Proprotein Convertase PC3 Is Not a Transmembrane Protein†

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ABSTRACT: Proprotein convertase PC3 (also known as PC1) is an endopeptidase involved in proteolytic processing of peptide hormone precursors in granules of the regulated secretory pathway of endocrine cells. Lacking any extended hydrophobic segments, PC3 was considered to be a secretory protein only peripherally attached to the granule membrane. Recently, evidence has been presented that PC3 is a transmembrane protein with a 115-residue cytoplasmic domain and a membrane-spanning segment containing eight charged amino acids [Arnaoutova, I., et al. (2003) *Biochemistry 42*, 10445−10455]. Here, we analyzed the membrane topology of PC3 and of a PC3 construct containing a conventional transmembrane segment of 19 leucines. Alkaline extraction was performed to assess membrane integration. Exposure to the cytosol or to the ER lumen was tested by addition of C-terminal tags for phosphorylation or glycosylation, respectively. Protease sensitivity was assayed in permeabilized cells. The results show that the C-terminus of PC3 is translocated across the endoplasmic reticulum membrane. Furthermore, the proposed transmembrane segment of PC3 and a similar one of carboxypeptidase E did not stop polypeptide translocation when inserted into a stop-transfer tester construct. PC3 is thus not a transmembrane protein. These results have implications for the mechanism of granule sorting of PC3 as well as for the topology of PC2 and carboxypeptidase E, which have been reported to span the lipid membrane by homologous charged sequences.

Intracellular processing and maturation of prohormones and proneuropeptides are achieved by common proteolytic mechanisms. The processing enzymes include a family of endopeptidases, the proprotein convertases (PCs),[1] that cleave hormone and neuropeptide precursors at dibasic sites (*1*−*3*), and a family of metallocarboxypeptidases that selectively remove the basic amino acids exposed at the new C-termini (*4*). PCs belong to the kexin/subtilisin family of serine proteases. Eight mammalian PCs have been identified. They share a common organization, illustrated in Figure 1A for PC3 (also known as PC1 or PC1/3), with an N-terminal signal peptide for targeting to the endoplasmic reticulum (ER), a propeptide, a catalytic domain, a P-domain, and a variable C-terminal segment. In the case of PC3, the prodomain is autocatalytically cleaved in the ER, converting the ∼95 kDa full-length precursor to an ∼85 kDa intermediate. In secretory granules, a further autoproteolytic cleavage occurs at the C-terminus of the P-domain, producing a ∼64 kDa form with full enzymatic activity.

Some members of the PCs (furin, PC5/6B, and PC7) contain an obvious transmembrane segment in their C-terminal domain and are thus type I membrane proteins. The other members of the family (PC1/3, PC2, PC4, PC5/6A, and PACE4) were generally assumed to be fully translocated into the ER lumen as soluble or peripherally membrane-associated proteins. Indeed, in vitro-translated PC2 and PC3 were shown to associate with TGN/secretory granule membranes and lipid rafts (*5*, *6*). In addition, PC2 and PC3 contain a C-terminal sequence potentially forming an amphipathic helix associating with membranes peripherally. However, evidence has recently been presented on the basis of protease protection, antibody binding, and chemical modification experiments with purified secretory granules, suggesting that PC3 is in fact a type I transmembrane protein with a cytoplasmic C-terminus (*7*). The sequence proposed to act as the membrane-spanning segment was identified as residues 619−638, directly adjacent to the P-domain (QNDRRGVEKMVDPGEEQPTQENPKENTLVS).

Similarly, residues 617−634 at the very end of the 40-amino acid C-terminal domain of PC2 were proposed to function as a transmembrane anchor mainly on the basis of membrane-impermeant biotinylation of purified chromaffin granules (*8*) (SKLAMSKKEELEEELDEAVERSLKSILNKN-COOH).

These sequences were initially identified by similarity to the C-terminal sequence of carboxypeptidase E (CPE), which on isolated secretory granules was found to be sensitive to carboxypeptidase Y digestion and accessible to antibodies against the C-terminus (*9*) (FELESFSERKEEEKEELMEWWKMMSETLNF-COOH).

The transmembrane topology of these proteins is particularly important, because these sequences and the potentially cytoplasmic domains are implicated in lipid raft association and sorting into the regulated secretory pathway. CPE was even proposed to function as a sorting receptor for regulated

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[1] Abbreviations: ER, endoplasmic reticulum; PC, proprotein convertase; CPE, carboxypeptidase E; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; TGN, trans-Golgi network.

