

Internalization of Proprotein Convertase PC7 from Plasma Membrane Is Mediated by a Novel Motif*

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Background: The proprotein convertase, PC7, cycles between the *trans*-Golgi network (TGN) and the plasma membrane.

Results: Internalization of PC7 is mediated by clathrin-coated vesicles and depends on a Pro-Leu-Cys (PLC) motif in the cytoplasmic tail.

Conclusion: The PLC motif is essential and sufficient for internalization of PC7 from the plasma membrane.

Significance: New insight in the trafficking of PC7 provides further information concerning its physiological function.

Proprotein convertase 7 (PC7) is a member of the subtilisin-like proprotein convertase family, which is involved in the endoproteolysis of a variety of precursor proteins. Under steady state conditions, PC7 is mainly localized in the *trans*-Golgi network, but a small fraction is found at the cell surface. So far, no sorting signals for membrane trafficking have been identified in PC7. In this study, we have examined the internalization of PC7 from the plasma membrane. Our results show that internalization of PC7 is mediated by clathrin-coated vesicles. After inhibition of clathrin-mediated endocytosis using hypertonic conditions or the small molecule inhibitor, Pitstop 2, PC7 accumulated at the plasma membrane. Furthermore, PC7 was present in isolated clathrin-coated vesicles. To determine the internalization motif, constructs were generated in which parts of the N and C terminus of the cytoplasmic tail of PC7 were deleted, and chimeric proteins were constructed consisting of the luminal and transmembrane domains of Tac (CD25) and parts of the cytoplasmic domain of PC7. Antibody uptake experiments as well as surface biotinylation experiments demonstrated that the region between Ala⁷¹³ and Cys⁷²⁶ in the cytoplasmic domain of PC7 is essential and sufficient for the internalization of PC7 but not for *trans*-Golgi network localization. Individual amino acids in this region were substituted with alanine, which identified Pro, Leu, and Cys as the essential amino acids. In conclusion, internalization of PC7 depends on a short transferable sequence in the cytoplasmic tail, which contains the three crucial amino acids PLC.

Proprotein convertase 7 (PC7),³ also called PC8 or lymphoma PC (LPC), is the most distinct member of the subtilisin-

like proprotein convertase family (1). Like the other family members PC1/3, PC2, furin, PC4, PC5/6, and PACE4, it cleaves precursors at the carboxyl-terminal side of basic amino acid residues motifs such as (K/R)X(K/R)R ↓ (2). PCs are multidomain enzymes, consisting of a propeptide followed by a subtilisin-like catalytic domain, a P-domain (also called a middle domain), and one or more carboxyl-terminal domains. The first three domains are essential and sufficient for catalytic activity, and the carboxyl-terminal domains control intracellular trafficking (3, 4).

Human PC7 was first identified in the chromosome breakpoint region of a high grade lymphoma carrying a t(11;14)(q23; q32) translocation (5). Independently, human (6), mouse (7), and rat (8) PC7 were cloned from cell lines. PC7 binds to BiP in the endoplasmic reticulum to prevent aggregation and dissociates after folding (9). Its propeptide is removed autocatalytically. PC7 undergoes additional post-translational modifications (10), such as palmitoylation of 2 cysteines in the cytosolic tail (11), sulfation (12), and *N*-glycosylation (7). PC7 is widely expressed and has been demonstrated in early embryonic stages (8) as well as in several adult tissues (5, 7). The PC7 knock-out mouse does not show any obvious phenotypic abnormalities (13), and this in sharp contrast to knock-out mice generated for other PCs (14). This might be explained by redundancy provided by other PCs in the ability to process certain precursor proteins (14). Immunohistochemical studies have shown that PC7 (10), furin (15), and PC5/6B (16) each concentrate in the *trans*-Golgi network (TGN). Thus, furin and/or PC5/6B might as such compensate for the absence of PC7. Indeed, cleavage of a set of prorenin mutants with different cleavage sites indicated similar specificity, although furin appeared to have a broader activity (10). In addition, many other substrates can be processed by PC7 and by furin, such as platelet-derived growth factor (PDGF)-AA (17), PDGF-BB (18), bone morphogenic protein (BMP) (19), and vascular endothelial growth factor (VEGF)-C (20), among others. On the other hand, furin and PC5/6 knock-out mice are lethal, suggesting that the degree of redundancy for those enzymes is limited (21, 22). Substrate specificity under physiological conditions is also dependent on cell biological properties of the enzymes. Potential substrates should co-localize at least temporarily with the

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³ The abbreviations used are: PC7, proprotein convertase 7; AS, alanine scan; CCV, clathrin-coated vesicle; PACS-1, phosphofurin acidic cluster sorting protein 1; PLC, proline, leucine, cysteine; PM, plasma membrane; TGN, *trans*-Golgi network; TRITC, tetramethylrhodamine isothiocyanate.

enzyme. It is therefore important to study the intracellular trafficking of the enzymes. Although the intracellular trafficking of furin and PC5/6B has been studied extensively, little is known about the trafficking of PC7.

