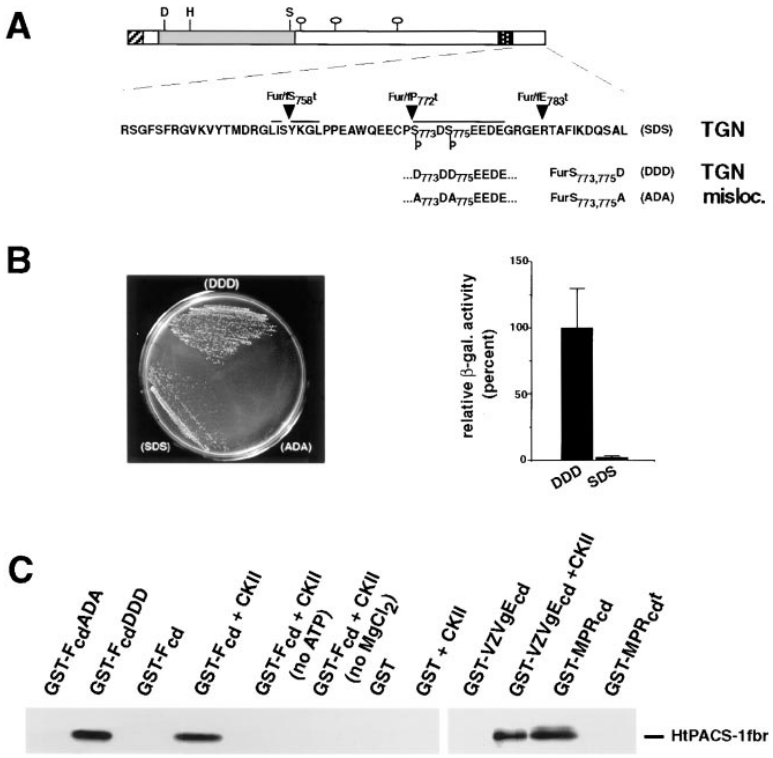

Figure 1. PACS-1 Binds to Acidic Cluster Sorting Motifs

(A) FLAG-tagged furin constructs and localization. The N-terminal cross-hatched box shows the FLAG epitope; the shaded area depicts the catalytic domain with active site residues; tailed circles depict N-linked carbohydrate; and the stippled box depicts the membrane-spanning domain. The 56-amino acid cd is shown. The Tyr-based and dileucine-like internalization motifs and the AC TGN localization motif are overlined.

(B) (Left) L40 yeast cells were cotransformed with pPACS (Leu^- selection) and bait plasmid (Trp^- selection) containing the furin-cd (SDS) or phosphorylation-state mutants (DDD, ADA) and then selected for growth on Leu^-/Trp^- YC minimal plates. Colonies from the Leu^-/Trp^- plates were streaked onto His^- plates and scored for growth. (Right) Measurement of β-gal activity provided a quantitative comparison of the binding affinity of PACS-1 for the native (SDS) and mutant (DDD) furin cd's (assay performed in triplicate, error bars = SD).

(C) (Left) HtPACS-1fbr was combined with either GST, GST-F_{cd}DDD, GST-F_{cd}ADA, GST-F_{cd}, or GST-F_{cd}, phosphorylated by CKII (Jones et al., 1995) in binding buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 2 mM $MgCl_2$, and 1% NP40) and incubated for 1 hr at RT. Protein complexes were recovered with glutathione-agarose beads (30 min, RT). Bound proteins were washed and analyzed by Western blot with antiserum 678 (PACS-1). Controls showed binding was strictly dependent on phosphorylation of GST-F_{cd}, since no binding occurred when either ATP or Mg^{2+} were omitted. (Right) Binding of PACS-1 to additional TGN/endosomal proteins. Binding of HtPACS-1 to GST-VZVgE_{cd} was examined as described above, using both nonphosphorylated and CKII-phosphorylated samples. Binding of PACS-1 to the CI-MPR cd was assessed using either a GST-MPR fusion protein containing both AC motifs (GST-MPR_{cd}) or a truncated construct lacking the C-terminal AC (GST-MPR_{cd}t; see Figure 7A). Results are representative of several independent experiments.

an itinerant TGN/endosomal membrane protein that, like furin, utilizes AC sorting motifs within its cd (Meresse and Hoflack, 1993; Mauxion et al., 1996; Chen et al., 1997). The presence of candidate PACS-binding motifs within many membrane protein cd's suggests a broad and important role for PACS-1 in protein sorting.

Results

Cloning of *PACS-1* by Two-Hybrid Screen

A yeast genetic screen was conducted to identify cytosolic proteins that bind directly to the phosphorylated furin-cd. Because furin-cd phosphorylation cannot be controlled in yeast, this approach required the generation of site-directed furin mutants that constitutively exhibit the phosphorylation-dependent localization of the endoprotease to the TGN (Figure 1A). Furin constructs with either the native sequence (fur/f) or a phosphorylation-mimic mutation, fur/f$S_{773,775}$D (DDD), are localized to the TGN, whereas fur/f$S_{773,775}$A (ADA), a construct that cannot be phosphorylated, is mislocalized (Takahashi et al., 1995; Dittié et al., 1997). Based on these data, a yeast two-hybrid analysis of a mouse embryo cDNA library was conducted that selected for clones that interact with the phosphorylation-mimic mutant $S_{773,775}$D and not the $S_{773,775}$A construct containing disrupted phosphorylation sites. The analysis for one such clone, *PACS-1*, is shown in Figure 1B. Both the native furin-cd and $S_{773,775}$D support growth in His^- media. However, only $S_{773,775}$D supports induction of β-galactosidase (β-gal). The higher affinity of PACS-1 for $S_{773,775}$D suggests the native furin-cd is phosphorylated inefficiently in yeast.

Protein–protein binding in vitro showed that the sequence encoded by the cloned *PACS-1* segment interacts directly with the phosphorylated furin-cd (Figure 1C). A His-tagged construct, HtPACS-1fbr, containing the 140–amino acid furin-binding region (fbr) was incubated with either GST, phosphorylated or nonphosphorylated GST-furin-cd (GST-F_{cd}), or constructs containing phosphorylation state mutations. In agreement with the two-hybrid analysis, the interaction between HtPACS-1fbr and GST-F_{cd}DDD was readily detected, whereas no interaction was observed with GST-F_{cd}, GST-F_{cd}ADA, or GST alone. However, phosphorylation of GST-F_{cd} by CKII promoted binding to HtPACS-1fbr. Similarly, HtPACS-1fbr bound the cd of VZV-gE in a phosphorylation-dependent manner, further suggesting a role for PACS-1 in directing the TGN localization of membrane proteins.

Although TGN localization of both furin and VZV-gE is strictly dependent upon the integrity of their cd ACs, additional proteins (e.g., CI-MPR) have similar AC motifs with important but less well-defined sorting functions (see Figure 7A). Indeed, HtPACS-1fbr also binds to the CI-MPR cd. Furthermore, binding requires the C-terminal AC, a motif important for receptor trafficking and AP-1 recruitment (Mauxion et al., 1996). Interestingly, whereas efficient binding of PACS-1 to the furin and

VZV-gE cd's requires phosphorylation, binding to the CI-MPR does not. Together, the binding assays indicate a broad role for PACS-1 in directing protein sorting in the TGN/endosomal system.

Screening of a rat brain cDNA library with the *PACS-1* segment yielded two cDNAs, representing splice variants of a single gene (Figure 2A). Both cDNAs encode complete open reading frames (ORFs) that contain the 140-amino acid fbr present in PACS-1(Val-115-Pro-255). The larger clone, *PACS-1a*, contains a 2886 nt ORF encoding a 961-residue protein. The smaller clone, *PACS-1b*, contains a 1680 nt ORF encoding a 559-residue protein. Northern blot hybridization with transcript-specific probes showed that, like furin, both *PACS-1* transcripts are expressed ubiquitously and the 4.4 kb *PACS-1a* transcript is present in great excess (>20-fold) over that of the 3.6 kb *PACS-1b* transcript (data not shown).

PACS-1 Has Properties of a Cytosolic Coat Protein

Analysis of homogenates from tissues and cultured cell lines revealed a 120 kDa anti-PACS immunoreactive protein that comigrated with recombinant PACS-1a (see Figure 3A). Consistent with the absence of a predicted transmembrane domain (Figure 2B, left), sedimentation at 100,000 × g resulted in 78% of the PACS-1a partitioning to the supernatant, while 22% remained membrane-associated but could be extracted with high salt (0.5 M) and carbonate (0.1 M, [pH 11]) (Figure 2B, right).