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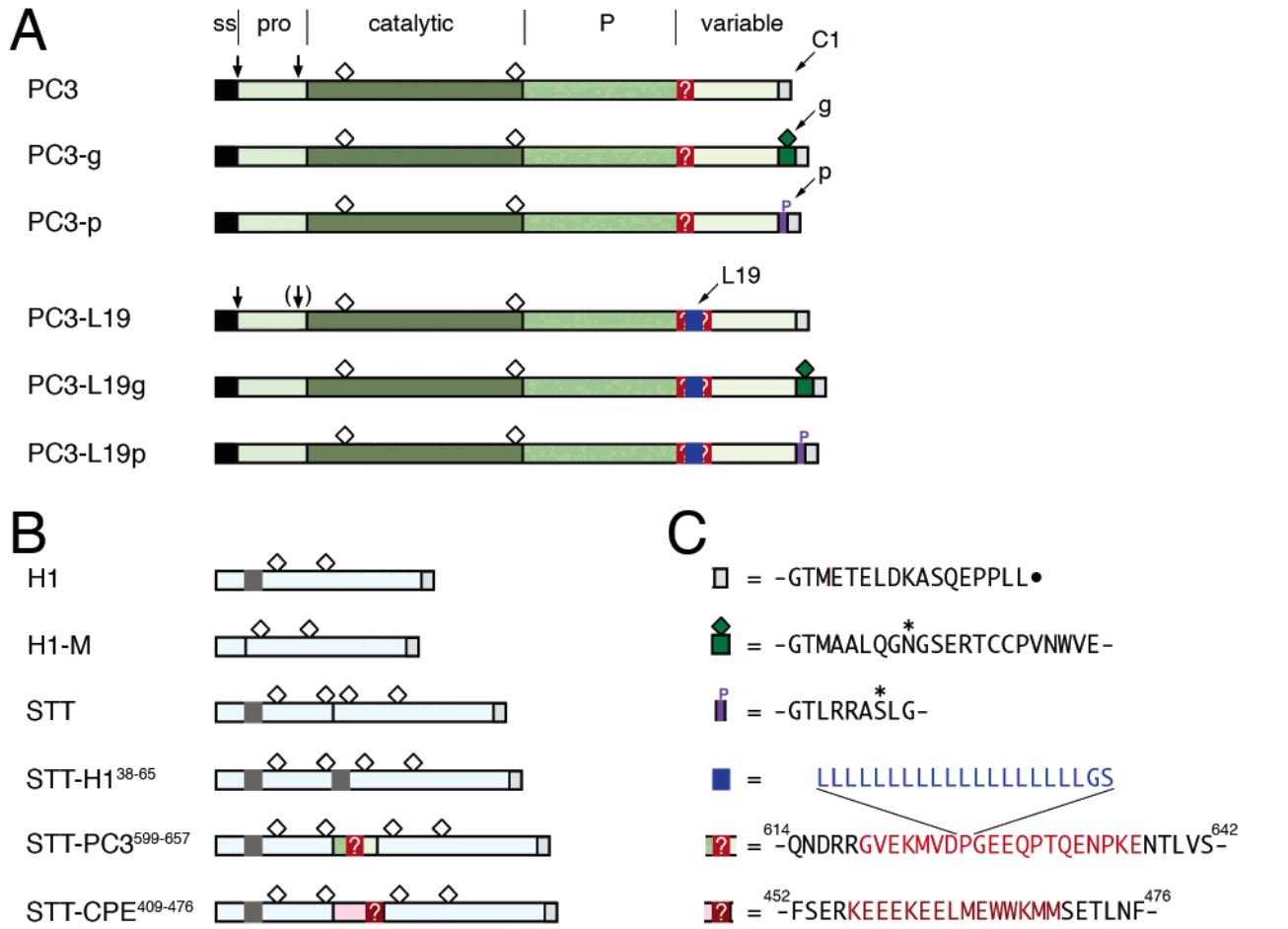

FIGURE 1: PC3 constructs for studying membrane topology. (A) The domain organization of PC3 and derived constructs are schematically shown. PC3 consists of a signal sequence (ss), the pro domain (pro), the catalytic portion, the P-domain, and a C-terminal segment that is variable among the PC family members. The proposed transmembrane segment (residues 619−638) is highlighted in red with a question mark. Potential glycosylation sites are denoted with diamonds. Vertical arrows indicate cleavage sites (in parentheses where cleavage is slowed by insertion of the L19 sequence). The leucine$_{19}$ sequence inserted into the proposed transmembrane segment, the C1 epitope tag, the glycosylation, and the phosphorylation tags are denoted with L19, C1, g, and p, respectively. (B) Schematic representations of the stop-transfer tester construct (STT) and its derivatives containing the signal−anchor sequence of the asialoglycoprotein receptor H1 (H1$^{38-65}$), residues 599−657 of PC3, or residues 409−476 of CPE are shown. (C) The amino acid sequences of the relevant elements in the PC3 and STT constructs shown in panels A and B are listed. A dot represents the C-terminus, and asterisks represent the residues modified by glycosylation or phosphorylation.

