Many peptide hormones and neuropeptides are processed by members of the subtilisin-like family of prohormone convertases (PCs), which are either soluble or integral membrane proteins. PC1 and PC2 are soluble PCs that are primarily localized to large dense core vesicles in neurons and endocrine cells. We examined whether PC1 and PC2 were active when expressed as membrane-tethered proteins, and how tethering to membranes alters the biosynthesis, enzymatic activity, and intracellular routing of these PCs. PC1 and PC2 chimeras were constructed using the transmembrane domain and cytoplasmic domain of the amidating enzyme, peptidylglycine α-amidating monoxygenase (PAM). The membrane-tethered PCs were rerouted from large dense core vesicles to the Golgi region. In addition, the chimeras were transiently expressed at the cell surface and rapidly internalized to the Golgi region in a fashion similar to PAM. Membrane-tethered PC1 and PC2 exhibited changes in pro-domain maturation rates, N-glycosylation, and in the pH and calcium optima required for maximal enzymatic activity against a fluorogenic substrate. In addition, the PC chimeras efficiently cleaved endogenous pro-opiomelanocortin to the correct bioactive peptides. The PAM transmembrane domain/cytoplasmic domain also prevented stimulated secretion of pro-opiomelanocortin products in AtT-20 cells.

The activation of peptides and neuropeptides is a multi-step process involving post-translational modifications such as endoproteolysis, phosphorylation, glycosylation, and amidation, as well as intracellular sorting, routing, and storage events. In many cases, endoproteolytic cleavage of pro-proteins is performed by the prohormone convertases (PCs) such as PC1, PC2, and furin (1–6). For example, the PCs have been shown to cleave pro-opiomelanocortin (POMC) (7, 8), proinsulin (9, 10), pro-β-nerve growth factor (11), proenkephalin (12), dynorphin (13), and thyrotropin-releasing hormone (14).

The PCs are expressed as inactivezymogens and undergo removal of the pro-domain (Fig. 1) which has been shown to occur via an autocatalytic mechanism (1, 6). The PCs have highly conserved catalytic domains consisting of catalytically essential Asp, His, and Ser residues (Fig. 1, D, H, and S). This region is followed by a conserved P domain that is thought to be involved in regulating the stability, calcium dependence, and pH dependence of the convertases (15, 16). The C-terminal regions of the PCs are less well conserved; PC1 and PC2 contain a C-terminal amphipathic α-helical region, whereas furin (1–6), PC5/PC6B (17, 18), and PC8/PC7 (19, 20) (also known as the lymphoma prohormone convertase) (21) contain a hydrophobic C-terminal transmembrane domain (TMD). Moreover, furin, PACE4, and PC5/PC6 contain a luminal cysteine-rich region. Although the exact function of this region is unknown, C-terminally truncated furin was found to be active (22), whereas pro-PACE4 cleavage was accelerated in a PACE4 mutant lacking the cysteine-rich region (23, 24).

Full activation of PC1 requires acidic pH and millimolar calcium concentrations (16, 25, 26), conditions found in the trans-Golgi network (TGN) and in immature secretory granules that have budded from the TGN (27–30). Consistent with the enzymatic studies, PC1 has been localized to the TGN as well as secretory granules (31–33). PC2 has a more acidic pH requirement than PC1, and its late actions on POMC and insulin suggest that it acts in a post-Golgi compartment or in secretory granules (8, 12, 34–38). In contrast, furin has a neutral pH optimum and at steady state is co-localized with the TGN marker protein, TGN38 (11, 39–42). Taken together, the subcellular localization and intracellular conditions required for optimal enzyme activity (16, 43) support the idea that PC1 and PC2 normally function in a different subcellular compartment than furin.

Following endoproteolytic cleavage of proteins by the PCs, C-terminal amino acids are trimmed by carboxypeptidase E (44, 45), and glycine-extended peptides are C-terminally amidated by peptidylglycine α-amidating monoxygenase (PAM) (28, 46, 47). Like furin, PAM is localized to the TGN at steady state; however, confocal light and electron microscopy demonstrated that PAM is located in a more distal subcompartment of the TGN than TGN38 (48). In addition, although the actions of PAM are initiated in the TGN, it primarily acts within immature secretory granules (28, 46–49) along with PC1 and PC2 (31–33, 50). The cytoplasmic domain (CD) of PAM contains routing information for recycling from LDCV and for retrieval via the endocytic pathway (51, 52). For example, the CD of integral membrane PAM (PAM-1) has been shown to redirect the interleukin 2 receptor α-chain, which normally resides on the cell surface, to the TGN (52). Similarly, neuropeptide Y, which is normally a resident of LDCV, was rerouted to the TGN by the addition of the PAM CD (53).
Although all members of the PC family show a requirement for calcium and cleave following basic amino acids, they differ in the presence or absence of a TMD as discussed above. Interestingly, phylogenetic analyses indicate that PC8, which contains a TMD, is the primordial mammalian PC (20, 54). This suggests that loss of the TMD in other members of this family of enzymes occurred later in evolution. It is known that removal of the TMD domain of furin results in a soluble active form of furin (22), but this is not true for all enzymes. For example, missense mutation in the transmembrane domain of the Lewis enzyme, an α(1,3/1,4)-fucosyltransferase involved in the synthesis of type-1 Lewis antigens, leads to decreased Golgi retention and reduced enzymatic activity (55). Conversely, peptidylglycine-α-hydroxylating monoxygenase (one of the two catalytically active domains of PAM) is more active when expressed as a soluble enzyme than in its membrane-bound form (56). To understand better the differences between the membrane-bound and soluble PCs, we asked what effects the addition of a TMD has on the biosynthesis and enzymatic activity of normally soluble PC1 and PC2. PC1 and PC2 were expressed as integral membrane proteins by fusing the C-terminal TMD and CD of PAM in-frame with each PC (Fig. 1). The TMD/CD of integral membrane PAM is a suitable candidate to address this question since PAM is expressed in the same compartments as the soluble PCs and the intracellular routing of PAM has been well characterized. We expressed the PC chimeras in endocrine (AtT-20) and non-endocrine (HEK-293) cells and examined whether PC1 and PC2 were enzymatically active as membrane-bound enzymes. The effects of the PAM TMD/CD on the intracellular localization, routing, protease activation, and substrate specificity of PC1 and PC2 were also examined.