Immunocytochemical studies supported the membrane fractionation analysis and demonstrated that PACS-1 has properties characteristic of a coatomer protein. Analysis of PACS-1 localization by immunofluorescence microscopy revealed a punctate staining pattern present in the paranuclear region that coincided with that of γ-adaptin and extended into the cell periphery (Figure 2C). Treatment of cells with AlF_4^- prior to fixation, which enhances coatomer association with membranes (Ikonen et al., 1997), increased the intensity of the paranuclear PACS-1 signal. Consistent with previous studies, AlF_4^- produced an enhanced TGN staining pattern for γ-adaptin, which was more condensed than in control cells and in close juxtaposition to the PACS-1 staining. By contrast, treatment of cells with brefeldin A (BFA), which blocks the ARF-directed coatomer association with cellular membranes, resulted in dispersed PACS-1 and γ-adaptin staining patterns. These results indicate that PACS-1 has a dynamic, ARF-dependent, TGN-proximal localization that is distinct, yet overlapping with γ-adaptin.

TGN Localization of Furin Requires PACS-1

The importance of PACS-1 in the sorting of furin in vivo was investigated using an antisense strategy. A7 melanoma cells, which contain detectable levels of endogenous furin, were stably transfected with either pCEP-4 plasmid containing *PACS-1* in reverse orientation or with an empty vector. Following drug selection, the level of PACS-1 in the clonal cell lines was determined by Western blot analysis (Figure 3A). Multiple antisense clones were obtained with levels of PACS-1 protein reduced 3- to 9-fold relative to a control protein (α-tubulin).

The effect of the antisense knockdown of PACS-1 levels on endogenous furin localization was determined by immunofluorescence microscopy (Figure 3B). Analysis of control cells showed a paranuclear staining pattern for endogenous furin characteristic of its TGN localization (Molloy et al., 1994; Schäfer et al., 1995). By contrast, endogenous furin staining was markedly dispersed in the PACS-1 antisense cells. Western blot analysis showed similar levels of furin in control and antisense cells, demonstrating that the weaker staining in the antisense cells was due to a mislocalization of the endoprotease and not to differences in expression and/or stability (inset).

The mislocalization of furin did not result from a gross corruption of the TGN, since localization of both TGN46, the human ortholog of TGN38, and γ-adaptin were unaffected by PACS-1 depletion (Figure 3B). These results also demonstrate that furin and TGN38/46 are localized to the TGN by separate mechanisms. Other secretory pathway markers, including those for the ER (SSR), Golgi cisternae (mannosidase II), and plasma membrane/endosomes (transferrin receptor), were also unaffected by PACS-1 depletion. These results demonstrate a role for PACS-1 in the sorting of select proteins within the TGN/endosomal system.

A direct link between mislocalization of furin and suppression of PACS-1 expression was provided by rescue experiments (Figure 3C). Due to the low level of endogenous furin, fur/f was expressed alone or in combination with PACS-1a in antisense (AS19) and control (C1) cells in order to increase the sensitivity of this analysis. Consistent with the distribution of endogenous furin, the staining pattern of fur/f was dramatically different in the control and antisense cells. However, when fur/f and PACS-1a (or PACS-1b, data not shown) were coexpressed in the antisense cells, the TGN localization of fur/f was restored.

Localization of CI-MPR Requires PACS-1

The binding of HtPACS-1fbr to the C-terminal CI-MPR AC (Figure 1C) suggested that PACS-1 may also direct sorting of the CI-MPR. To examine this possibility, the localization of CI-MPR was compared in C1 and AS19 cells (Figure 4). In control cells, both CI-MPR and TGN46 colocalized with γ-adaptin (Figure 4A). Furthermore, in agreement with Figure 3, TGN46 in AS19 cells remained colocalized with γ-adaptin in the paranuclear region. By contrast, the CI-MPR staining pattern in AS19 cells was more distended relative to that of γ-adaptin and was redistributed to an endosomal population where it overlapped with internalized dextran. The extent of CI-MPR redistribution was quantified by morphometric analysis (Figure 4B). These data demonstrate that the routing of CI-MPR is directed by PACS-1. Furthermore, the uncoupling of CI-MPR and γ-adaptin staining patterns argues that recruitment of AP-1 to the TGN is independent of cargo protein. Finally, these data support a broad and important role for the PACS family in protein sorting within the secretory pathway.

TGN Retrieval, but Not Retention, Is Mediated by Furin-cd Acidic Cluster

The requirement of furin-cd phosphorylation for the TGN localization of furin suggested that the furin-cd AC directs either the retention or the retrieval of the endoprotease to the TGN. Furthermore, phosphorylation of the

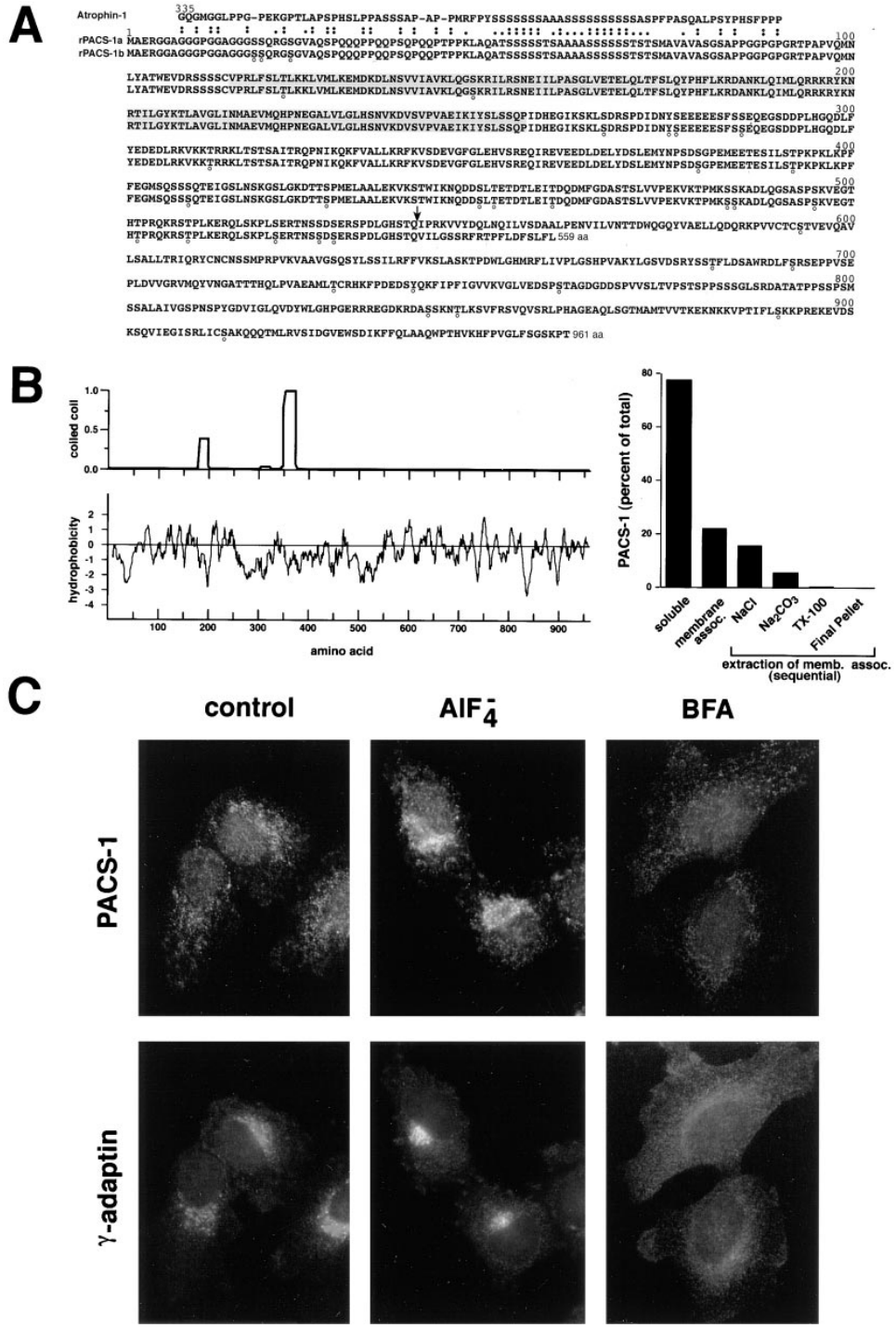

Figure 2. Predicted Amino Acid Sequence and Subcellular Localization of PACS-1

(A) Analysis of *PACS-1a* and *PACS-1b* cDNAs. The predicted translation start site in both open reading frames complies with Kozak's rules (Kozak, 1991) and is 93 nts downstream from an in-frame stop codon. The predicted splice site (|) follows Gln-540. Hydropathy analysis (Kyte-Doolittle) predicts both proteins are hydrophilic and lack membrane-spanning domains. The fbr is shaded. A single amino acid difference is present in mouse PACS-1fbr (Ala-144→Gly). The N-terminal region shares limited homology with atrophin-1 (shown). Sequences between Leu-164 and the PACS-1a C terminus share 31% identity with the *Drosophila* gene product KrT95D. EST clones containing PACS-1 sequences were found for human (gbAA381307, gbH85491, embZ44004, gbAA296369, embZ40056, gbR50269, gbT11563, gbT11564, and gbAA159946), *C. elegans* (D36982, D37521), and rice (D48009). Open circles, potential phosphorylation sites; double closed circles, identical amino acids; closed circles, conserved amino acids.