secretion (*10*), although this has remained controversial (*11*). The C-terminal portion of CPE was shown to interact with ADP-ribosylation factor 6 (ARF6) in yeast two-hybrid assays and pull-down experiments (*12*). A fusion protein of the lumenal portion of the IL-2 receptor α subunit and the 25 C-terminal residues of CPE behaved as a raft-associated protein showing ARF6-dependent recycling from the plasma membrane to the TGN (*12*), supporting a membrane-spanning topology of the protein.

However, the three proposed transmembrane segments are highly unusual, since they are composed of 40−60% charged residues, predominantly glutamates. Conventional transmembrane sequences consist of 16−25 uncharged, mainly hydrophobic residues. In single-span membrane proteins, charged residues are virtually absent in the core of the lipid bilayer (*13*, *14*). In the exceptional case of individual intramembrane charges in transmembrane segments, they may cause ER retention and degradation, unless neutralized by hetero-oligomerization with a complementary partner protein (*15*, *16*).

Because of the physiological importance of the question of whether these prohormone processing enzymes are membrane-spanning proteins due to a novel type of transmembrane segment, we set out to test the membrane disposition of PC3 using methods developed for analysis of membrane protein topology. We chose PC3 as the protein to be investigated, because the proposed membrane-spanning segment is further inside the polypeptide chain. Modifications at the C-terminus of PC3 are 115 amino acids downstream of the proposed transmembrane domain, and the possibility that they affect membrane insertion and topology can therefore be excluded. We also analyzed the proposed transmembrane segments of PC3 and CPE in a stop-transfer tester construct for their ability to stop translocation of a polypeptide across the ER membrane. The results consistently show that these sequences, unlike a hydrophobic Leu$_{19}$ sequence, are unable to integrate into the ER membrane and that PC3 is not a transmembrane protein.

MATERIALS AND METHODS

DNA Constructs. The cDNA of human PC3 was a generous gift from W. van de Ven (Flanders Institute of Biotechnology, Leuven, Belgium). To insert a classical transmembrane domain of 19 consecutive leucines,

a BglII site was introduced by polymerase chain reaction (PCR) in front of the sequence encoding the oligoleucine segment of H1ΔQLeu19 (*17*) using the mutagenic primer GCGAGATCTGTTGCTTTTGCTGCTG and fused in-frame to the 5′ portion of PC3 up to the BamHI site at codon 625. The 3′ portion of PC3 was amplified with the primer CGCAGATCTGGGGAGGAGCAGCCC introducing a BglII site before codon 627 and ligated in-frame into the BamHI site at the end of the oligoleucine sequence. In the wild-type PC3 sequence and the oligoleucine insertion mutant, the stop codon was replaced with the sequence of an Asp718 site by PCR using the antisense primer CGCGGTACCAGAATTTTCCTCATTCAGAATGTC and fused via Asp718 to the 14 C-terminal codons of the asialoglycoprotein receptor H1, which encode the C1 epitope recognized by a rabbit anti-peptide antiserum (*18*).

To introduce a C-terminal glycosylation tag, the 40 C-terminal codons of H1ΔL22[110] (*19*), which encode residues 141−160 of H1 with an N-glycosylation site and the C1 epitope, were amplified with a primer introducing a 5′ BrsGI site to be ligated into the Asp718 site at the 3′ end of the PC3 or PC3-L19 sequences. Similarly, the sequence of the C1 epitope was amplified with a primer containing a BsrGI site, and the sequence encoding the consensus heptapeptide sequence for phosphorylation by protein kinase A and ligated to the PC3 or PC3-L19 sequences. The resulting C-terminal peptide extensions of PC3 are shown in Figure 1C.

To generate a size marker for unprocessed PC3, the segment encoding the signal sequence (codons 2−26) was deleted from the cDNA of C1-tagged PC3 by PCR using the primer GCGAAGCTTACCATGGCGAAAAGGCAATTTGTC (the HindIII site used for subcloning is underlined). cDNA constructs encoding H1 and H1-M (lacking the transmembrane domain) were described previously (*20*).