Furin is concentrated in the TGN under steady state conditions. Nevertheless, it cycles between this compartment and the cell surface through the endocytic pathway. The cytoplasmic domain of furin is necessary and sufficient to direct TGN localization (15, 23) and to recycle the enzyme (24). The exit from the TGN to endosomes occurs via clathrin-coated vesicles (CCVs) and depends on the phosphorylation state of a pair of serines within a cluster of acidic residues, a tyrosine motif, and a leucine-isoleucine signal, which are all involved in the interaction of the cytoplasmic domain with AP-1 and its μ 1 subunit (23, 25). TGN localization of furin is mediated by the interaction of the phosphorylated cytoplasmic domain with the connector protein PACS-1 (26). Recycling of furin from the plasma membrane occurs via an AP-2/clathrin-dependent pathway. The interaction of the cytoplasmic domain of furin with AP-2 depends on a tyrosine-based sorting signal, a phenylalanine signal, and to a lesser extent, on the leucine-isoleucine motif (24).

In contrast to furin, PC5/6B accumulates in a brefeldin A-dispersible and BaCl₂-responsive perinuclear compartment and in the tips of AtT-20 cells. Thus, it has a distinct distribution within the TGN/endosomal pathway (27). Endocytosis is predominantly directed by a canonical tyrosine-based motif. Furthermore, two clusters of acidic amino acids within the cytoplasmic domain of PC5/6B contain differential sorting information. The membrane-proximal acidic amino acids direct TGN localization via PACS-1 but are not phosphorylation-dependent. The membrane-distal acidic amino acids promote the localization characteristic for full-length PC5/6B as described above. The differential localization of PC5/6B and furin may help explain the inability of PC5/6B to compensate for the absence of furin in knock-out mice (21) because it suggests that they might activate proproteins in distinct subcellular compartments. Similarly, a considerable proportion of PC7 is found in TGN-derived vesicles devoid of furin (28).

Trafficking of PC7 has not yet been studied into detail. It has been demonstrated that PC7 reaches the cell surface via the conventional route as well as via a brefeldin A- and coat protein complex II (COPII)-independent unconventional secretory pathway (12). Furthermore, it has been suggested that pro-EGF processing by full-length membrane-bound PC7 does not occur at the cell surface but that it requires endocytosis into clathrin-coated pits (29). In this study, the endocytosis of PC7 from the plasma membrane was investigated by means of cell fractionation, antibody uptake experiments, and cell surface biotinylation.

EXPERIMENTAL PROCEDURES

Plasmids and Mutagenesis—The cloning of the 2.6-kb human PC7 cDNA in the mammalian expression vector pcDNA3 (Invitrogen), the introduction of the FLAG epitope (amino acids DYKDDDDK) between the pro- and catalytic domain, and the mutants f-PC7- Δ cyt (lacking the cytoplasmic tail; stop codon after Ser⁶⁸⁹) and f-PC7-H⁷⁰⁸ (stop codon after His⁷⁰⁸) have been described previously (10, 11). PC7 constructs

carrying the FLAG epitope are indicated as f-PC7. New mutations were generated using the QuikChange site-directed mutagenesis kit (Stratagene) according to the guidelines of the supplier, standard polymerase chain reactions, or a combination of both. Mutations were confirmed by nucleotide sequence analysis, and an overview of the crucial constructs is given in Fig. 1. To facilitate the subcloning of the mutants, an XhoI site was created from the Leu⁶⁸⁴-Glu⁶⁸⁵ codons, making use of codon degeneracy. In mutants f-PC7-L⁷²⁵, f-PC7-C⁷²⁶, and f-PC7-D⁷³⁰, a stop codon was introduced immediately following the indicated amino acid. Construct f-PC7-A⁷¹³-D⁷³⁰ encodes a PC7 mutant in which amino acids Ala⁷¹³ until Asp⁷³⁰ are fused to the carboxyl terminus of f-PC7- Δ cyt, and in the f-PC7^{L725A} residue, Leu⁷²⁵ is replaced by an alanine using full-length f-PC7 as template.