(B) (Left) Lupas coiled-coil determination (window = 21 residues) (Lupas et al., 1991) and Kyte-Doolittle hydrophobicity plot (window = 11 res-

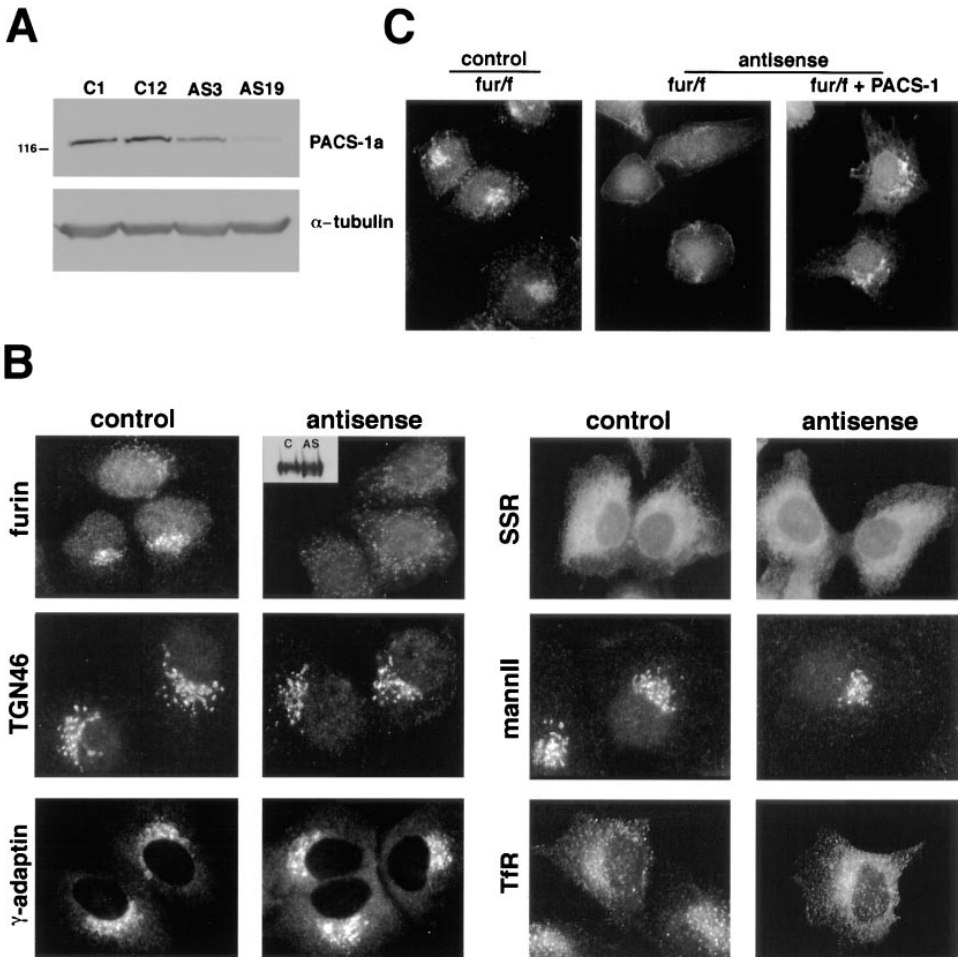

Figure 3. PACS-1 Is Required for TGN Localization of Furin

(A) An equivalent aliquot of lysate from control (C1,C12) and antisense (AS3,AS19) cell lines was resolved by SDS-PAGE and analyzed by Western blot using antiserum 7704 (PACS-1) and MAb N356 (α-tubulin), followed by chemiluminescent detection. Signals were quantified using NIH Image software. M_r standard (kDa) is shown on left. Similar results were obtained for suppression of PACS-1b (data not shown).

(B) Parallel plates of C1 and AS19 cells were fixed, permeabilized, and stained with MAb MON152 (furin), anti-mannosidase II, anti-SSR, anti-TGN46, and MAb 100/3 (γ-adaptin). TfR was visualized by incubation of cells with rhodamine transferrin (r-Tf, 40 ng/ml) for 1 hr. The selective mislocalization of endogenous furin was observed in several independent PACS-1a lines. (Inset) Membrane samples from equal amounts of C1 and AS19 cells were separated by SDS-PAGE and analyzed by Western blot using a furin-cd antiserum (ABR).

(C) Parallel plates of C1 and AS19 cells were infected with VV recombinants expressing fur/f (m.o.i. = 3) or fur/f (m.o.i. = 3) and PACS-1a (m.o.i. = 3). At 4 hr postinfection, cells were fixed, permeabilized, and fur/f visualized with MAb M2. Coexpression of fur/f with wild-type VV had no effect on fur/f trafficking (data not shown).

CI-MPR-cd by CKII has been reported to recruit AP-1 to the TGN (Mauxion et al., 1996), suggesting that the furin-cd AC may also modulate TGN budding. To distinguish between these possibilities, a cell-free budding assay was performed on FLAG-tagged furin constructs expressed in AtT-20 cells (Figure 5). AtT-20 cells were used in this assay because the different N-glycosylated forms are readily resolved by SDS-PAGE. The efficacy of the budding reaction was demonstrated by showing that budding of TGN (i.e., sialylated) furin constructs—both the membrane-anchored construct and a soluble form generated by cleavage within the lumenal domain—required addition of cytosol, ATP, and hydrolyzable GTP (Figure 5A). Furthermore, budding was specific for TGN-accumulated furin, since a Golgi-stack form of furin (i.e., nonsialylated) was not released during the incubation. The relative efficiency of budding of each furin construct was determined quantitatively by normalization to the amount of soluble sialylated furin released in each sample (Figure 5B). Budding of furin from

idues) of PACS-1a ORF. (Right) Membrane fractionation of endogenous PACS-1a analyzed by Western blotting using antiserum 7704 (PACS-1).

(C) A7 cells were either fixed immediately or treated with AlF_4^- (50 μM $AlCl_3$, 3 μM NaF) or 10 μM BFA for 30 min prior to fixation. Cells were then permeabilized and double labeled with affinity-purified antiserum 678 (PACS-1) and MAb 100/3 (γ-adaptin). Identical results were obtained with antiserum 7704 (PACS-1).