The stop-transfer tester constructs STT and STT-H1$^{38-65}$ were derived from plasmids pSA0 and pSAA described previously (*21*, *22*). Codons 599−657 of PC3 and codons 409−476 of CPE were amplified using primers providing a 5′ AccI site and a 3′ ClaI site for ligation into the ClaI site of pSA0. All constructs were verified by sequencing and inserted into the pECE expression plasmid (*23*).

In Vivo Expression and Labeling. COS-1 cells were grown in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C with 7.5% CO_2. Transient transfection was performed with lipofectin (Life Technologies) according to the manufacturer's protocol in six-well clusters. The cells were processed 2 days after transfection.

Transfected cells were starved for 30 min in methionine-free medium, labeled with 100 μCi/mL [^{35}S]methionine in starvation medium for 30 min at 37 °C, and washed with cold phosphate-buffered saline (PBS). The cells were lysed and immunoprecipitated using a rabbit antiserum directed against the C1 epitope. The immune complexes were isolated with protein A−Sepharose and analyzed by SDS−polyacrylamide gel electrophoresis and phosphorimaging. For deglycosylation, immune complexes were incubated in 50 mM potassium phosphate, 25 mM EDTA, 2% Trition X-100, 0.2% SDS, and 1% 2-mercaptoethanol, in the presence or absence of 0.2 or 5 milliunits of endoglycosidase H (for partial or complete deglycosylation, respectively) for 1 h at 37 °C.

For labeling with [^{32}P]phosphate, cells were starved in phosphate-free medium and labeled for 30 min with 100 μCi/mL [γ-^{32}P]ATP in the presence of 20 μM forskolin. Cells were lysed in the presence of phosphatase inhibitors (500 μM nitrophenyl phosphate, 50 μM sodium orthovanadate, 1 mM sodium fluoride, and 1 mM EDTA) and further processed as described above.

For Western analysis, cells were boiled in SDS sample buffer and proteins were separated by gel electrophoresis and transferred to a polyvinylidene fluoride membrane that was then incubated in blocking buffer (PBS with 0.1% Tween 20 and 5% nonfat dry milk) for 60 min and with anti-C1 antibody in blocking buffer for 60 min at 4 °C. Bound antibody was detected using horseradish peroxidase-conjugated anti-rabbit immunoglobulin secondary antibody and the ECL kit (Amersham Biosciences).

Alkaline Extraction and Protease Protection. [^{35}S]Methionine-labeled cells were extracted under alkaline conditions as described previously (*24*) by homogenization in Hepes buffer (pH 11.5) for 15 min on ice. One-half of each sample was centrifuged through a sucrose cushion, and the membrane pellet, the supernatant, and the untreated sample were immunoprecipitated separately and analyzed. For protease protection, [^{35}S]methionine-labeled cells were swollen in 15 mM Hepes-KOH (pH 7.2) and 15 mM KCl, scraped, pelleted, and resuspended in 1.2 mL of PBS. Aliquots were incubated for 30 min on ice with or without 200 μg/mL trypsin or with trypsin and 1% Triton X-100. Reactions were stopped with 400 μg/mL soybean trypsin inhibitor before immunoprecipitation and analysis by SDS gel electrophoresis and autoradiography.

RESULTS

To test membrane insertion of PC3, its sequence was extended by short carboxy-terminal diagnostic tags, as shown in Figure 1A, to reveal the disposition of the C-terminus of the protein. PC3-g has a 22-residue extension with an efficient site for N-glycosylation to report exposure of the C-terminus to the ER lumen. In contrast, PC3-p is tagged with a nine-amino acid consensus sequence for phosphorylation by cAMP-dependent protein kinase in the cytosol (*25*−*27*). These two constructs and the wild-type sequence were furthermore provided with a 16-residue C1 epitope for efficient immunoprecipitation. As a further control, a conventional transmembrane domain composed of an uninterrupted stretch of 19 leucine residues (L19) was inserted into the region of the proposed stop-transfer sequence of PC3, generating PC3-L19, PC3-L19g, and PC3-L19p (Figure 1A,C).

PC3 Is Solubilized upon Alkaline Extraction. Under strongly alkaline conditions (pH >11), biological membranes have been shown to be converted to open membrane sheets. Soluble and peripheral membrane proteins are released, whereas integral membrane proteins generally remain embedded in the lipid bilayer and are pelletable (*28*). This method proved to be a useful empirical procedure for identifying integral membrane proteins.