**Materials and Methods**

**Plasmid Construction and Generation of Stable Cell Lines**—The mammalian expression vector pCI.neo (Promega) was used to generate expression constructs for membrane-tethered versions of PC1 and PC2. A Myc epitope tag (MEQKLISEEDLNG), followed by 5 glycine residues, was inserted C-terminal to the PCs (57) (Fig. 1). The resulting PC1 and PC2 chimeras were termed PC1-Myc-CD and PC2-Myc-CD, respectively. To create PC2-Myc-CD, full-length PC2 was fused in-frame to the Myc epitope tag plus the Gly5 linker followed by the terminal 116 amino acid residues of PAM-1 (53). This region of PAM-1 consisted of 9 luminal residues followed by the TMD and CD. The PC1-Myc-CD chimera was created using rat PC1 C-terminally truncated at residue 616 (PC1ΔC) (Fig. 1) to reduce the possibility of endoproteolytic cleavage between PC1 and the C-terminal tag during protein maturation (56).

As a control for the effects of the Myc tag on the rerouting of the PC chimeras, a construct encoding only the signal peptide (residues 1–26) and the N-terminal pro-region of PAM (residues 27–50) was fused in-frame directly to the Myc tag followed by the Gly5, spacer, and the TMD/CD of PAM-1. This construct was termed Myc-TMD/CD (Fig. 1). Two additional TMD/CD constructs were also examined as controls. The first construct contained only the pre- and pro-domains of PAM (terminating at amino acid 35), followed by a Myc tag and the TMD/CD of PAM. The second expression construct also used the pro-domain of PAM but contained the hemagglutinin epitope tag (YPYDVPDYA) (58) instead of the Myc tag. All expression constructs were transfected into mouse pituitary (AtT-20) and human embryonic kidney (HEK-293) cells. Stable cell lines were selected by resistance to G418 (0.5 mg/ml). All cell lines were grown and maintained in Dulbecco’s modified Eagle’s medium/F12 containing 10% fetal bovine serum and 10% NuSerum (Collaborative Research, Bedford, MA).

**Antibodies**—Rabbit polyclonal antisera raised against rPAM-1-(898–
Rerouting of PC1 and PC2 by PAM CD

976) (antibody 571) and rPAM-1(463–864) (antibody 471) were used to detect the cytoplasmic domain (CD) and peptidyl-α-hydroxylysin e α-amidating lyase (PAL) domains of PAM, respectively (60). Monoclonal antibody 6E6 was raised against rPAM-1 CD (48). Polyclonal rabbit antiserum JH93 was raised against ACTH (1–17) (8). JH888 antiserum was raised against synthetic peptide 30% isoupryl alcohol, 0.5% dithiothreitol (DTT) and 0.05% 3-mercaptopropanoic acid in 10 mM NaH2PO4 (pH 7.5), 0.5% Nonidet-P-40, 10 mM β-mercaptoethanol at 37 °C overnight. Immunoprecipitated proteins were resolved by SDS-PAGE (8), fixed and stained with Coomassie blue. Immunoprecipitated POMC products were also resolved on 12% SDS-Page gel in borate/acetate buffer (8).