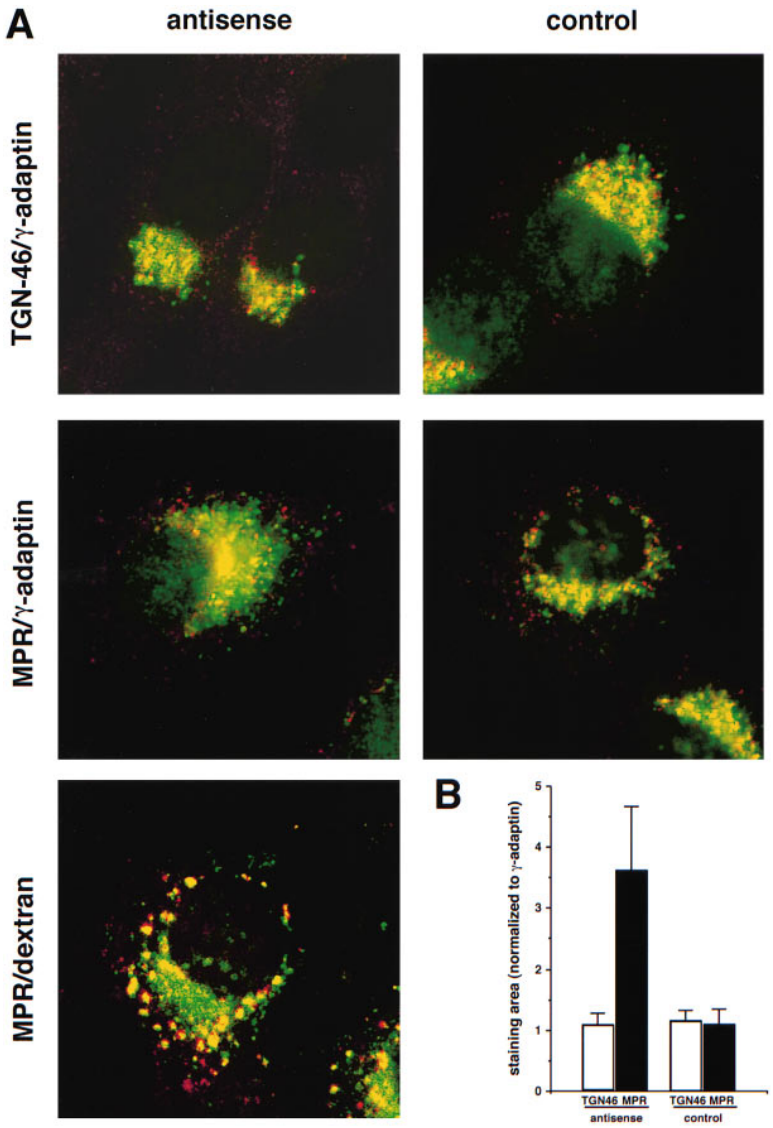

Figure 4. Localization of CI-MPR is PACS-1-Dependent

(A) Parallel plates of C1 and AS19 cells were fixed, permeabilized, and double-labeled with anti-TGN46 or anti-CI-MPR antisera and MAb 100/3 (γ-adaptin). Antibody staining was visualized with anti-rabbit-FITC (TGN46 and MPR, green) and anti-mouse IgG-TXR (γ-adaptin, red). Additional cells were incubated with TXR-dextran for 1 hr to label endocytic compartments prior to fixation and staining with anti-CI-MPR antiserum.

(B) CI-MPR mislocalization was assessed quantitatively by comparing the staining area of TGN46 and CI-MPR relative to γ-adaptin, using morphometric analyses (Scion Image 1.62). The summation includes only positive staining pixels (n = 50, error bars = SD).

the TGN was independent of cd phosphorylation state (fur/fDDD, fur/fADA) as well as the presence (fur/f, fur/fE_{783}t) or absence (fur/fP_{772}t) of the AC. By contrast, deletion of the tyrosine-based internalization signal (fur/fS_{758}t) blocked budding of furin. These results show that efficient budding of furin from the TGN is independent of the AC but requires furin-cd sequences containing the tyrosine-based motif previously shown to bind AP-1 directly (Ohno et al., 1996). Furthermore, the furin-cd AC has no effect on retention of the endoprotease to the TGN. Rather, these data argue that phosphorylated AC-dependent localization of furin to the TGN is mediated by a PACS-1-dependent retrieval mechanism. These findings are consistent with those in Figure 2, demonstrating a predominantly non-TGN localization of PACS-1, and Figure 4, showing that localization of the CI-MPR can be uncoupled from γ-adaptin.

PACS-1 Connects Furin-cd to Clathrin Adaptors

The selective binding of the PACS-1fbr to phosphorylated furin (Figure 1C), together with the observation that phosphorylation of the furin-cd increases its binding to γ-adaptin (Dittié et al., 1997), suggests that PACS-1 directs the TGN retrieval of furin by linking the endoprotease to components of the clathrin-sorting machinery. We tested this hypothesis by performing a series of protein binding assays (Figure 6). These analyses showed that GST-F_{cd}DDD, but not GST-F_{cd}ADA, bound endogenous PACS-1a in bovine brain cytosol (bbc) (Figure 6A). This selective binding was coupled with an enhanced association of γ-adaptin. Furthermore, γ-adaptin was also selectively recovered from bbc using HtPACS-1fbr but not the control protein HtABP-280 (containing sequences of the furin-sorting protein APB-280 that binds the furin-cd, independent of its phosphorylation state [Liu et al., 1997]) (Figure 6B). These results suggest that the association of AP-1 with the phosphorylated furin-cd is mediated by PACS-1.

Studies with cultured cells further supported our hypothesis that PACS-1 links furin to the clathrin-sorting machinery. Endogenous γ-adaptin could be recovered from cell extracts using Ni-agarose beads only in the

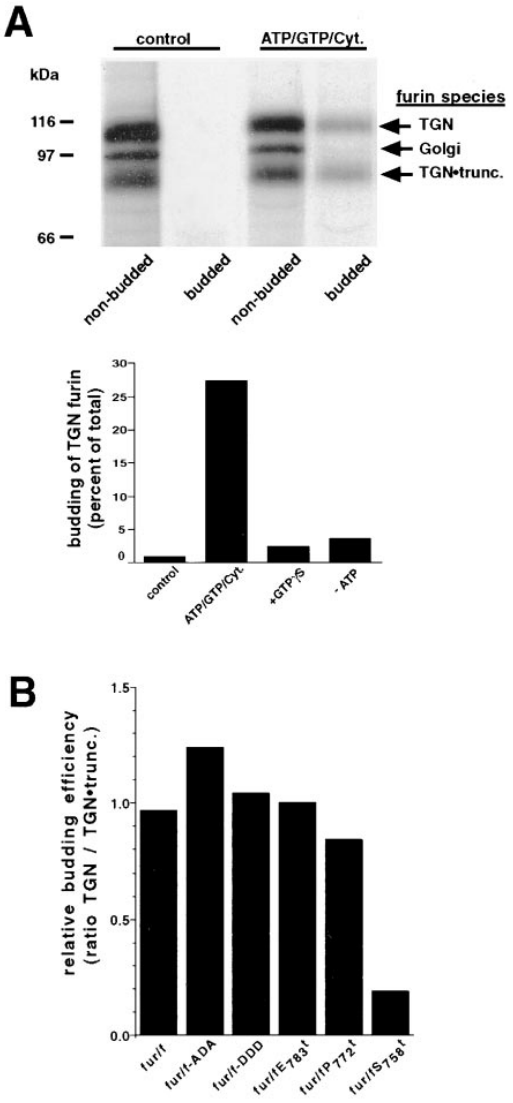

Figure 5. The Furin-cd Acidic Cluster Is Not Required for Efficient TGN Budding

(A) AtT-20 cells expressing fur/f were pulse-labeled with [^{35}S]Met/Cys, placed on ice, and scraped. Washed cells were incubated (37°C, 60 min) in the absence (control) or presence of budding mix and bbc (ATP/GTP/Cyt.) then placed on ice and released TGN-budded vesicles separated from broken cells by centrifugation. FLAG-tagged forms of furin were immunoprecipitated with MAb M1, separated by SDS-PAGE, and detected by fluorography. Only sialylated (TGN localized) furin was able to bud. The positions of sialylated, membrane-anchored furin (TGN) and sialylated, soluble furin proteolytically clipped in vivo, (TGN·trunc.), as well as endoglycosidase H-resistant, neuraminidase-insensitive furin (Golgi) are indicated. Control experiments showed that budding was ATP- and time-dependent and sensitive to GTPγS. Protease sensitivity demonstrated that budded fur/f was in the correct orientation. Molecular weight markers (kDa) are shown.

(B) The budding efficiency of a series of C-terminal furin truncations (Figure 1A) and CKII phosphorylation site mutants (DDD, ADA) was compared to native furin as described above. Budding efficiency is plotted as percentage of membrane bound/percentage soluble furin in the vesicle fraction. Results are representative of several independent experiments.

presence of coexpressed His-tagged PACS-1b (Figure 6C). Furthermore, GST-F_{cd}DDD bound substantially more γ-adaptin in extracts of control C1 cells than in extracts from PACS-1-depleted AS19 cells, despite equal levels of adaptor expression (Figure 6D). Together, our results show that PACS-1 directs the retrieval of phosphorylated furin to the TGN by connecting the endoprotease to the clathrin-sorting machinery in vivo.

Discussion

In this report, we describe PACS-1 as a member of a novel gene family of cytosolic connector proteins required for localization of membrane proteins to the TGN. PACS-1 is a partially membrane-associated cytosolic protein (Figure 2) that binds selectively to phosphorylated furin and VZV-gE cd's (Figure 1C), a modification required for their TGN localization (Takahashi et al., 1995; Alconada et al., 1996; Dittié et al., 1997). Antisense expression shows PACS-1 is required for TGN localization of furin but not TGN46 (Figure 3), demonstrating multiple sorting systems are used to maintain itinerant proteins in the TGN. In addition, PACS-1 binds to the CI-MPR-cd and is required for its correct localization (Figure 4), suggesting that PACS-1 directs the routing of a potentially large number of membrane proteins containing AC sorting motifs within their cd's (Figure 7A). Binding assays in vitro and coprecipitation studies in vivo show PACS-1 localizes furin to the TGN by connecting the endoprotease to the clathrin-sorting machinery (Figure 6). These studies, coupled with a demonstration of the lack of effect of the furin-cd AC on TGN retention or budding (Figure 5), argue that PACS-1 localizes the endoprotease to the TGN by a phosphorylation-dependent retrieval mechanism.