PC3 and PC3-L19 were transiently expressed in COS-1 cells in parallel with the asialoglycoprotein receptor H1, a

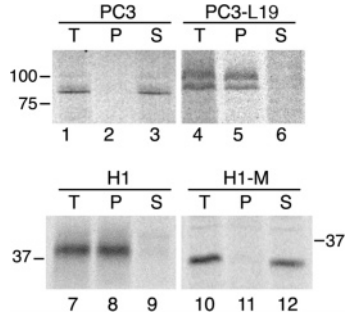

FIGURE 2: PC3 is released from the membrane upon alkaline extraction. Transfected COS-1 cells expressing PC3, PC3-L19, H1, or H1-M were labeled with [^{35}S]methionine for 30 min and subjected to alkaline extraction and centrifugation to separate soluble and membrane-integrated proteins. The total before centrifugation (T) and the supernatant (S) and membrane pellet (P) after centrifugation were analyzed by immunoprecipitation, SDS gel electrophoresis, and autoradiography. The positions of marker proteins with their molecular masses in kilodaltons are indicated.

single spanning type II membrane protein, and H1-M, the same protein lacking its transmembrane segment, as controls. Cells were labeled with [^{35}S]methionine for 30 min and then extracted at pH 11.5. Extracted material and membranes were separated by centrifugation through a sucrose cushion. The total material, pellet, and supernatant were analyzed by immunoprecipitation, SDS gel electrophoresis, and autoradiography (Figure 2). As expected, H1 was recovered exclusively in the membrane pellet (lanes 7−9) and H1-M in the soluble fraction (lanes 10−12). PC3 was produced as a single species with the expected apparent molecular mass of ~85 kDa, corresponding to the protein lacking its N-terminal pro domain (lane 1). It was efficiently extracted into the supernatant (lanes 1−3). Expression of PC3-L19 generated two products differing in size by ~8 kDa (lane 4). The two species correspond to the pro form and the N-terminally processed form. The presence of the oligoleucine sequence appears to slow autocatalytic cleavage of the pro domain. Both forms of PC3-L19 remained completely membrane associated due to the oligoleucine sequence (lanes 4−6). The proposed transmembrane segment of PC3 thus cannot anchor the protein in the lipid bilayer under alkaline conditions. However, while alkaline extraction as an empirical method provides quite reliable results for conventional membrane proteins, the proposed PC3 transmembrane segment rich in glutamate and aspartate under harsh deprotonating conditions may follow different rules.

The C-Terminus of PC3 Is Not Accessible to Cytosolic Protein Kinase. To test for the cytosolic exposure of the C-terminus, the PC3 construct with a C-terminal phosphorylation site (PC3-p), wild-type PC3, and the corresponding oligoleucine-containing constructs (PC3-L19p and PC3-L19) were expressed in COS-1 cells, labeled for 30 min with [^{32}P]phosphate, and analyzed by immunoprecipitation against the C1 epitope, SDS gel electrophoresis, and autoradiography. As a control, transfected cells were analyzed by Western blotting to reveal steady-state levels of PC3 constructs in the cells. PC3 and PC3-p were not detectably labeled with [^{32}P]phosphate (Figure 3, lanes 1 and 3). In contrast, PC3-L19p was strongly phosphorylated (lane 4), supporting a transmembrane disposition of the protein with the oligoleucine segment as a transmembrane domain. Even PC3-L19 without the phosphorylation tag was labeled (lane 2),

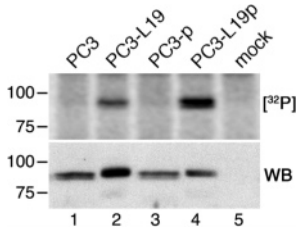

FIGURE 3: PC3 is not phosphorylated at a C-terminal phosphorylation site. Transfected COS-1 cells expressing PC3, PC3-L19, or the corresponding construct PC3-p, or PC3-L19p with a C-terminal site for phosphorylation by protein kinase A in the cytosol, were either labeled for 30 min with [^{32}P]phosphate ([^{32}P]) and analyzed by immunoprecipitation, gel electrophoresis, and autoradiography or subjected to Western blot analysis (WB) as a measure of steady-state expression levels. In parallel, mock-transfected cells were analyzed. Since generally the steady-state levels of PC3 and PC3-p were lower than those of the corresponding L19 constructs, 3 times more sample was loaded in lanes 1 and 3. The positions of marker proteins with their molecular masses in kilodaltons are indicated.