The human cDNA encoding the α -chain of the interleukin-2 receptor, Tac (CD25), was a gift from Dr. Bonifacino (Bethesda, MD) and was subcloned as an EcoRI-XbaI fragment in pcDNA3. In Tac-PC7cyt, only the cytoplasmic tail was swapped (Leu⁶⁸⁴-Cys⁷⁸⁵). TacPC7cyt-D⁷³⁰ and TacPC7cyt-A⁷¹³-D⁷³⁰ contained only parts of the cytoplasmic tail of PC7 (Leu⁶⁸⁴-Asp⁷³⁰ and Ala⁷¹³-Asp⁷³⁰, respectively). The alanine-scan of residues Lys⁷¹⁴-Cys⁷²⁶ was performed using TacPC7cyt-D⁷³⁰ as template and the mutants named AS (alanine scan) with the substituted amino acid(s) indicated (e.g. AS^{C726}). Amino acids were individually substituted by alanines, except for AS^{KEE716} and AS^{GTE719}, in which three amino acids were replaced simultaneously.⁴

Cell Lines and DNA Transfer—Medium, serum, and supplements used for the maintenance of cells were obtained from Invitrogen. Generation of the CHO-DHFR⁻ cell line stably overexpressing PC7 (CHO-PC7) has been described before (10). The cells were cultured in minimal essential medium α containing ribonucleosides and deoxyribonucleosides supplemented with 10% fetal calf serum and 250 μ g/ml G418. CHO-K1 cells were grown in Dulbecco's modified Eagle's medium/Ham's F12 (1:1) supplemented with 10% fetal calf serum.

CHO-K1 cells were plated 1 day before transfection. 8–10 \times 10⁵ cells/10-cm² culture plates were transfected with 2 μ g of DNA and 6 μ l of FuGENE (Roche Diagnostics) and used for experiments the next day. All experiments were performed at least twice.

Antibody Uptake/Immunofluorescence Experiments—Transfected CHO-K1 cells were washed and incubated with serum-free medium containing 1 μ g/ml anti-FLAG antibody M2 (Sigma) or anti-Tac (BIOSOURCE) for 30 min at 4 °C followed by 15 or 60 min at 37 °C, as indicated. The effect of sucrose and the small molecule inhibitor of clathrin-mediated endocytosis (Pitstop 2, Ascent Scientific) was studied by preincubation of the cells with 0.3 M sucrose (final concentration) in serum-free medium or with serum-free medium containing 30 μ M Pitstop 2 for 30 min at 37 °C. Subsequently, the cells were incubated for 30 min at 4 °C and 15 min at 37 °C in the same solutions con-

⁴ The multiple mutants used in this study are defined as follows: AS^{KEE716} = AS^{K714A,E715A,E716A}, and AS^{GTE719} = AS^{G717A,T718A,E719A}.

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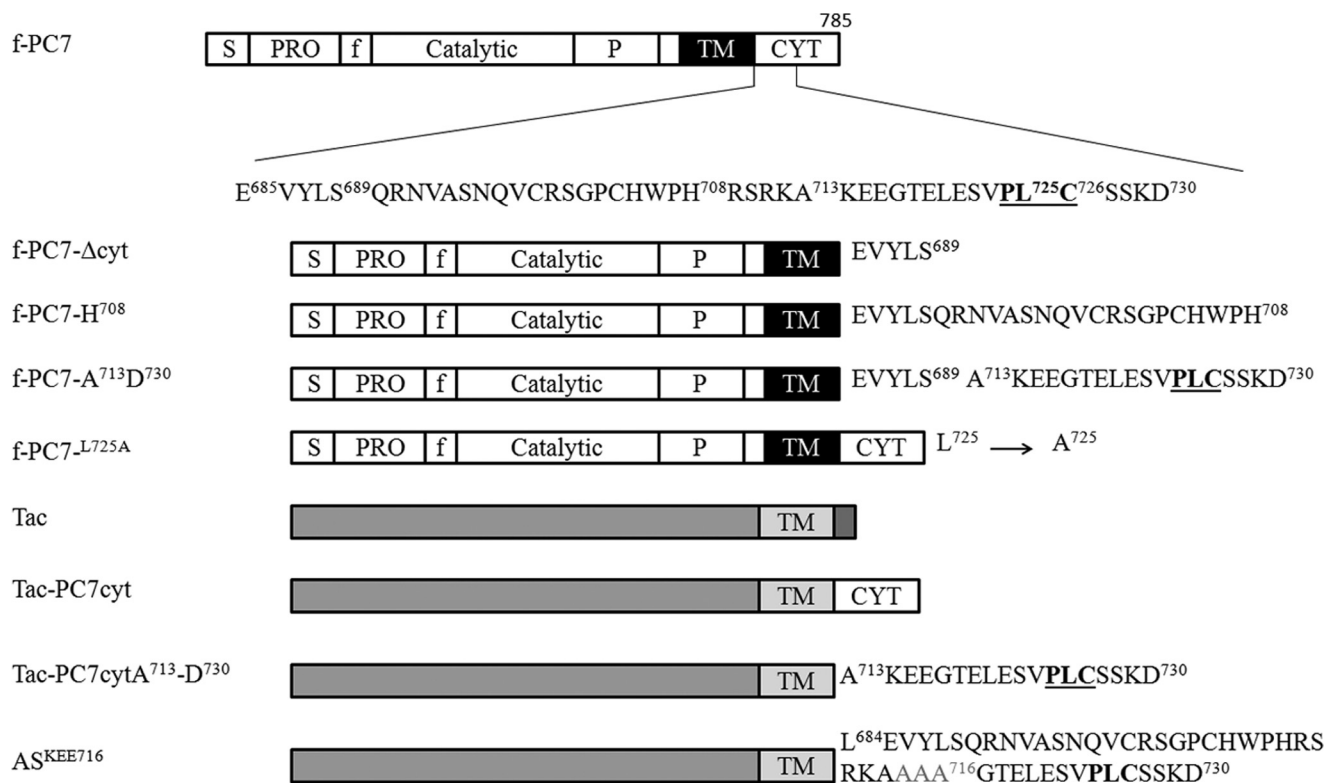