A Model for PACS-1-Mediated TGN Localization of Membrane Proteins

Based on our results, we propose a model for the localization of furin and other membrane proteins (e.g., CI- and CD-MPRs and VZV-gE) to the TGN in mammalian cells (Figure 7B). This model explains TGN localization by a selective retrieval mechanism, as opposed to the retention process used by resident membrane proteins of the Golgi cisternae (Munro, 1995). The initial step consists of the efficient budding of membrane-anchored furin from the TGN by virtue of its tyrosine motif interacting with AP-1. Evidence for this step is supported by several findings, including (1) the requirement for the tyrosine motif, but not the AC, for TGN budding of furin (Figure 5), (2) the ability of the furin-cd tyrosine motif to bind AP-1 (and AP-2) by two-hybrid analysis (Ohno et al., 1996), (3) the ability of VZV-gE-cd constructs containing only the tyrosine motif and not the AC to recruit AP-1 to TGN membranes (Alconada et al., 1996), and (4) the demonstration that export of lamp-1 from the TGN to clathrin-coated vesicles is directed by the binding of AP-1 to a tyrosine-based motif within the lamp-1 cd (Honing et al., 1996). The direct binding of AP-1 to tyrosine-based motifs does not, however, preclude the participation of additional AP-1-associated proteins (Mallet

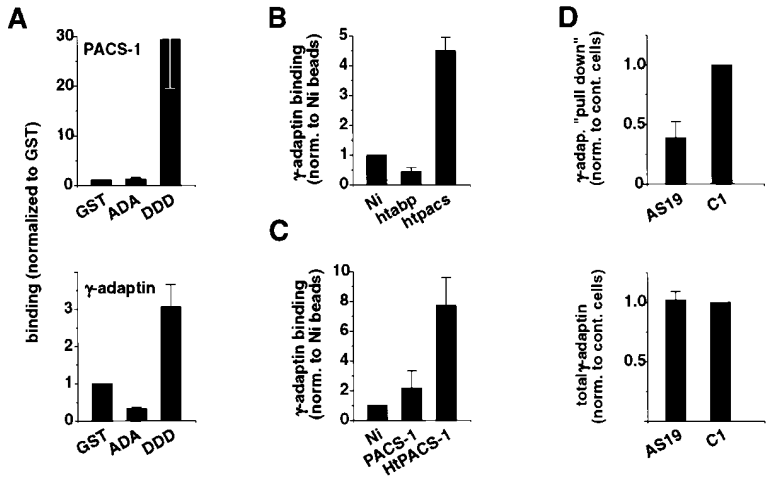

Figure 6. PACS-1 Mediates Association of Furin-cd with AP-1

(A) Aliquots of bbc were incubated with either GST, GST-F_{cd}ADA, or GST-F_{cd}DDD (37°C, 30 min). Bound proteins were recovered with glutathione-agarose beads and analyzed by Western blot.

(B) Aliquots of bbc were incubated (37°C, 30 min) in the absence or presence of either HtABP-280 or HtPACS-1fbr prebound to Ni-agarose in binding buffer containing 8 mM imidazole. Proteins were washed with binding buffer containing 50 mM imidazole, eluted in SDS-SB, analyzed by Western blot, and quantified.

(C) BSC-40 cells were infected with either wild-type VV or VV recombinants expressing either PACS-1b or HtPACS-1b (m.o.i. = 5). Clarified lysates (prepared in 50 mM Tris [pH 7.5], 150 mM NaCl, 2 mM MgCl2, 1% NP-40, 0.2 mM PMSF, and 5 μg/ml leupeptin) were incubated with Ni-agarose beads in the presence of 3 mM imidazole (37°C, 30 min) and washed as described in (B), and bound proteins were analyzed by Western blot and quantified.

(D) Equivalent amounts of high-speed supernatant solution (290 μg total protein) containing equal amounts of γ-adaptin (bottom) were prepared from parallel plates of C1 and AS19 cells and incubated with GST-F_{cd}DDD (4°C, 16 hr; top). Protein complexes were recovered with glutathione agarose beads, analyzed by Western blot, and quantified. All data are averaged from at least three independent experiments and were quantified with NIH image software (error bars = SD).

and Brodsky, 1996; Seaman et al., 1996). Once incorporated into a post-TGN endosome, furin may either be exported to the cell surface or retrieved to the TGN. Export to the cell surface would require the activity of a furin phosphatase (S. S. M. and G. T., submitted), whereas retrieval to the TGN requires phosphorylation of the furin-cd AC by CKII (Jones et al., 1995; Takahashi et al., 1995; Dittié et al., 1997). Like coatomer proteins, PACS-1 is recruited to the endosome by ARF-1 (Figure 2), where it binds to phosphorylated furin and connects the endoprotease to the clathrin/adaptor complex (Figure 6). Phosphorylated furin is then retrieved back to the TGN. While we have demonstrated an interaction between PACS-1 and AP-1, the participation of other adaptor complexes (e.g., AP-3 [Simpson et al., 1997]) is not excluded. Thus, our model distinguishes the role of the tyrosine-based sorting motif (TGN budding) from the AC motif (retrieval). This phosphorylated AC-dependent retrieval step is consistent with the AP-1-associated removal of phosphorylated furin from clathrin-coated immature secretory granules (ISGs) (Dittié et al., 1997).

PACS-1 Homologs Are Broadly Expressed in Metazoans

Analysis of EST databases reveals at least three human PACS family members (Figure 2, and unpublished results). In addition, PACS homologs are also expressed in a wide variety of metazoans, including other mammals, nematodes, insects, and plants. PACS-1a shares significant sequence similarity with KrT95D, a Krüppel-regulated *Drosophila* gene product of unknown function (Hartmann and Jäckle, 1997). Sequence alignment of PACS-1a and KrT95D shows greater than 31% sequence identity between Leu-164 and Thr-961. Interestingly, the PACS-1 N-terminal region, composed of a glycine (G)-rich domain followed by domains rich in proline/glutamine (P/Q) and serine/alanine (S/A), is absent from the KrT95D sequence but shares limited homology with atrophin-1 (see Figure 2A), the gene product mutated in the neurodegenerative disease Dentatorubral-pallidoluysian atrophy (DRPLA, Nagafuchi et al., 1994). In addition to PACS-1 and atrophin-1, P/Q- and S/A-rich regions are present in many transcription factors and appear to direct protein–protein interactions (Gerber et al., 1994), suggesting a potential regulatory role for this domain of PACS-1. Despite the presence of PACS-1 homologs in evolutionarily distant organisms, none were identified in a search of the yeast genome database, suggesting metazoan cells require sorting steps not performed in yeast.

Functional Parallels between PACS-1 and Cell Surface Connectors

PACS-1 is functionally related to a small yet diverse set of cytosolic proteins that connect membrane proteins to the clathrin-sorting machinery. Previously identified members of this group, including HIV-1 Nef (Foti et al., 1997), Eps15 (Benmerah et al., 1998), and β-arrestin (Lin et al., 1997), mediate internalization of cell surface receptors (CD4, EGF, and G protein–coupled receptors, respectively) by connecting them to the clathrin/AP-2-sorting machinery. By contrast, PACS-1 connects membrane proteins to the clathrin/AP-1-sorting machinery. Interestingly, the regulation of protein sorting by PACS-1 and β-arrestin share many features. First, both connectors bind to membrane proteins phosphorylated on serine residues (Sohlemann et al., 1995, and Figure 1). The β-AR is phosphorylated by the β-adrenergic receptor kinase (βARK) to initiate receptor down-regulation (desensitization), whereas the furin-cd is phosphorylated by CKII to localize the endoprotease to the TGN. Second, PACS-1 and β-arrestin connect the membrane proteins to the clathrin-sorting machinery. β-arrestin connects the β-AR directly to clathrin-heavy chains (Goodman et