In Vitro Protease Assays—Non-transfected cells and cells expressing the PAM-1 CD were grown to confluency in 100-mm dishes and collected by scraping into a hypotonic solution containing the protease inhibitors as described under membrane preparations. Cells were homogenized using a hand-held homogenizer, subjected to several cycles of freeze/thawing, and centrifuged at 1,000 × g for 5 min in a crude membrane preparation. The pellet was resuspended and solubilized in 100 mM Tris acetate (pH 7.0) containing 1% Thesit (Roche Molecular Biochemicals) and protease inhibitors. Cell lysates were then incubated at 4 °C, rotating for 1 h with protein G resin which had been cross-linked with monoclonal antibody to the Myc epitope (1 ml of Myc ascites to 1 g of protein G) (71). sPC2 was purified from spent medium of AT-20 cells by binding to PC2 polyclonal antiserum cross-linked to protein A resin (5 ml of PC2 antisera to 1 g of protein A) (48). The antibodies were blocked for 10 min at room temperature prior to use. Following binding, the resin was washed twice in 100 mM Tris acetate (pH 7.0), containing 0.25 mM NaCl, followed by washing in 20 mM Tris acetate (pH 7.0). Enzyme reaction mixtures for PC1-Myc-CD and PC2-Myc-CD contained 75 mM TES, 75 mM sodium acetate (pH 4.0–8.0), 5 mM CaCl2, protease inhibitors and 0.2 mM pyrogulutamyl-Arg-Thr-Lys-Arg-methylcymoaryl-7-amide (62). In vitro enzymes were performed by adding the enzyme reaction mixture directly to the washed antibody-resin complex and incubating at 37 °C for 20 h. Assays were performed in duplicate in 0.5-ml microcentrifuge tubes in a total volume of 100 μl. Following incubation the resin was pelleted by centrifugation, and the supernant collected, transferred to a 96-well plate, and assayed for fluorescence. To generate the standard curve, serial dilutions of the fluorescent 7-amino-4-methylcymoarmin compound were used. Fluorescence was measured using 370 nm excitation and 460 nm emission wavelengths on a 1420 VICTOR2 multilabel counter (EG & G Wallac, Turku, Finland) or a Microflow fluorometer (365/450 nm) (Dynatech). Enzyme activity (pmol/ng protein/h) was calculated for each PC and the resin blank subtracted. Protein content was calculated using the BCA protein determination reagent (Pierce). Cell extracts and proteins bound to the antibody resin were assessed by SDS-PAGE and Western blot analysis using antiserum to PC1, PC2, or the PAM CD.

Stimulated Secretion of ACTH—Duplicate wells of non-transfected and stably transfected AT-20 cells were grown on poly-l-lysine-coated tissue culture plates. Prior to media collections, cells were equilibrated with CSFM for a total of 2 h (media changed and discarded every 30 min). Cells were incubated in fresh medium. Medium was collected for two 30-min periods under basal conditions (CSFM only) followed by one 30-min collection in the presence of 1 mM BaCl2 (72). Harvested medium was centrifuged to remove non-adherent cells, and protease inhibitors were added prior to storage at –80 °C. Cells were extracted with TES containing 1% Triton X-100 for the measurement of Peptidylglycine-α-hydroxylating monooxygenase activity or 10 mM mannitol containing 15 mM potassium chloride for measurement of immunoreactive ACTH. The ACTH immunounassay used C-terminal ACTH antiserum (Kathy) which reacts equally with ACTH biosynthetic intermediate and ACTH but not with intact POMC (28, 49).125I-ACTH (18–39) was used as the tracer, and human ACTH (1–39) peptide (0–20 ng) (Ciba Pharmaceutical Corp.) was used to generate the standard curve. Media samples were prepared in duplicate and assayed in triplicate for ACTH.

RESULTS

Subcellular Distribution of Tethered PCs—To identify the intracellular sites of expression of PCs, stably transfected AT-20 cell lines were examined using immunofluorescence microscopy (Fig. 2). Immunostaining of non-transfected AT-20 cells with antiserum to PC1 revealed a dispersed expression of endogenous PC1, with some accumulation within the perinuclear region (Fig. 2A, arrows). Intense staining was also ob-
arrows staining and tip staining are indicated by stained with the monoclonal antibody to the PAM CD (respectively. AtT-20 cells expressing Myc-TMD/CD were immuno-
stained using polyclonal PC1-specific (sPC2 (respectively.

Comparison of immunofluorescent staining of AtT-20 cells (arrowhead), indicative of expression within secretory granules. Non-transfected AtT-20 cells were also immunostained with antiserum to PC2 (Fig. 2B), and although they do not express significant levels of PC2 mRNA or protein (73), faint cross-reactivity was observed with the DAR-Cy3 secondary antibody (Fig. 2B) but not with the fluorescein isothiocyanate-conjugated goat anti-
rabbit secondary antibody (not shown). Immunofluorescent staining of AtT-20 cells stably expressing sPC2 revealed intense staining of PC2 at the tips of processes (Fig. 2C, arrow-
head) and a diffuse ER-like staining pattern, with lesser perinuclear accumulation. Comparison of immunofluorescent staining of AtT-20 cells stably expressing the PC chimeras revealed a distinctly different staining pattern compared with non-transfected cells. At steady state, PC1-Myc-CD was predominantly localized to the perinuclear region of AtT-20 cells (Fig. 2D, arrow), with decreased staining at the tips of processes, indicative of expres-
sion within LDG (Fig. 2D, arrowhead). Unexpectedly, decreased endogenous PC1 staining in cell processes was observed in cells overexpressing the PC1-Myc-CD chimera (Fig. 2D) compared with non-transfected AtT-20 cells (Fig. 2A). Perinuclear (Fig. 2E, arrow) and tip expression (Fig. 2E, arrow-
head) of PC2-Myc-CD was also observed in stably transfected AtT-20 cells, in contrast to the ER-like staining seen in cells expressing sPC2 (Fig. 2C). Perinuclear staining was also ob-
served in AtT-20 cells expressing the Myc-TMD/CD chimera (Fig. 2F), although with negligible tip staining.