FIGURE 1. **Schematic overview of different types of constructs.** The different domain structures are indicated (*S* = signal peptide; *PRO* = propeptide; *P* = propeptide domain, *f* = FLAG tag; *TM* = transmembrane; *CYT* = cytoplasmic). In deletion constructs, a stop codon was introduced after the indicated amino acid (f-PC7-H⁷⁰⁸ is included as example). In the chimeric proteins, the cytoplasmic tail of Tac was swapped with (part of) the cytoplasmic tail of PC7. In the AS constructs, individual amino acids or groups of three amino acids were substituted with alanine. AS^{KEE716} is shown as example.

taining 1 $\mu\text{g/ml}$ anti-FLAG antibody M2. Anti-TGN38 antibody was a generous gift of Dr. Luzio (Cambridge, UK).

After incubation with antibody, the cells were washed in ice-cold PBS, fixed in 4% freshly prepared formaldehyde, and quenched with 50 mM NH_4Cl . To facilitate rapid and conclusive screening of the mutants, two different methods were used for the processing of anti-FLAG (M2) and anti-Tac antibody uptake for immunofluorescence. The cells incubated with M2 were incubated for 1 h at room temperature with a rabbit anti-mouse Fab fragment (Jackson ImmunoResearch Laboratories) in PBS-B (PBS containing 0.5% blocking reagent (Roche Diagnostics)) before permeabilization with PBS-BT (PBS-B containing 0.2% Triton X-100). Finally, the cells were incubated with FITC-conjugated donkey anti-rabbit and TRITC-conjugated sheep anti-mouse antibodies (GE healthcare). Using this two-step method, surface-localized M2 was labeled with FITC, whereas internalized M2 was labeled with TRITC. Cells incubated with anti-Tac antibody were immediately permeabilized with PBS-BT and incubated only with TRITC-conjugated sheep anti-mouse antibodies. Slides were analyzed with a Zeiss Axiophot microscope equipped with UV optics. A large number of positive cells were analyzed (>100), and representative cells are shown in the figures. Only cells overexpressing low to moderate levels of recombinant protein were analyzed. Images were recorded with a CE2000A charge-coupled device camera (Photometrics).

Surface Biotinylation—8–10 $\times 10^5$ transfected CHO-K1 cells were placed on ice, washed four times with PBS, and incubated for 20 min with 1 mg/ml sulfo-NHS-LC-biotin (Pierce) in

PBS. Unreacted sulfo-NHS-LC-biotin was quenched with complete culture medium (10 min). After two additional washes with PBS, cells were lysed in PBS containing 1% Triton X-100 and Complete protease inhibitor mixture (Roche Diagnostics). Biotinylated proteins were precipitated using ImmunoPure immobilized streptavidin (Pierce), and nonbiotinylated proteins in the supernatant were precipitated using 4 volumes of methanol. Both samples were separated on SDS-PAGE and analyzed by Western blotting using either M2 ($n = 5$ –10) or anti-Tac ($n = 4$ –6) antibody. Quantification of biotinylated and unbiotinylated PC7 or Tac chimeras was performed using the Image Station 440 (Kodak Digital Science).