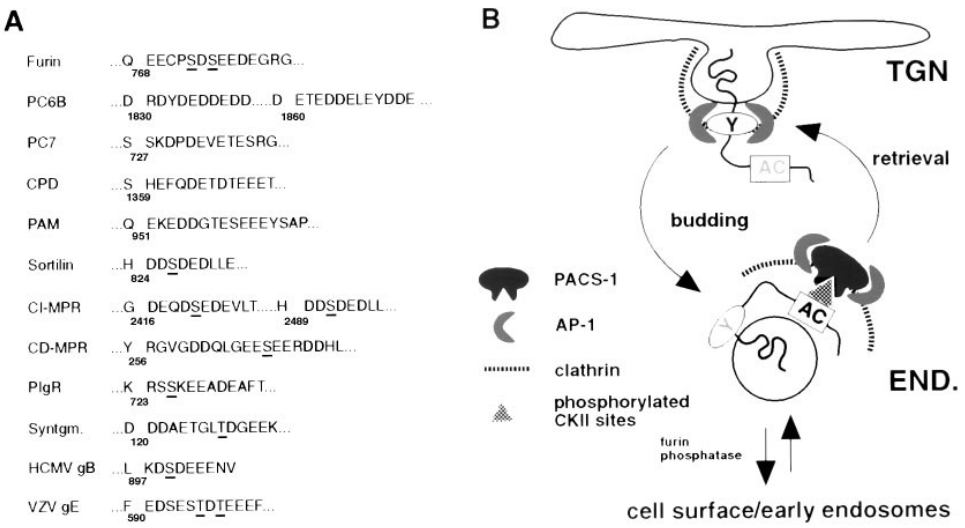

Figure 7. A Model for Acidic Cluster–Dependent Protein Trafficking

(A) Shown are (phosphorylatable) ACs of furin (Jones et al., 1995), PC6B (Nakagawa et al., 1993), PC7/8 (Bruzzaniti et al., 1996), carboxypeptidase D (Xin et al., 1997), PAM (Stoffers et al., 1991), sortilin (Petersen et al., 1997), CI-MPR (Meresse and Hoflack, 1993), CD-MPR (Korner et al., 1994), polymeric immunoglobulin receptor (pIgR) (Okamoto et al., 1994), synaptotagmin (Bennett et al., 1993), cytomegalovirus (HCMV) gB (Norais et al., 1996), and VZV-gE (Yao et al., 1993). Phosphorylated residues are underlined.

(B) A model for PACS-1-dependent TGN localization of furin. Budding of furin from the TGN is mediated by direct interaction of the furin-cd tyrosine motif (Y) with AP-1. The phosphorylated furin-cd acidic cluster (AC) is "silent" at this step (lightly shaded). The AC silencing is likely not due to lack of phosphate, since furin is phosphorylated as early as the endoplasmic reticulum (Jones et al., 1995). Rather, it may reflect exclusion of PACS-1 from TGN membranes. Once in a post-TGN endosome, furin is either shuttled to the cell surface/early endosomes or is retrieved to the TGN. Transit to the cell periphery would require dephosphorylation of the furin-cd (Jones et al., 1995; Dittié et al., 1997). By contrast, TGN retrieval requires binding of PACS-1 to the CKII-phosphorylated furin-cd AC and recruitment of AP-1. AP-1 is unable to bind the furin-cd AC directly and requires PACS-1 to serve as a connector protein. In this step, the Y motif is "silenced." The silencing mechanism may result from steric interference by PACS-1, compartment-specific modification of AP-1 (Wilde and Brodsky, 1996), or perhaps the exclusion from this compartment of accessory proteins that mobilize the Y-bound AP-1 molecules into clathrin coats (Mallet and Brodsky, 1996; Seaman et al., 1996).

al., 1997), whereas PACS-1 connects furin to AP-1 (Figure 6). Our results do not exclude the possibility that PACS-1 connects furin (or other itinerant membrane proteins containing PACS-1-binding motifs [e.g., CI-MPR, Figure 4]) directly to clathrin or to other adaptor complexes (e.g., AP-3). Third, both connectors are phosphorylated in vivo (Lin et al., 1997, and data not shown). While phosphorylation directs recruitment of β-arrestin onto cellular membranes, the role of PACS-1 phosphorylation has not been determined.

Regulation of Furin Trafficking by Phosphorylation

The sorting of furin through the TGN/endosomal system is dynamic and regulated by several factors, including identified components of the cytoskeleton (ABP-280; Liu et al., 1997) and the phosphorylation state of the furin-cd. In addition to localizing furin to the TGN, phosphorylation by CKII modulates localization of the endoprotease to an early endosome/cell surface cycling loop (S. S. M. and G. T., submitted) and, in neuroendocrine cells, the AP-1-coupled retrieval of furin molecules from ISGs (Dittié et al., 1997). By contrast, movement of furin between endosomes and the TGN is regulated by dephosphorylation of the furin-cd by specific PP2A isoforms (Jones et al., 1995, and S. S. M. and G. T., submitted). The phosphorylation-dependent trafficking of furin suggests that formation of furin-containing proprotein-processing compartments within the TGN/endosomal system is dynamic and acutely regulated by signal transduction pathways. The role of furin-cd phosphorylation in regulating the localization of the endoprotease to several cellular compartments suggests multiple sorting steps mediated by PACS-1. Indeed, in addition to localizing furin to the TGN, PACS-1 is also required for localization of the endoprotease to early endosomes (S. S. M. and G. T., submitted). Whether retrieval of furin from ISGs requires PACS-1 is currently being investigated. The importance of PACS-1 for localizing furin to multiple compartments is consistent with its distribution to vesicular compartments throughout the cytoplasm (Figure 2C).

A Broad Role for PACS Family Members in Protein Sorting

The presence of (phosphorylatable) ACs within the cd's of a large number of secretory pathway membrane proteins (Figure 7A) suggests PACS family members have a broad and important role in the organization of and trafficking between secretory pathway compartments. This is supported by the binding of PACS-1 to the VZV-gE and CI-MPR cd's as well as the requirement of PACS-1 for CI-MPR localization (Figure 4). Indeed, the TGN localization of VZV-gE, as well as the sorting of both the CI- and CD-MPRs, is directed by AC-sorting domains (Meresse and Hoflack, 1993; Alconada et al.,

1996; Mauxion et al., 1996; Chen et al., 1997). Very recently, a 47 kDa protein was identified that binds to a hydrophobic sorting signal in the CI- and CD-MPR cd's and participates in endosome-to-TGN sorting (Diaz and Pfeffer, 1998). Whether PACS-1 and TIP47 function in the same or in different sorting steps remains to be determined.

Our identification of a novel gene family of sorting proteins required for the phosphorylation state-mediated TGN localization of furin delineates core components of a novel signaling system used to sort proteins within the mammalian cell secretory pathway. How these signaling systems are used to regulate the dynamic localization of furin, and other itinerant membrane proteins, to specific compartments is currently being studied.

Experimental Procedures

Yeast Two-Hybrid Screen and Cloning

All methods used for yeast two-hybrid analysis are described elsewhere (Liu et al., 1997). The mouse embryo cDNA library was subcloned into pVP16 (HSV activation domain). DNA sequences encoding the native furin-cd or the DDD and ADA mutants were amplified by PCR and subcloned into pBTM116 (LexA DNA-binding domain). Quantitative β-gal assays were performed using dual transfected cells. Washed cells were resuspended in phosphate buffer and frozen/thawed, and clarified supernatants were incubated with ONPG (0.75 mg/ml) at 30°C and measured for β-gal activity (A-420). Cloning procedures were performed as described (Sambrook et al., 1989). The λgt10 rat brain cDNA library was provided by J. Adelman.

Bacterial Fusion Protein Production

pGEX-3X plasmids expressing either the native furin-cd or the DDD and ADA mutants are described elsewhere (Jones et al., 1995). pGEX-3X expressing the GST-CI-MPR cd's were provided by S. Tooze. The VZV-gE cd was obtained by PCR amplification (5′ primer-CGCGGATCCGGGTTAAAGCCTATAGGGTAG and 3′ primer-CGGAATTCCGGGTCTTATCTATATACACCGTG) of pTM1-VZVgE(wt) and subcloned into pGEX-3X following digestion with BamHI and EcoRI. Methods for isolation of GST-fusion proteins were performed as described (Pharmacia). HtPACS-1fbr was generated by PCR using pVP16:PACS as the template (5′ primer-GGAATTCCATATGCCCAG GCTCTTCAGCTTAAC; 3′ primer-CGGGATCCATGGGCTGGCTGGA TAAAG). The PCR product was subcloned into pET16b (Novagen), digested with NdeI and BamHI, expressed in *E. coli* BL-21 lysS, and purified by Ni-chromatography. HtABP-280 containing amino acids P-1490→A-1607 of human ABP-280 is described elsewhere (Liu et al., 1997).

Cell Culture and Vaccinia Virus

BSC-40, AtT-20, and A7 cells were grown as described (Liu et al., 1997). To generate antisense cell lines, pZVneo:PACS-1b (below) was digested with BamHI and XhoI, and the DNA insert was subcloned in reverse orientation in pCEP-4. A7 cells were transfected with either pCEP-4:PACS-1as (antisense clones) or empty vector (control clones). Stable clones were selected with 300 μg/ml hygromycin B (Sigma).