Co-localization with TGN38 and WGA—To better characterize the subcompartments where the membrane-tethered chimeras were expressed, double immunofluorescent staining was performed using antisera to detect the PCs, and antisera to the TGN marker protein (TGN38), or fluorescently labeled wheat germ agglutinin (WGA) to detect the Golgi apparatus (30) (Fig. 3). At steady state, overlapping perinuclear staining of Myc and TGN38 was observed in AtT-20 cells expressing PC1-Myc-CD (Fig. 3A) or Myc-TMD/CD (Fig. 3B). Similarly, overlapping expression of PC2-Myc-CD and WGA staining was observed (Fig. 3C). In contrast, sPC2 cells exhibited a dispersed expres-
sion throughout the cell which was not restricted to the Golgi region (Fig. 3D). PC1-Myc-CD, PC2-Myc-CD, and Myc-
TMD/CD were also localized to the TGN region when expressed in HEK-293 cells (see Fig. 4). Thus, PC1-Myc-CD, PC2-Myc-
CD, and Myc-TMD/CD were largely localized within the TGN of AtT-20 cells at steady state. Moreover, the results indicated that most of PC2-Myc-CD was localized to a different subcom-
artment of AtT-20 cells than sPC2.

The PC chimeras utilized residues 1–50 of PAM and thus contained Cys42 that could potentially form disulfide bonds with endogenous proteins and thus affect the routing of the chimeras. We therefore constructed an additional chimera (Myc-TMD/CD) using only the prepro-domain of PAM (amino acids 1–35) and examined the routing of this protein in AtT-20 cells. The shorter Myc-TMD/CD construct was also localized to the TGN in AtT-20 cells (not shown) indicating that the presence of Cys42 does not alter trafficking. Similarly, substitution of the Myc tag by the hemagglutinin tag also resulted in TGN localization (not shown), demonstrating that the Myc tag was not responsible for the rerouting effects observed in AtT-20 cells.

Co-localization of Different Domains of the Chimeric Mole-
cules—Maturation of wild-type PC1 involves several endoprote-
olytic steps, one of which results in C-terminal truncation of the protein. The PC1-Myc-CD chimera used in this study lacked the potential C-terminal cleavage site (Fig. 1); however, it was still necessary to determine whether the different do-
 mains of the chimeric proteins were predominantly localized to the same region of the cells. To address this, double immuno-
fluorescent labeling was performed using PC-specific antiserum in combination with Myc-specific antisera or CD-specific an-
siserum (Fig. 4). Immunostaining of PC1-Myc-CD expressing AtT-20 cells revealed co-localization of the PC-specific antibody and CD-specific antibody primarily within the perinuclear re-
gion of the cell; the PC1 antibody detects both endogenous PC1 and PC1-Myc-CD (Fig. 4, A and B). Similarly, immunostaining of AtT-20 cells expressing PC2-Myc-CD revealed co-localization of the PC-specific antibody with the Myc-specific antibody and with the CD-specific antibody (Fig. 4, C and D). Co-localization of antibodies to the Myc and CD domains was also observed in AtT-20 cells expressing Myc-TMD/CD (Fig. 4E).

The PC chimeras and the Myc-TMD/CD protein were also stably expressed in non-endocrine HEK-293 cells. Immunostaining of HEK-293 cells revealed a predominantly perinu-
clear expression as in AtT-20 cells (Fig. 4, F and G). Co-locali-
zation of antibodies to the different chimeric domains was also observed in HEK-293 cells at steady state. In summary, co-
localization of antibodies with specificities for the different domains of the PC chimeras suggested that the chimeras stayed intact when expressed in AtT-20 cells and in HEK-293 cells. However, immunostaining alone does not exclude the possibility of some cleavage, especially in the case of PC2-
Myc-CD which was expressed largely in the TGN region with barely detectable amounts at the tips of cells (see Fig. 4C). In addition, immunostaining revealed that the PC chimeras were expressed in comparable amounts to the soluble PCs. This was supported by Western blot analyses (see below).

Tethered PCs Reach the Cell Surface and Are Internal-
ized—It was previously established that integral membrane PAM proteins reach the cell surface of AtT-20 and HEK-293 cells—and are rapidly internalized via endosomes (46, 47, 51). This effect was mediated via signals present within the cyto-
plasmic domain, since PAM-1 truncated immediately after the TMD failed to internalize efficiently and accumulated on the cell surface (47, 51). To determine if PC1-Myc-CD and PC2-Myc-CD underwent trafficking events similar to PAM-1, antibody internalization assays were performed. Cells were incubated in serum-free medium containing BSA and PC-specific antiserum, washed, and chased before visualizing with fluorescently tagged secondary antibody (Fig. 5).

Following internalization, punctate vesicular staining localized to the perinuclear region and some tip staining was observed in AtT-20 cells expressing PC1-Myc-CD, suggesting that a small proportion of the internalized PC1 antibody reached secretory granules (Fig. 5A). This observation was not simply the result of staining of endogenous wild-type PC1 since internalization of the PC1 antibody was not observed in non-transfected AtT-20 cells (not shown). Perinuclear staining was also observed in PC2-Myc-CD cells following internalization assays using the PC2 antibody (Fig. 5B). The punctate perinuclear staining of the PC chimeras was similar to the internalization pattern observed for PAM-1-expressing AtT-20 cells incubated with a polyclonal antibody to the PAL domain (Fig. 5C) (47). By using a similar internalization paradigm, the Myc monoclonal antibody was also internalized from the cell surface of HEK-293 cells (Fig. 5D) and AtT-20 cells (not shown) expressing the Myc-TMD/CD protein. For all of the PC chimeras examined, internalization from the cell surface was only observed in cell lines expressing high levels of the chimeras, suggesting that surface expression could be the rate-limiting step.