Isolation of CCVs—CCVs were isolated from CHO-K1 cells stably overexpressing PC7 (10) essentially as described previously (23). Briefly, cells were washed in PBS containing 0.1 mM CaCl_2 and 1 mM MgCl_2 , scraped, and spun for 10 min at $2000 \times g$. The cell pellet was resuspended in vesicle buffer (140 mM sucrose, 75 mM potassium acetate, 10 mM MES, pH 6.7, 1 mM EGTA, 0.5 mM magnesium acetate) containing 1 mM dithiothreitol, 1 mM PMSF, and Complete protease inhibitor mixture and homogenized by 15 passages through a 24-gauge needle. From a postnuclear supernatant, a postmitochondrial supernatant was prepared, which was fractionated by two gradient centrifugations. First a microsomal fraction was prepared on a 10-ml continuous gradient of 10–90% (w/v) $^2\text{H}_2\text{O}$ (Sigma) in vesicle buffer followed by a 10-ml continuous gradient from 2% (w/v) Ficoll, 9% (w/v) $^2\text{H}_2\text{O}$ to 20% Ficoll, 90% $^2\text{H}_2\text{O}$ in vesicle buffer. 1-ml fractions were collected from the bottom of the

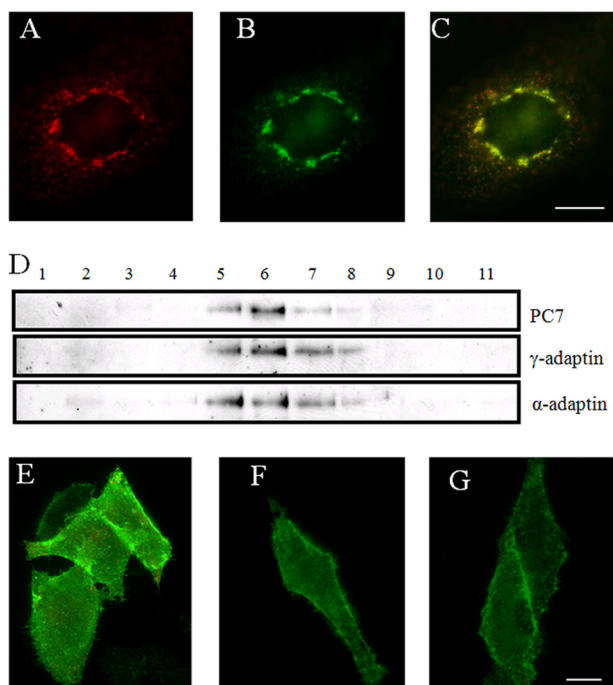


FIGURE 2. PC7 recycles from PM to TGN via CCVs. *A*, CHO-K1 cells transfected with f-PC7 were incubated with the anti-FLAG antibody M2 for 30 min at 4 °C and 1 h at 37 °C, fixed, and stained with a TRITC-conjugated secondary antibody to visualize PC7. *B*, immunofluorescent post-fix staining of the same cells for TGN38 to visualize the TGN. *C*, merged picture of those cells demonstrates that f-PC7 was transported from the PM to the TGN. *D*, CCVs were purified using standard procedures as described under "Experimental Procedures." Each fraction from the second density gradient was separated by SDS-PAGE and analyzed by Western blot analysis for α - and γ -adaptin and PC7. *E*, antibody uptake experiment (30 min 4 °C, 15 min 37 °C) in hypertonic medium on CHO-K1 cells transfected with f-PC7 using the anti-FLAG antibody M2. A double immunofluorescent staining was performed to visualize intracellular PC7 in red and to visualize cell surface-localized PC7 in green. PC7 mainly localizes at the PM under these conditions. *F*, antibody uptake experiment (30 min 4 °C, 15 min 37 °C) on CHO-K1 cells transfected with f-PC7 and incubated with 30 μ M Pitstop 2, using the anti-FLAG antibody M2. A double immunofluorescent staining was performed to visualize intracellular PC7 in red and to visualize cell surface-localized PC7 in green. PC7 mainly localizes at the PM under these conditions. *G*, CHO-K1 cells transfected with f-PC7 were incubated for 30 min at 4 °C with serum-free medium containing anti-FLAG antibody M2. A double immunofluorescent staining was performed to visualize intracellular PC7 in red and cell surface-localized PC7 in green. PC7 mainly localizes at the PM under these conditions. Scale bar = 10 μ m.

second gradient, precipitated with trichloroacetic acid (10% final concentration), and analyzed by Western blotting.