Vaccinia virus (VV) recombinants expressing fur/f, fur/f$S_{773,775}$D, fur/f$S_{773,775}$A, fur/fE_{783}t, fur/fP_{772}t, and fur/fS_{758}t have been described (Molloy et al., 1994; Jones et al., 1995). To generate VV recombinants expressing PACS-1a or PACS-1b, the cDNA inserts were excised from pGEM7Zf(+)PACS-1a and pGEM7Zf(+)PACS-1b by digestion with SphI and EcoRI, blunt-ended with Klenow and T4 DNA polymerase, and subcloned into pZVneo. Vaccinia recombinants expressing PACS-1a and PACS-1b were generated by standard methods (Liu et al., 1997). To generate the VV recombinant expressing HtPACS-1b, a 10× His C tail was inserted into the *PACS-1b* cDNA by PCR [5′ primer-TACGACAGTCTAGAAATGTAC, 3′ primer- GGAATTCATCAA $(TGA)_{10}$CCCAAGATTACCTGTGTGC]. The mutant DNA fragment was cut with XbaI and EcoRI and subcloned into pGEM7Zf(+)PACS-1b. The *HtPACS-1b* cDNA was then subcloned into pZVneo as described above, and a VV recombinant was generated. HtPACS-1b contains all but the C-terminal 15 residues of PACS-1b ORF, which are replaced by the His-tag.

In Vitro Binding Assays

bbc was prepared as described (Dittié et al., 1997). Binding assays contained 200 μl bbc (4 μg/ml) combined with GST- or His-tagged proteins and were performed in 25 mM HEPES, 25 mM KCl, 2.5 mM KOAc (pH 7.2) (supplemented with 0.5 mM PMSF and 5 μg/ml leupeptin), except where indicated in the figure legends. Proteins were recovered with glutathione- or Ni-agarose, eluted with 2× SDS-sample buffer (SDS-SB), resolved by SDS-PAGE, and analyzed by Western blot with either MAb 100/3 (γ-adaptin) or antiserum 7704 (PACS-1) and HRP-conjugated secondary antibodies. Chemiluminescent signals were quantified using NIH Image software.

Immunofluorescence Microscopy and Antibodies

Immunofluorescence analyses were performed as previously described (Molloy et al., 1994). FLAG-tagged furin was detected with MAb M2 (IBI). Endogenous furin was detected with MAb MON152 (W. van de Ven) or a polyclonal antiserum against the furin-cd (ABR, Liu et al., 1997); γ-adaptin was detected using MAb 100/3 (Sigma). The anti-mannosidase II antiserum was provided by K. Moremen, the anti-TGN46 antiserum was provided by V. Ponnambalam, and the anti-CI-MPR antiserum was provided by B. Hoflack. Alpha-tubulin was detected with MAb N356 (Amersham). Staining of primary antibodies was visualized with species- or subtype-specific secondary antibodies (Fisher) conjugated to FITC or TXR. r-Tf and TXR-dextran internalization assays were performed as described (Liu et al., 1997).

To generate antiserum 678, the 422 nt DNA fragment encoding the fbr was amplified from pPACS-1a by PCR (5′ primer-AGCTCGGATCCGGCCGCTCAG; 3′ primer-GTTTTCCCAGTCACGAC), digested with BamHI and EcoRI and subcloned into pGEX-3X. The GST fusion protein was expressed in *E. coli* strain BL21, purified by glutathione agarose followed by SDS-PAGE, and the emulsified acrylamide gel was used to immunize rabbits. To generate antiserum 7704, the 859 nt fragment encoding residues Met-403 to the PACS-1b C-terminus (including 121 amino acids common to both PACS-1a and PACS-1b) was amplified by PCR (5′ primer- GGAATTCCATATGCCCAGGC TCTTCAGCTTAAC; 3′ primer- CGGGATCCATGGGCTGGCTGGATA AAG), using pGEM7Zf(+)PACS-1b as template. The fragment was digested with NdeI and BamHI and subcloned into pET16B. The His-tagged fusion protein was used to immunize rabbits as described above.

TGN Budding Assays

Methods were adapted from established protocols (Xu and Shields, 1993). AtT-20 cells (7×10^6) were infected with VV recombinants expressing either fur/f, fur/fDDD, fur/fADA, or one of the fur/f truncation mutants. At 5 hr postinfection, the cells were labeled with [^{35}S]Met/Cys for 30 min, chased in complete medium (90 min, 20°C), placed on ice, and rinsed two times with cold PBS and one time with hypotonic swelling buffer (15 mM KCl, 10 mM HEPES, [pH 7.2]). Cells were permeabilized by scraping in "breaking" buffer (90 mM KCl, 10 mM HEPES, [pH 7.2]) and were then pelleted at low speed and washed two times with assay buffer. The cell pellet was resuspended in 300 μl total volume including buffer, budding mix (ATP regenerating system and GTP), and bbc (~3 mg/ml final). The budding reaction was incubated (37°C, 60 min) and then placed on ice and the broken cells separated from post-TGN vesicles by centrifugation (15,000 × g, 2 min). The supernatant and pellet were diluted into mRIPA and the FLAG-tagged furin constructs were immunoprecipitated with MAb M1 and then resolved by SDS-PAGE.

Acknowledgments

We thank J. Nelson, F. Jean, E. Anderson, and S. Arch for helpful discussions and critical reading of the manuscript. We thank B. Hoflack, V. Ponnambalam, and W. van de Ven for antisera; S. Tooze for plasmids; and C. Grose for VZV DNA. This work was supported

by NIH grants DK37274, DK44629 (G. T.), NRSA DK09394 (G. L.), and T32 DK07680 (S. L. R.).

Received March 17, 1998; revised June 15, 1998.

References

Alconada, A., Bauer, U., and Hoflack, B. (1996). A tyrosine-based motif and a casein kinase II phosphorylation site regulate the intracellular trafficking of the varicella-zoster virus glycoprotein I, a protein localized in the trans-Golgi network. EMBO J. *15*, 6096–6110.

Benmerah, A., Lamaze, C., Begue, B., Schmid, S.L., Dautry-Varsat, A., and Cerf-Bensussan, N. (1998). AP-2/Eps15 interaction is required for receptor-mediated endocytosis. J. Cell Biol. *140*, 1055–1062.

Bennett, M.K., Miller, K.G., and Scheller, R.H. (1993). Casein kinase II phosphorylates the synaptic vesicle protein p65. J. Neurosci. *13*, 1701–1707.

Bruzzaniti, A., Goodge, K., Jay, P., Taviaux, S.A., Lam, M.H., Berta, P., Martin, T.J., Moseley, J.M., and Gillespie, M.T. (1996). PC8, a new member of the convertase family. Biochem. J. *314*, 727–731.

Chen, H.J., Yuan, J., and Lobel, P. (1997). Systematic mutational analysis of the cation-independent mannose 6-phosphate/insulin-like growth factor II receptor cytoplasmic domain. An acidic cluster containing a key aspartate is important for function in lysosomal enzyme sorting. J. Biol. Chem. *272*, 7003–7012.

Cole, N.B., and Lippincott-Schwartz, J. (1995). Organization of organelles and membrane traffic by microtubules. Curr. Opin. Cell Biol. *7*, 55–64.

Diaz, E., and Pfeffer, S.R. (1998). TIP47: a cargo selection device for mannose 6-phosphate receptor trafficking. Cell *93*, 433–443.

Dittié, A.S., Thomas, L., Thomas, G., and Tooze, S.A. (1997). Interaction of furin in immature secretory granules from neuroendocrine cells with the AP-1 adaptor complex is modulated by casein kinase II phosphorylation. EMBO J. *16*, 4859–4870.

Foti, M., Mangasarian, A., Piguet, V., Lew, D.P., Krause, K.H., Trono, D., and Carpentier, J.L. (1997). Nef-mediated clathrin-coated pit formation. J. Cell Biol. *139*, 37–47.

Gerber, H.P., Seipel, K., Georgiev, O., Hofferer, M., Hug, M., Rusconi, S., and Schaffner, W. (1994). Transcriptional activation modulated by homopolymeric glutamine and proline stretches. Science *263*, 808–811.

Goodman, O.B., Jr., Krupnick, J.G., Gurevich, V.V., Benovic, J.L., and Keen, J.H. (1997). Arrestin/clathrin interaction. Localization of the arrestin binding locus to the clathrin terminal domain. J. Biol. Chem. *272*, 15017–15022.

Hartmann, C., and Jäckle, H. (1997). Putative Krüppel target gene of novel sequence expressed in Drosophila VO5 muscle precursors. Dev. Genes Evol. *207*, 186–193.