**Western Blot Analysis of PC1-Myc-CD and PC2-Myc-CD**—Analysis of AtT-20 cells stably expressing the chimeric proteins was performed by SDS-PAGE followed by Western blot analysis using PC-specific or PAM-1 CD-specific antisera (Fig. 6). Mature PC1 (82 kDa) and C-terminally truncated PC1ΔC (66 kDa) were the predominant forms of endogenous PC1 detected in non-transfected AtT-20 cells (Fig. 6A, NT). In contrast, only one major protein species was detected with the CD-specific antiserum in AtT-20 cells expressing PC1-Myc-CD (Fig. 6A, PC1-Myc-CD). This was presumed to be a mixture of pro-PC1-Myc-CD (84 kDa) and mature PC1-Myc-CD (79 kDa) which co-migrated due to the presence of a large number of N-linked sugars, as discussed below.
Western blot analysis revealed two major protein species corresponding to pro-sPC2 (76 kDa) and mature sPC2 (64 kDa) in AtT-20 cells expressing sPC2, whereas in stably transfected AtT-20 cells expressing PC2-Myc-CD, pro-PC2-Myc-CD (98 kDa), and mature PC2-Myc-CD (86 kDa) were observed (Fig. 6B). Western blot analyses performed using PC2-specific antiserum also revealed a low molecular mass protein species of approximately 30 kDa. Although this was of low abundance compared with full-length PC2-Myc-CD, it suggested some C-terminal cleavage of PC2-Myc-CD in AtT-20 cells (data not shown).

Tethered PCs Are Membrane-bound—To establish whether the PC chimeras were membrane-associated proteins when expressed in AtT-20 cells, stably transfected cells were homogenized and separated into soluble and crude particulate membrane fractions by centrifugation. Membrane fractions were further washed with high pH carbonate buffer to remove peripheral proteins. Aliquots of each fraction were solubilized, resolved by SDS-PAGE, and analyzed by Western blot analysis using polyclonal antisera to detect PC1-Myc-CD, sPC2, and PC2-Myc-CD were pulse-labeled for 20 min, chased for 2 h, and analyzed as above. Immunoprecipitated samples were treated without (−) or with (+) N-glycosidase F (N-gly, overnight at 37 °C. Arrows indicate size changes following deglycosylation. Data are representative of three independent experiments. Apparent molecular masses are in kDa.

MATURATION OF PC1-MYCD AND PC2-MYCD—To examine early maturation events, stable cell lines expressing the PCs were metabolically labeled for 5 min and chased for 5, 10, or 15 min in CSFM without the radioactive isotope. Radiolabeled PCs were immunoprecipitated with PC-specific or CD-specific antiserum (not shown). The PC1 antibody detected immature and processed forms of wild-type PC1 (88, 82, and 67 kDa). B, AtT-20 cells expressing sPC2 or PC2-Myc-CD were labeled for 20 min, chased for 30, 60, or 120 min, and harvested as in Fig. 5. Immunoprecipitation of sPC2 was performed using PC2-specific antiserum, and PC2-Myc-CD was immunoprecipitated with CD-specific antiserum. Asterisk indicates an intermediate form of PC2-Myc-CD. C, non-transfected AtT-20 cells and stably transfected AtT-20 cells expressing PC1-Myc-CD, sPC2, and PC2-Myc-CD were pulse-labeled for 20 min, chased for 2 h, and analyzed as above. Immunoprecipitated samples were treated without (−) or with (+) N-glycosidase F (N-gly), overnight at 37 °C. Arrows indicate size changes following deglycosylation. Data are representative of three independent experiments. Apparent molecular masses are in kDa.

Efficient conversion of pro-sPC2 (76 kDa) to the mature form (64 kDa) was observed in AtT-20 cell extracts following a 2-h chase (Fig. 7B, left panel), consistent with the delayed maturation of sPC2 (8, 35, 61, 68–70, 75). As with sPC2, inefficient processing of pro-PC2-Myc-CD (98 kDa) to mature PC2-Myc-CD (86 kDa) was evident following the 2-h chase period (Fig. 7B, right panel). An intermediate processed form of PC2-Myc-CD (91 kDa), probably the result of cleavage after the first tetrabasic Lys-Arg-Arg-Arg site, was also observed in
PC2-Myc-CD expressing AtT-20 cells (Fig. 7B, asterisk). An intermediate processed form of sPC2 was also detected in longer exposures of SDS-PAGE gels (not shown). Increasing the chase times to 4 and 8 h did not lead to a substantial increase in the processing of PC2-Myc-CD (data not shown).