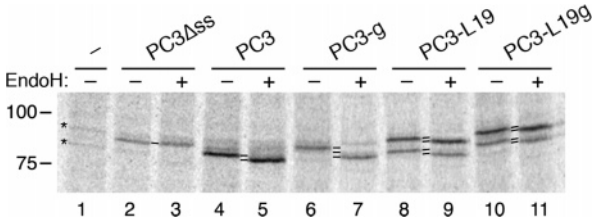

FIGURE 4: C-Terminus of PC3 is exposed to the ER lumen. Transfected COS-1 cells expressing PC3, PC3-L19, or the corresponding construct PC3-g, or PC3-L19g with an additional site of N-glycosylation at the C-terminus, were labeled with [^{35}S]methionine for 30 min and subjected to immunoprecipitation. The immunoprecipitates were split in half and incubated with (+) or without (−) endoglycosidase H (EndoH) before gel electrophoresis and autoradiography. In parallel, untransfected cells (−) were analyzed and cells transfected with PC3Δss lacking the signal sequence, as a size marker. Asterisks denote background bands. Mobility shifts due to deglycosylation are indicated by lines. The positions of marker proteins with their molecular masses in kilodaltons are indicated.

indicating that the C-terminal region of PC3, which is rich in serines and threonines, is unspecifically phosphorylated when exposed to the cytosol. These results thus indicate that the C-terminus of PC3 is not exposed to the cytosol in intact cells.

The C-Terminus of PC3 Is Exposed to the ER Lumen. N-Linked glycosylation is a lumenal ER modification frequently used to assay protein topology, since it provides positive evidence for lumenal localization of glycosylation sites. Human PC3 contains two potential sites for N-glycosylation, both within the catalytic domain. Upon expression in COS-1 cells and labeling for 30 min with [^{35}S]methionine, a major species with the expected apparent molecular mass of ~85 kDa was produced (Figure 4, lane 4). This form corresponds to the processed protein lacking the 83-residue pro domain, which is autocatalytically removed. Deglycosylation of the protein by endoglycosidase H digestion results in a shift of ~3 kDa (lane 5), indicating that PC3 is glycosylated at a single site. As a size marker, a truncation construct lacking the signal sequence (PC3Δss) was expressed in parallel (lanes 2 and 3). The resulting uncleaved and unglycosylated pro-PC3 was larger than the deglycosylated PC3 product by ~8 kDa, consistent with the size of the pro domain.

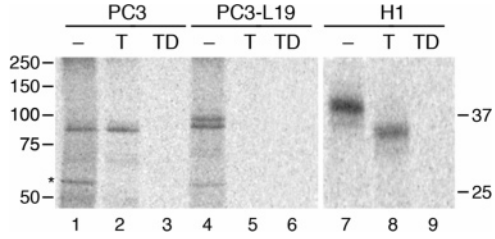

FIGURE 5: C-Terminus of PC3 is protected from protease digestion by the ER membrane. Transfected COS-1 cells expressing PC3, PC3-L19, or the control protein H1 were labeled with [^{35}S]-methionine for 30 min, broken by swelling and scraping, and incubated on ice with or without trypsin (T) or detergent (D). After addition of trypsin inhibitor, samples were lysed, immunoprecipitated, and analyzed by gel electrophoresis and autoradiography. An unspecific background band is denoted with an asterisk. The positions of marker proteins with their molecular masses in kilodaltons are indicated.

PC3-g, because of the additional glycosylation tag, exhibited an electrophoretic mobility like PC3Δss (Figure 4, lane 6). Deglycosylation generated twice the mobility shift observed for PC3 (lane 7), indicating that PC3-g was glycosylated twice: in the catalytic domain and in the C-terminal extension. The C-terminus of PC3 is thus exposed to oligosaccharyl transferase in the ER lumen, contradicting a transmembrane topology. Expression of PC3-L19 and PC3-L19g generated two products each, with and without the pro domain (Figure 4, lanes 8 and 10). Upon deglycosylation, all forms shifted by only ~3 kDa, indicating a single glycosylation. The additional glycosylation site at the C-terminus of PC3-L19g was therefore not translocated into the ER lumen, consistent with a type I membrane-spanning topology.

PC3 Is Protected from Exogenous Protease by the ER Membrane. An alternative method of assaying for cytosolic protein domains is protease protection. Using isolated secretory granules, the 86 kDa form of PC3 was previously found to be sensitive to trypsin digestion (*7*). We applied this method to PC3 while it was still in the ER. Transfected COS-1 cells were labeled for 30 min with [^{35}S]methionine, swollen, and scraped to break the plasma membrane. The permeabilized cells were then incubated with or without trypsin, or with trypsin and detergent before immunoprecipitation and analysis. H1, which was analyzed as a control, was resistant to trypsin digestion except for its N-terminal cytoplasmic domain of 40 amino acids (Figure 5, lanes 7−9). PC3 was completely protected from hydrolysis, except when the membrane was dissolved with detergent (lanes 1−3). In contrast, PC3-L19 disappeared upon trypsin treatment, indicating that the C-terminal epitope was exposed on the outside of the microsomal membranes. The results support the conclusion that PC3 resides entirely within the ER lumen.