RESULTS

PC7 Shuttles from Plasma Membrane (PM) to TGN—Under steady state conditions, PC7 is concentrated in the TGN, frequently associated with coated buds and vesicles also positive for the AP-1 subunit γ -adaptin (10). To demonstrate that PC7, like furin and PC5/6B, recycles from the PM to the TGN, antibody uptake experiments were performed. CHO-K1 cells were transfected with f-PC7. 24 h later, the cells were incubated with the anti-FLAG antibody M2 for 30 min at 4 °C to allow binding of the antibody without internalization and for 1 h at 37 °C to allow internalization of the membrane-bound PC7. After incubation with the antibody, the cells were fixed, and PC7 was visualized with a TRITC-conjugated secondary antibody (Fig. 2*A*). After 1 h of endocytosis, the majority of PC7 had been internalized and transported to the TGN as demonstrated by its

co-localization with TGN38, an endogenous marker protein for the TGN (Fig. 2, *B* and *C*). This demonstrates that PC7 can recycle from the plasma membrane to the TGN.

PC7 Recycles to TGN via Clathrin-mediated Endocytosis—Within 1 h, the majority of PC7 recycled from the PM to the TGN. This recycling might occur through clathrin-mediated endocytosis, which can occur within a time frame of 4 min (30). Therefore, to demonstrate that PC7 localizes to CCVs, CCVs were prepared from CHO-K1 cells stably overexpressing PC7 using a standard fractionation protocol (23). The last density gradient of the purification procedure was analyzed by Western blot analysis. PC7 was mainly observed in fractions 5–7. These fractions also contained γ -adaptin and α -adaptin, which are marker proteins for CCVs (Fig. 2*D*), indicating that PC7 is present in CCVs.

To substantiate that PC7 recycles from the plasma membrane to the TGN via CCVs, antibody uptake experiments were performed on CHO-K1 cells transfected with f-PC7 in the presence or absence of hypertonic medium (0.3 M sucrose). Hypertonic treatment induces abnormal clathrin polymerization into empty microcages, rendering clathrin unavailable for assembly, and as such, the clathrin-coated pits disappear, inhibiting endocytosis (31). If PC7 recycles to the TGN via CCVs, PC7 should accumulate at the PM upon hypertonic treatment. Surface-localized PC7 was stained green using an FITC antibody, whereas intracellular PC7 was stained red using a TRITC antibody, using a double labeling method as described by Franzusoff *et al.* (32). Upon treatment of the cells with hypertonic medium, PC7 accumulated at the PM (Fig. 2*E*), whereas without sucrose treatment, the majority of PC7 either was internalized or had accumulated in punctate structures at the cell surface, probably clathrin-coated pits (Fig. 3). Although the 15-min incubation at 37 °C is long enough for endocytosis, it is too short for recycling to the TGN. Hypertonic solution is not specific for the disruption of clathrin-mediated endocytosis. Therefore, to formally prove the involvement of clathrin-mediated endocytosis, we selectively blocked endocytic ligand association with the clathrin terminal domain, using the small molecule inhibitor Pitstop 2 (33). Similarly as for cells treated with hypertonic solution, PC7 also accumulated at the PM upon incubation with Pitstop 2 (Fig. 2*F*). As a negative control, cells were incubated for 30 min at 4 °C with anti-FLAG M2 antibody without any chase period. In those experiments, no internalization of PC7 could be observed (Fig. 2*G*). Taken together, these experiments demonstrate that PC7 is endocytosed via CCVs.

Cytoplasmic Domain of PC7 Is Required for Recycling of PC7 from PM to TGN—In the case of furin and PC5/6B, the motif required for the trafficking of proteins from the PM to the TGN is located in the cytoplasmic tail (24, 27). To investigate whether this is also the case for PC7, an antibody uptake experiment was performed using a construct lacking the cytoplasmic domain of PC7 (Fig. 3). f-PC7 Δ cyt accumulated at the plasma membrane with only a few weakly stained internalized vesicles. Moreover, surface staining was mostly diffuse with few punctate structures such as f-PC7. This demonstrates that the cytoplasmic domain is indeed essential for endocytosis. To determine the region within the cytoplasmic domain that is required for endocytosis of PC7 from the PM, antibody uptake experi-