Wild-type PC1 and PC2 have three predicted N-glycosylation sites (2, 76–78). To examine whether PC1-Myc-CD and PC2-Myc-CD were N-glycosylated, cell extracts from a 2-h chase period were immunoprecipitated and deglycosylated with N-glycosidase F. PC1ΔC (66 kDa) was the major form of endogeneous PC1 detected in the 2-h chase samples and decreased in size by approximately 2 kDa following digestion with N-glycosidase F (Fig. 7C). In contrast, a 7–10-kDa decrease in the size of PC1-Myc-CD was observed following deglycosylation (Fig. 7C). The increased glycosylation of PC1-Myc-CD compared with the wild-type PC1 was unexpected given that wild-type PC1 contained three potential N-glycosylation sites, whereas PC1-Myc-CD, which was constructed using PC1ΔC, contained only two potential N-glycosylation sites (Asn173 and Asn401). This suggests that PC1-Myc-CD was exposed to the glycosylat- ing enzymes for a longer period and/or was exposed to a different set of glycosylating enzymes (79). The immature and processed sPC2 and PC2-Myc-CD were both sensitive to treatment with N-glycosidase F (Fig. 7C). However, sPC2 decreased in size by approximately 6 kDa, whereas PC2-Myc-CD underwent a 12-kDa decrease in molecular mass following deglycosylation. The increased glycosylation of PC2-Myc-CD may reflect glyco- sylation at additional sites, since not all potential N-glycosyla- tion sites are equally used in PC2 (1, 7, 8, 16, 80).

Wild-type PC1 and sPC2 were easily recovered from spent medium following the 2-h chase period using PC-specific antisera (not shown). However, immunoprecipitation using PC-specific or Myc-specific antisera failed to recover any protein from the spent media of AtT-20 cells expressing PC1-Myc-CD or PC2-Myc-CD, even following longer chase times (up to 4 h; data not shown). Thus, the PC chimeras are not cleaved and therefore cannot be secreted under basal conditions.

In vitro Enzymatic Activity of Tethered PCs—Since AtT-20 cells endogenously express wild-type PC1, as well as other PCs such as furin and PC8, it was necessary to purify PC1-Myc-CD and PC2-Myc-CD prior to analysis of enzymatic activity. Cells were collected by scraping, lysed by freeze/thawing, and mem- brane fractions prepared and solubilized. The PC chimeras were then immunosolated using the Myc monoclonal antibody cross-linked to protein G resin (Fig. 8). Western blot analysis revealed that the PC chimeras remained bound to the antibody resin after washing, whereas wild-type PC1 was removed (Fig. 8A). By using a similar immunoprecipitation procedure, sPC2 was purified from spent medium of stably transfected AtT-20 cells using PC2 antibody cross-linked to protein A resin (16, 63) (not shown). Soluble PC1 (sPC1) was collected from the spent media of HEK-293 cells expressing PC1.

Metabolic labeling studies (see Fig. 7) demonstrated that the PC chimeras underwent maturation of the pro-region while membrane-tethered. It was therefore possible that the PC chimeras would be catalytically active while bound to protein G, thus eliminating the need for high salt washes and the extreme pH values required to elute the PCs from the antibody resin. In vitro enzyme assays were performed by adding the synthetic fluorogenic substrate directly to the PC-antibody-resin complex. Fluorescence was measured and enzymatic activity of the PC chimeras determined (Fig. 8B). The results clearly demonstrated that PC1-Myc-CD and PC2-Myc-CD isolated from AtT-20 cells were active in cleaving the pyroglutamyl-Arg-Thr-Lys-Arg-methylcoumaryl-7-amide substrate while still bound to the antibody resin.

We next examined whether expressing the PCs as trans- membrane proteins altered their pH and calcium require- ments. First, the enzymatic activities of the PC chimeras and the soluble PCs were compared at different pH values (Fig. 8, C and D). Concordant with a previous study (16), the pH optimum of sPC1 collected from spent medium of HEK-293 cells was pH 5.5–6.0 (Fig. 8C). Interestingly, PC1-Myc-CD had a much broader pH optimum (pH 6.0–7.0) than sPC1 (Fig. 8C). This difference was most apparent at neutral pH where PC1- Myc-CD was fully active and sPC1 exhibited only 25% of its maximum activity. As expected, integral membrane PC2- Myc-CD and sPC2 were active at more acidic pH values than PC1; PC2-Myc-CD and sPC2 exhibited a pH optimum pH 5.0 (Fig. 8D). However, unlike PC1-Myc-CD, both PC2-Myc-CD and sPC2 exhibited a narrower pH range where they were maximally active, although PC2-Myc-CD was more active than sPC2 at more neutral pH values (Fig. 8D).

By using the optimal pH values for each of the PCs, the calcium requirements for maximal enzymatic activity of the soluble and integral membrane PCs were compared. Both sol- able and membrane-tethered PCs exhibited a requirement for calcium (Fig. 8, E and F). PC1-Myc-CD exhibited half-maximal activity (K0.5) at 2.5 mM calcium (Fig. 8E), whereas sPC1 had a K0.5 of 0.8 mM calcium. The calcium requirements for half- maximal activity of PC2-Myc-CD and sPC2 were both 1 mM (Fig. 8F).

Effects of PC Chimeras on Cleavage of Endogenous POMC—It was previously established that PC1 and PC2 expressed in AtT-20 cells act sequentially to cleave POMC, generating products that are representative of processing events seen in intermediate pituitary melanotropes (7, 8, 35, 61, 73, 81, 82). Since PC1-Myc-CD and PC2-Myc-CD are catalytically active in vitro, the effects of the PC chimeras on cleavage of endogenous POMC were examined by metabolic labeling followed by immu- noprecipitation with an antibody directed against the N- terminal portion of ACTH (Fig. 9). As expected, POMC was cleaved primarily to ACTH and glycosylated ACTH in non- transfected AtT-20 cells. However, increased production of ACTH and glycosylated ACTH was observed in cells expressing either PC1-Myc-CD or PC2-Myc-CD. In addition, an increased amount of ACTH-(1–13)-NH2 was detected in medium collected from PC1-Myc-CD- and PC2-Myc-CD-expressing cells. These findings were reproduced using two independent cell clones for each chimera. Additional evidence for the catalytic activity of both the soluble and membrane-tethered PCs on endogenous POMC was obtained using an ACTH radioimmunoassay with antiserum directed against ACTH-(11–24) which detects POMC precursor and ACTH but is unable to detect the ACTH- derived products, ACTH-(1–13)-NH2 and CLIP (data not shown).