The Proposed Transmembrane Segments of PC3 and CPE Do Not Function as Stop-Transfer Sequences. We previously created a stop-transfer tester construct, STT, on the basis of sequences of H1 (Figure 1B) (*21*, *22*). It consists of the N-terminal cytosolic domain, the internal signal−anchor sequence, and a partially duplicated C-terminal domain of H1 with four sites for N-linked glycosylation. The signal−anchor sequence targets the protein to the ER and induces translocation of the C-terminal domain. Upon expression in COS-1 cells and [^{35}S]methionine labeling, STT is found as a major species with four glycans and a minor one with three

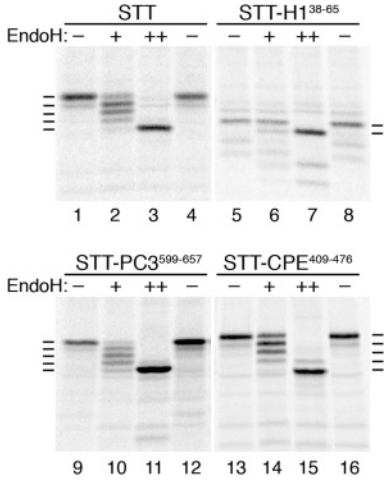

FIGURE 6: Proposed transmembrane segments of PC3 and CPE do not stop polypeptide translocation in the ER. Transfected COS-1 cells expressing the stop-transfer tester (STT) constructs without an insertion, with the signal−anchor sequence of H1, with residues 599−657 of PC3, or with residues 409−470 of CPE were labeled with [^{35}S]methionine for 30 min and subjected to immunoprecipitation. Aliquots of the immunoprecipitates were incubated with 0 (−), 0.2 (+), or 5 milliunits (++) of endoglycosidase H (EndoH) and analyzed by gel electrophoresis and autoradiography.

(Figure 6, lanes 1−4). This is verified by partial deglycosylation with endoglycosidase H, yielding a ladder of intermediates corresponding to polypeptides with zero to four *N*-glycans. Sequences inserted between the second and third glycosylation sites can be tested for their ability to stop further translocation of the polypeptide. If they do, like in the case of a second copy of the signal−anchor sequence of H1 in STT-H1$^{38-65}$ (lanes 5−8), maximally two glycosylation sites will reach the ER lumen. As observed previously (*21*, *22*), the resulting protein is glycosylated only once, because the second site is too close to the second transmembrane segment.

In the construct STT-PC3$^{599-657}$, the proposed transmembrane domain of PC3 together with approximately 20 flanking residues on both sides was inserted into STT. When expressed in COS-1 cells, the products were glycosylated 3- and 4-fold (Figure 6, lanes 9−12), just like STT without an insertion. Similarly, the 68 C-terminal residues of CPE, including the segment of residues 457−471 that had been implicated to span the membrane, produced 3- and 4-fold glycosylation (lanes 13−16). The results indicate that both sequences cannot stop protein transfer in the ER. In summary, the results demonstrate that PC3 is not a type I membrane protein and that the proposed transmembrane segments of PC3 and CPE are not functional as stop-transfer sequences in the ER.

DISCUSSION

Because PC2, PC3, and CPE lack a conventional hydrophobic segment in their sequence, they were initially considered to belong to the class of secretory proteins. All three proteins were found, however, to associate with membranes, particularly with lipid rafts, a feature that was proposed to mediate their sorting into secretory granules (*5*, *6*, *29*, *30*). Membrane association thus qualified them for classification as peripheral membrane proteins. The proposal that these three proteins are integral membrane proteins

spanning the lipid bilayer (*7*−*9*) challenged this conventional model as well as the traditional concepts on the properties of transmembrane segments.