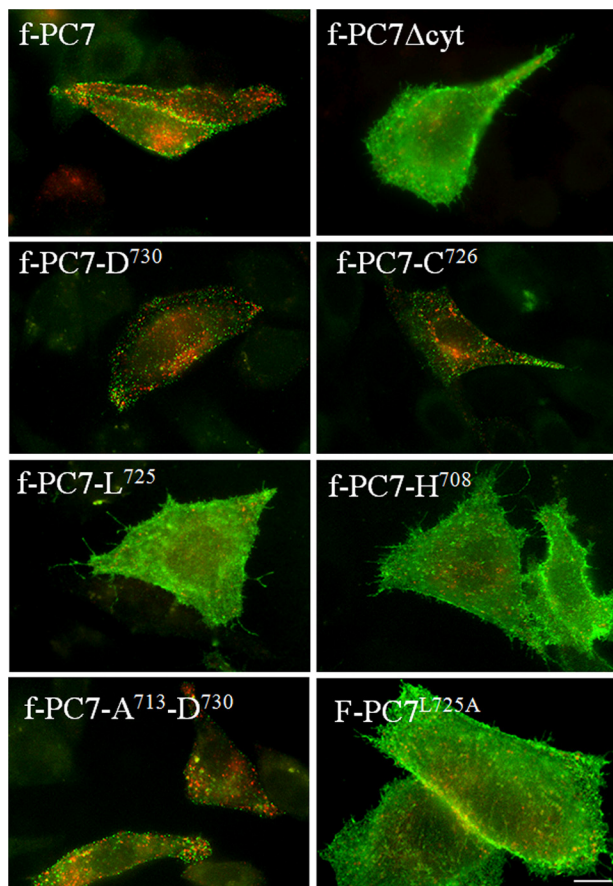


FIGURE 3. Region between Ala⁷¹³ and Cys⁷²⁶ in cytoplasmic domain of PC7 is essential for endocytosis of PC7. Antibody uptake experiments (30 min 4 °C, 15 min 37 °C) using the anti-FLAG antibody M2 were performed on CHO-K1 cells transfected with f-PC7 or different truncation constructs in which parts of the cytoplasmic tail of PC7 were deleted. Construct f-PC7^{L725A} contains the entire cytoplasmic tail in which Leu⁷²⁵ was substituted with alanine. Double immunofluorescent staining was performed to stain cell surface-localized PC7 in green and to stain internalized PC7 in red. Scale bar = 10 μm.

ments were performed using constructs in which parts of the cytoplasmic domain were truncated. Antibody uptake experiments performed with f-PC7-D⁷³⁰ and f-PC7-C⁷²⁶ showed efficient endocytosis of PC7, similar to that of the full-length f-PC7 construct (Fig. 3). In contrast, when one extra amino acid was deleted from the C terminus (construct f-PC7-L⁷²⁵) or when even more amino acids were removed using the construct f-PC7-H⁷⁰⁸, PC7 accumulated at the PM (Fig. 3). To further narrow down the region within the cytoplasmic domain that contains the signal for endocytosis, a construct encoding a PC7 mutant in which amino acids Ala⁷¹³ until Asp⁷³⁰ are fused to the carboxyl terminus of f-PC7-Δcyt was generated. This construct was also endocytosed (Fig. 3). Finally, when Leu⁷²⁵ was mutated to alanine using full-length f-PC7 as template, PC7 accumulated at the PM, suggesting that this amino acid is an essential part of the major, if not only, endocytic motif of PC7 (Fig. 3). Altogether, those results demonstrate that a motif in the region between Ala⁷¹³ and Cys⁷²⁶ in the cytoplasmic domain of PC7 is essential for the trafficking of PC7 from the PM to the TGN.

To confirm the results obtained by the immunofluorescence experiments, surface biotinylation experiments were per-

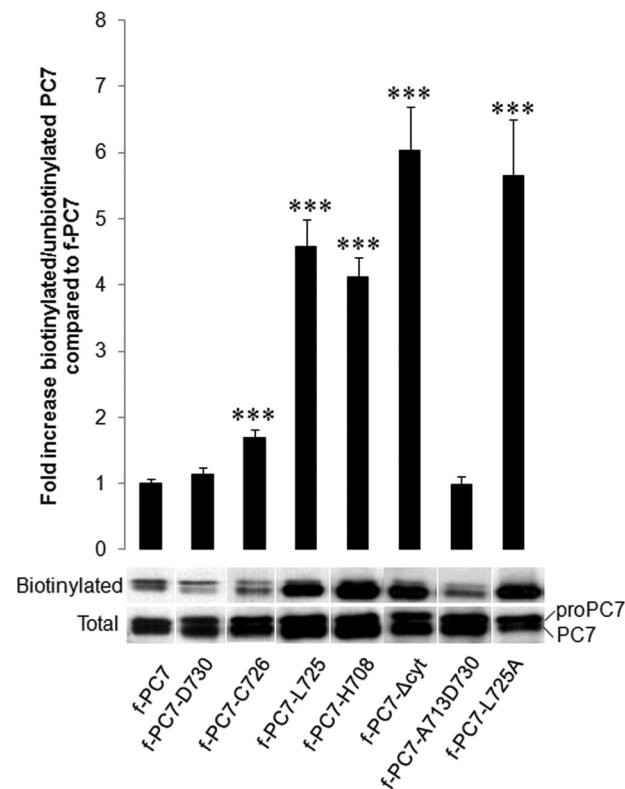


FIGURE 4. Truncation of cytoplasmic tail of PC7 beyond Ser⁷²⁷ results in increased surface localization. CHO-K1 cells transfected with the same constructs as in Fig. 3. Surface molecules were biotinylated with sulfo-NHS-LC-biotin, and the ratio of biotinylated/unbiotinylated PC7 was determined by Western blot analysis. A representative figure of this Western blot analysis is shown. Quantification of biotinylated and unbiotinylated PC7 was performed using the Image Station 440 (Kodak Digital Science). Statistical analysis was performed using the Student's *t* test. Values were considered to be significantly different from f-PC7 when *p* value < 0.05. ***, *p* value < 0.001. Error bars indicate S.E.