Honing, S., Griffith, J., Geuze, H.J., and Hunziker, W. (1996). The tyrosine-based lysosomal targeting signal in lamp-1 mediates sorting into Golgi-derived clathrin-coated vesicles. EMBO J. *15*, 5230–5239.

Ikonen, E., de Almeid, J.B., Fath, K.F., Burgess, D.R., Ashman, K., Simons, K., and Stow, J.L. (1997). Myosin II is associated with Golgi membranes: identification of p200 as nonmuscle myosin II on Golgi-derived vesicles. J. Cell Sci. *110*, 2155–2164.

Jones, B.G., Thomas, L., Molloy, S.S., Thulin, C.D., Fry, M.D., Walsh, K.A., and Thomas, G. (1995). Intracellular trafficking of furin is modulated by the phosphorylation state of a casein kinase II site in its cytoplasmic tail. EMBO J. *14*, 5869–5883.

Korner, C., Herzog, A., Weber, B., Rosorius, O., Hemer, F., Schmidt, B., and Braulke, T. (1994). In vitro phosphorylation of the 46-kDa mannose 6-phosphate receptor by casein kinase II. Structural requirements for efficient phosphorylation. J. Biol. Chem. *269*, 16529–16532.

Kozak, M. (1991). Structural features in eukaryotic mRNAs that modulate the initiation of translation. J. Biol. Chem. *266*, 19867–19870.

Letourneur, F., Gaynor, E.C., Hennecke, S., Demolliere, C., Duden, R., Emr, S.D., Riezman, H., and Cosson, P. (1994). Coatomer is essential for retrieval of dilysine-tagged proteins to the endoplasmic reticulum. Cell *79*, 1199–1207.

Lin, F.T., Krueger, K.M., Kendall, H.E., Daaka, Y., Fredericks, Z.L., Pitcher, J.A., and Lefkowitz, R.J. (1997). Clathrin-mediated endocytosis of the beta-adrenergic receptor is regulated by phosphorylation/dephosphorylation of beta-arrestin1. J. Biol. Chem. *272*, 31051–31057.

Liu, G., Thomas, L., Warren, R.A., Enns, C.A., Cunningham, C.C., Hartwig, J.H., and Thomas, G. (1997). Cytoskeletal protein ABP-280 directs the intracellular trafficking of furin and modulates proprotein processing in the endocytic pathway. J. Cell Biol. *139*, 1719–1733.

Lupas, A., Van Dyke, M., and Stock, J. (1991). Predicting coiled coils from protein sequences. Science *252*, 1162–1164.

Mallet, W.G., and Brodsky, F.M. (1996). A membrane-associated protein complex with selective binding to the clathrin coat adaptor AP1. J. Cell Sci. *109*, 3059–3068.

Mauxion, F., Le Borgne, R., Munier-Lehmann, H., and Hoflack, B. (1996). A casein kinase II phosphorylation site in the cytoplasmic domain of the cation-dependent mannose 6-phosphate receptor determines the high affinity interaction of the AP-1 Golgi assembly proteins with membranes. J. Biol. Chem. *271*, 2171–2178.

Meresse, S., and Hoflack, B. (1993). Phosphorylation of the cation-independent mannose 6-phosphate receptor is closely associated with its exit from the trans-Golgi network. J. Cell Biol. *120*, 67–75.

Molloy, S.S., Thomas, L., VanSlyke, J.K., Stenberg, P.E., and Thomas, G. (1994). Intracellular trafficking and activation of the furin proprotein convertase: localization to the TGN and recycling from the cell surface. EMBO J. *13*, 18–33.

Munro, S. (1995). An investigation of the role of transmembrane domains in Golgi protein retention. EMBO J. *14*, 4695–4704.

Munro, S., and Pelham, H.R.B. (1987). A C-terminal signal prevents secretion of luminal ER proteins. Cell *48*, 899–907.

Nagafuchi, S., Yanagisawa, H., Ohsaki, E., Shirayama, T., Tadokoro, K., Inoue, T., and Yamada, M. (1994). Structure and expression of the gene responsible for the triplet repeat disorder, dentatorubral and pallidoluysian atrophy (DRPLA). Nat. Genet. *8*, 177–182.

Nakagawa, T., Murakami, K., and Nakayama, K. (1993). Identification of an isoform with an extremely large Cys-rich region of PC6, a Kex2-like processing endoprotease. FEBS Lett. *327*, 165–171.

Norais, N., Hall, J.A., Gross, L., Tang, D., Kaur, S., Chamberlain, S.H., Burke, R.L., and Marcus, F. (1996). Evidence for a phosphorylation site in cytomegalovirus glycoprotein gB. J. Virol. *70*, 5716–5719.

Ohno, H., Fournier, M.C., Poy, G., and Bonifacino, J.S. (1996). Structural determinants of interaction of tyrosine-based sorting signals with the adaptor medium chains. J. Biol. Chem. *271*, 29009–29015.

Okamoto, C.T., Song, W., Bomsel, M., and Mostov, K.E. (1994). Rapid internalization of the polymeric immunoglobulin receptor requires phosphorylated serine 726. J. Biol. Chem. *269*, 15676–15682.

Petersen, C.M., Nielsen, M.S., Nykjaer, A., Jacobsen, L., Tommerup, N., Rasmussen, H.H., Roigaard, H., Gliemann, J., Madsen, P., and Moestrup, S.K. (1997). Molecular identification of a novel candidate sorting receptor purified from human brain by receptor-associated protein affinity chromatography. J. Biol. Chem. *272*, 3599–35605.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning, Second Edition, Vol. 1–3, C. Nolan, ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Schäfer, W., Stroh, A., Berghofer, S., Seiler, J., Vey, M., Kruse, M.L., Kern, H.F., Klenk, H.D., and Garten, W. (1995). Two independent targeting signals in the cytoplasmic domain determine trans-Golgi network localization and endosomal trafficking of the proprotein convertase furin. EMBO J. *14*, 2424–2435.

Schekman, R., and Orci, L. (1996). Coat proteins and vesicle budding. Science *271*, 1526–1533.

Schmid, S.L. (1997). Clathrin-coated vesicle formation and protein sorting: an integrated process. Annu. Rev. Biochem. *66*, 511–548.

Seaman, M.N.J., Sowerby, P.J., and Robinson, M.S. (1996). Cytosolic and membrane-associated proteins involved in the recruitment of AP-1 adaptors onto the trans-Golgi network. J. Biol. Chem. *271*, 25446–25451.

Simpson, F., Peden, A.A., Christopoulou, L., and Robinson, M.S. (1997). Characterization of the adaptor-related protein complex, AP-3. J. Cell Biol. *137*, 835–845.

Sohlemann, P., Hekman, M., Puzicha, M., Buchen, C., and Lohse, M.J. (1995). Binding of purified recombinant beta-arrestin to guanine-nucleotide-binding-protein-coupled receptors. Eur. J. Biochem. *232*, 464–472.

Stoffers, D.A., Quafik, L.H., and Eipper, B.A. (1991). Characterization of novel mRNAs encoding enzymes involved in peptide α-amidation. J. Biol. Chem. *266*, 1701–1707.

Takahashi, S., Nakagawa, T., Banno, T., Watanabe, T., Murakami, K., and Nakayama, K. (1995). Localization of furin to the trans-Golgi network and recycling from the cell surface involves Ser and Tyr residues within the cytoplasmic domain. J. Biol. Chem. *270*, 28397–28401.

Wilde, A., and Brodsky, F.M. (1996). In vivo phosphorylation of adaptors regulates their interaction with clathrin. J. Cell Biol. *135*, 635–645.

Wong, S.H., and Hong, W. (1993). The SXYQRL sequence in the cytoplasmic domain of TGN38 plays a major role in trans-Golgi network localization. J. Biol. Chem. *268*, 22853–22862.

Xin, X., Varlamov, O., Day, R., Dong, W., Bridgett, M.M., Leiter, E.H., and Fricker, L.D. (1997). Cloning and sequence analysis of cDNA encoding rat carboxypeptidase D. DNA Cell Biol. *16*, 897–909.

Xu, H., and Shields, D. (1993). Prohormone processing in the trans-Golgi network: endoproteolytic cleavage of prosomatostatin and formation of nascent secretory vesicles in permeabilized cells. J. Cell Biol. *122*, 1169–1184.

Yao, Z., Jackson, W., and Grose, C. (1993). Identification of the phosphorylation sequence in the cytoplasmic tail of the varicella-zoster virus Fc receptor glycoprotein gpI. J. Virol. *67*, 4464–4473.

Brookhaven Protein Data Bank ID Codes

The PACS-1a and PACS-1b sequences have been deposited in the Brookhaven Protein Data Bank under ID codes AF076183 and AF075184, respectively.