Secretion of PC2, PC3, or CPE activity into the medium is mainly attributed to forms cleaved at the end of the P-domain, releasing the functional protein without its putative membrane anchor. In the case of PC3, however, secretion of an 85 kDa form that includes its C-terminal portion has been observed in the medium of cultured cells (*31*, *32*). This indicated that at least a fraction of PC3 is not spanning the membrane. This leaves the possibility of partial anchoring of the polypeptide by an inefficient stop-transfer sequence. An example in which partial transmembrane insertion was observed is the prion protein (*33*, *34*). It is normally synthesized with an N-terminally cleaved signal and a C-terminal glycosyl phosphatidylinositol attachment sequence as a translocated form called ^{Sec}PrP. A fraction of the proteins, however, inserts into the bilayer via an internal segment to produce membrane-spanning forms ^{Ntm}PrP and ^{Ctm}PrP. The transmembrane segment is mildly hydrophobic, which explains its behavior as an "inefficient" stop-transfer sequence in a conventional way. In artificial proteins, the minimal hydrophobicity to stop polypeptide translocation was found to correspond to a stretch of 19 alanines or nine leucines (*35*).

The proposed transmembrane domains of PC2, PC3, and CPE violate the rule that polypeptides crossing the hydrophobic core of the membrane have to be predominantly apolar. As an explanation, it has been put forward that at acidic pH, as in the TGN or secretory granules, glutamic and aspartic acid residues are partially protonated, and that within the lipid bilayer the pK_a may dramatically increase (*9*). Yet, the energy for increasing the pK_a by 1 pH unit calculated according to the formula $\Delta G^\circ = RT \times 2.303\Delta pK_a$ amounts to ~5.7 kJ/mol. This may be compensated by the transfer of neighboring apolar residues into the hydrophobic environment of the bilayer. In the proposed transmembrane segments, potentially charged residues outnumber hydrophobic residues by far. In addition, even for the transfer of protonated acidic side chains into an apolar environment, one must account for the energy for removing hydration water. Energetically, insertion of the proposed sequences into the bilayer thus appears to be highly unfavorable.

In the case of PC3, spontaneous membrane insertion in an acidic organelle can be excluded, because the translocation of 115 residues would have to be accomplished. For this reason, it was suggested that the transmembrane orientation of PC3 is established during synthesis as the protein enters the rough ER (*7*). The proposed transmembrane sequence, upon interacting with the ER membrane, would stop further translocation of the remainder of the C-terminal domain, which would stay on the cytosolic side of the membrane. This is what we have experimentally tested in this study. We have used COS-1 cells as a convenient cell system. The machinery for membrane integration in the ER is unlikely to differ between cell types. Indeed, PC3 and PC3-g expressed in Neuro2a cells, a neuroendocrine cell line, behaved exactly as in COS-1 cells (data not shown). In our experiments, we found PC3 to be released from membranes upon alkaline extraction (Figure 2), an empirical method for distinguishing integral membrane proteins form peripheral or secretory proteins. We could not detect any phosphorylation of a C-terminal target sequence of protein kinase A (Figure 3), but efficient glycosylation of a C-terminally attached glycosylation site (Figure 4). The protein was furthermore resistant to protease digestion in permeabilized cells (Figure 5). The proposed transmembrane segment and its flanking sequences also scored negative as part of a construct designed to test for stop-transfer activity (Figure 6). In all tests, PC3 behaved as expected for a completely translocated protein, whereas PC3 with an inserted Leu_{19} sequence exhibited all the hallmarks of a type I integral membrane protein. The results lead to the conclusion that neither residues 619−638 nor any other sequence in PC3 spans the lipid bilayer in a significant fraction of the molecules.

The simplest explanation for rationalizing the discrepancies between our experiments and those described by Arnaoutova et al. (*7*) is contamination of secretory granules by PC3, in particular the 85 kDa form, from a broken ER or Golgi during granule purification. This material might be peripherally bound to the outside of the granule membrane and account for antibody binding, biotinylation, and protease sensitivity. In contrast, the phosphorylation and glycosylation assays used here reflect the situation in intact, living cells. For protease protection, the cells' plasma membrane was broken by swelling and scraping without further treatment before incubation with or without trypsin.

The conclusion that PC3 is not a membrane-spanning protein has implications on potential sorting mechanisms of the protein, since direct interaction with cytosolic factors can be excluded. Interaction with lipid rafts and/or raft-associated components is limited to the exoplasmic surface of the TGN and granule membranes. With respect to the proposed transmembrane segment of CPE, our stop-transfer test shows that it is not able to halt translocation of a polypeptide chain in the ER. Spontaneous integration in a later compartment of the secretory pathway cannot be excluded from the data, but the energetics of membrane integration do not make it seem likely. For polypeptide segments crossing the lipid membrane, there is no convincing alternative to hydrophobic sequences, certainly not in single spanning proteins.

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