Effects of PAM TMD/CD on Secretion of POMC Products—Since the metabolic labeling experiments suggested that expression of the chimeras affected secretion of newly synthe- sized POMC products (Fig. 9), we examined the effects of chimera expression on constitutive and BaCl2-stimulated se- cretion of POMC-derived products (Fig. 10). Secreted ACTH was quantified using an ACTH-specific antiserum as described previously (72). Results demonstrated little difference in the total amount of POMC products detected in basal media samples collected from all of the cell lines examined (Fig. 10). As expected, non-transfected AtT-20 cells exhibited significant ACTH secretion following BaCl2 stimulation (approximately 10-fold) (Fig. 10). AtT-20 cell expressing sPC2 also exhibited significant ACTH secretion following BaCl2 (approximately 5-fold). PAM-1 is known to block the ability of BaCl2 to stimu- late ACTH secretion using an AtT-20 line engineered for in-
ducible PAM-1 expression (83). Unexpectedly, stimulated secretion of ACTH was also inhibited in AtT-20 cells expressing PC1-Myc-CD, PC2-Myc-CD, or Myc-TMD/CD. Inhibition of regulated ACTH secretion was also observed using an ACTH radioimmunoassay with antiserum directed against ACTH-(11–24) (data not shown). Combined, the data suggest that the TMD/CD of PAM-1 had dramatic effects on the routing of several POMC products within AtT-20 cells independent of the lumenal domain.

**DISCUSSION**

**TGN Localization of the PC Chimeras**—PC1 and PC2 are soluble secretory granule proteins expressed in endocrine and neuroendocrine cells, whereas other PCs such as furin, PC8, and PC6B are membrane-anchored and are TGN-localized. As a means to understand better the differences between the soluble and membrane-bound PCs, we investigated the effects of membrane tethering on the biosynthesis, trafficking, and enzymatic activity of soluble PC1 and PC2. At steady state, PC1-Myc-CD, PC2-Myc-CD, and Myc-TMD/CD occupied a similar perinuclear compartment, coincident with the endogenous expression of TGN38 (Figs. 2 and 3). The distribution of all three proteins closely mimicked the steady state distribution of PAM-1, largely TGN and immature LDCV (48). At steady state, much of the sPC2 in AtT-20 cells remains in the ER (Fig. 2C), and although functional in LDCV (8, 12, 36, 37), significant accumulation of sPC2 in LDCV was not observed (Fig. 2C).

Unlike sPC2, PC2-Myc-CD exhibited TGN accumulation similar to PAM-1, suggesting more rapid exit of PC2-Myc-CD from the ER than sPC2. It has previously been reported that soluble PC2 can undergo C-terminal truncation within insulin secretory granules (84). Although the current data do not support C-terminal cleavage of sPC2, limited cleavage of the PC2 domain within the PC2-Myc-CD chimera cannot be discounted. However, no significant secretion of PC2-Myc-CD was observed. Thus, attachment of the TMD/CD domain of PAM-1 was sufficient to reroute the PCs from LDCV to the TGN. The use of C-terminally truncated PC1 in the construction of the PC1-Myc-CD chimera may have contributed to its rerouting in
Pro-domain Maturation of Tethered PC Chimeras—

The tethered PC chimeras underwent pro-domain cleavage and post-translational N-linked glycosylation (Fig. 7), indicating they moved from the ER to the secretory pathway of AtT-20 cells. Pro-PC1-Myc-CD conversion to mature PC1-Myc-CD occurred faster than conversion of pro-PC1, suggesting that the TMD/CD played a role in accelerating pro-PC1 folding, which is a pre-requisite for pro-domain cleavage. The addition of the TMD/CD to PC2 did not appear to alter the maturation rate of PC2-Myc-CD; both pro-sPC2 and pro-PC2-Myc-CD were inefficiently processed (Fig. 7B). However, increased N-linked glycosylation of PC2-Myc-CD, which presumably occurred in the ER, was observed compared with sPC2 (Fig. 7C). Similarly, increased glycosylation of PC1-Myc-CD compared with endogenous PC1 was observed. N-Glycosylation has been reported to be important in the intracellular stability of the PCs (95). However, in the current study, the membrane-tethered PC chimeras were stable over 4 h.

Enzymatic Activity of the Tethered PC Chimeras; in Vitro Cleavage of a Small Substrate—Although pro-domain maturation of the PC chimeras was demonstrated, it was important to establish that the PC chimeras were functionally active when expressed in AtT-20 cells. This was essential since removal of the pro-region is necessary, but not sufficient, for enzyme activation (96). Recycling of the PC chimeras (Fig. 5) could also potentially lead to their inactivation. Moreover, the possibility existed that the C-terminal Myc tag and PAM TMD/CD altered the conformation of the PCs such that catalytic activity, but not pro-region cleavage, was prevented due to the proximity of the PC catalytic core to the membrane. An in vitro enzymatic assay was therefore used to examine the activity of the PC chimeras. Results demonstrated that PC1-Myc-CD and PC2-Myc-CD were indeed active in cleaving a synthetic peptide substrate. The ability of PC2-Myc-CD to cleave the fluorogenic substrate also suggested that the transient association of PC2 with 7B2 (70, 75, 97) was not adversely affected by addition of the C-terminal tether. In support of this, PC2-Myc-CD (not shown) and sPC2 expressed in HEK-293 cells (63, 70, 75, 97) did not appear to alter the maturation rate of PC2-Myc-CD; both pro-sPC2 and pro-PC2-Myc-CD were inefficiently processed (Fig. 7B). However, increased N-linked glycosylation of PC2-Myc-CD, which presumably occurred in the ER, was observed compared with sPC2 (Fig. 7C). Similarly, increased glycosylation of PC1-Myc-CD compared with endogenous PC1 was observed. N-Glycosylation has been reported to be important in the intracellular stability of the PCs (95). However, in the current study, the membrane-tethered PC chimeras were stable over 4 h.

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agreement with earlier reports showing that the intervening sequence between the P domain and the C-terminal TMD is not essential for catalytic activity of furin (80), although the lumenal domain may play a role in targeting (98).

Interestingly, both PC chimeras exhibited a more neutral pH optimum than their corresponding soluble PCs. This was particularly evident for PC1-Myc-CD which exhibited a broad pH optimum (Fig. 8C) strongly resembling that reported for furin (99). From the current studies it is difficult to determine to what degree the change in the pH optimum of PC1-Myc-CD compared with sPC1 was due to the use of PC1ΔC in the construction of the chimera. Furthermore, spent medium from HEK-293 cells contained a mixture of mature PC1 and C-terminally truncated PC1. However, similar to our findings, PC1ΔC expressed in HEK-293 cells was maximally active at a more neutral pH (pH 6.0) than full-length PC1 (15), whereas in other studies, C-terminally truncated PC1 expressed in Chinese hamster ovary cells exhibited a narrower, more acidic pH range (pH 5.0–5.5) than full-length PC1 (16). Like PC1-Myc-CD, PC2-Myc-CD was active over a broader pH range than sPC2 (Fig. 8D). These findings were surprising given that PC2 has long been considered to be able to act only within mature secretory granules that have an acidic pH environment. Overall, differences in the pH conditions required for maximal activity of the chimeras indicate that the conformation of the catalytic core of tethered PCs, especially in the case of PC1-Myc-CD, was potentially altered by the PAM TMD/CD, although the essential requirement for calcium was maintained.

**Enzymatic Activity of the Tethered PC Chimeras; Cleavage of POMC within the Secretory Pathway**—All of the cleavages of POMC occur at pairs of basic amino acids and are mediated by the catalytic core of tethered PCs, especially in the case of PC1-Myc-CD. Within secretory granules, which are known to have an acidic internal pH. Conversely, the neutral pH optimum of PC1-Myc-CD (pH 6.5) (Fig. 8C) may argue against the actions of PC1-Myc-CD within secretory granules.

**PAM TMD/CD Inhibits Stimulated Secretion of POMC-derived Peptides**—Previous studies from this laboratory demonstrated that overexpression of integral membrane PAM by means of inducible and constitutive expression systems decreased cellular content of ACTH and PC1 and increased constitutive-like secretion of cleaved POMC products (83, 100). In addition, PAM-1 induction resulted in inhibition of stimulated secretion of ACTH. In the current study, stimulated secretion of ACTH was inhibited in AtT-20 cells expressing PAM-1 and also in cells expressing either PC1-Myc-CD, PC2-Myc-CD, or Myc-TMD/CD (Fig. 10). Since regulated secretion of ACTH was observed in non-transfected AtT-20 cells and sPC2-expressing cells, the results suggest that the TMD/CD of PAM-1 caused this effect. It remains to be seen whether these effects are due to changes in filamentous actin, adaptor proteins, or events mediated through integrins or cell-surface expressed proteins. Although minimal luminal domain was used in the construction of the chimeras, and replacement of the Myc epitope with the hemagglutinin epitope had no effect (data not shown), it is conceivable that inhibition of regulated secretion of ACTH was partly due to luminal interactions, since soluble PAM can aggregate with other soluble LDCV proteins (101). Examination of cell lines with different expression levels of the Myc-TMD/CD chimera may be useful to elucidate further the potential mechanisms involved in rerouting of POMC products.

**Conclusions**—This study demonstrated that the TMD/CD of integral membrane PAM caused the rerouting of PC1 from secretory granules, and PC2 from the ER, to the TGN. Membrane tethering altered the maturation rate of the PC1-Myc-CD and caused changes in the pH optimum and calcium requirements for optimal enzymatic activity of both PC1-Myc-CD and PC2-Myc-CD. Expression of normally soluble PCs as integral membrane proteins did not adversely affect their enzymatic activity as both PC1-Myc-CD and PC2-Myc-CD were active in cleaving a short synthetic substrate. Despite their altered steady state locations, both membrane-tethered PCs cleaved POMC to several of its known bioactive peptides within the secretory pathway. These findings suggest that the C-terminal domains of the PCs are important in modulating the enzymatic activity of the PCs as well as determining their intracellular localization.

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