formed. Therefore, CHO-K1 cells were transfected with the same constructs, and the surface proteins were biotinylated using sulfo-NHS-LC-biotin. The ratio between biotinylated and unbiotinylated PC7 was determined as described under "Experimental Procedures." If PC7 accumulates at the PM, more biotinylated PC7 should be detected, resulting in an increased ratio of biotinylated/unbiotinylated PC7. Indeed, this ratio significantly increased severalfold after transfection with the constructs f-PC7-L⁷²⁵, f-PC7-H⁷⁰⁸, f-PC7-Δcyt, and f-PC7^{L725A} (Fig. 4). This confirmed the results obtained by the immunofluorescent staining. A small but significant increase was observed with construct f-PC7-C⁷²⁶, indicating that truncation after this cysteine already affected the internalization signal slightly. This minor effect could not be observed in the antibody uptake experiment.

Region Encoded by Amino Acids Ala⁷¹³–Asp⁷³⁰ Is Essential and Sufficient for Endocytosis—The above described experiments demonstrated that the region between Ala⁷¹³ and Cys⁷²⁶ in the cytoplasmic domain of PC7 is essential for endocytosis of PC7. To demonstrate that this region is also sufficient for endocytosis, chimeric proteins were constructed consisting of the luminal and transmembrane domains of Tac (CD25) and parts of the cytoplasmic domain of PC7. The Tac luminal and transmembrane domains have been used previously to assess the

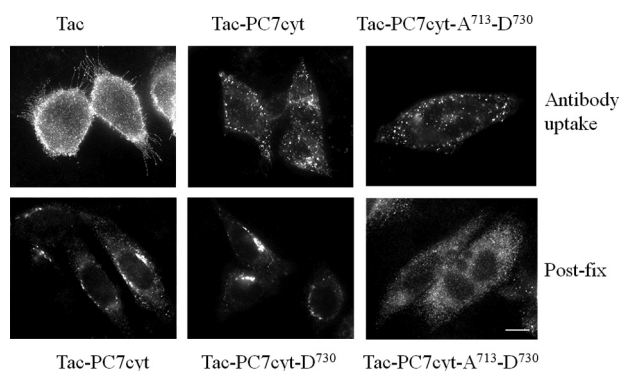


FIGURE 5. Region encoded by amino acids Ala⁷¹³–Asp⁷³⁰ is sufficient for endocytosis but not for TGN localization. *Upper panels*, Chimeric proteins were constructed composed of the luminal and transmembrane domains of Tac (CD25) and (parts of) the cytoplasmic domain of PC7, and antibody uptake experiments were performed (30 min 4 °C, 15 min 37 °C). When the cytoplasmic tail of Tac was swapped with the cytoplasmic tail of PC7 or only with part of the cytoplasmic tail of PC7 (between Ala⁷¹³ and Asp⁷³⁰), PC7 was still endocytosed. *Lower panels*, transfected cells were fixed, and Tac was subsequently visualized by immunofluorescent staining. Both Tac-PC7cyt and Tac-PC7cyt-D⁷³⁰ were found to be concentrated in the TGN, but Tac-PC7cyt-A⁷¹³-D⁷³⁰ was not. Scale bar = 10 μm.

sorting capacity of cytoplasmic sequences (34). Tac is the α -chain of the IL-2 receptor. It is a type 1 transmembrane protein present on the PM of activated T and B cells, some thymocytes, myeloid precursors, and oligodendrocytes. Antibody uptake experiments using an anti-Tac antibody indeed demonstrated that Tac was mainly localized at the plasma membrane (Fig. 5). However, when the cytoplasmic tail of Tac was swapped with the cytoplasmic tail of PC7, Tac-PC7cyt was endocytosed, as demonstrated by antibody uptake experiments (Fig. 5). Similar results were obtained when the cytoplasmic tail of Tac was swapped with only part of the cytoplasmic tail of PC7 (between Ala⁷¹³ and Asp⁷³⁰) (Fig. 5). This demonstrates that this part of the cytoplasmic tail of PC7 is not only essential but also sufficient for endocytosis. To determine whether this region is also sufficient to confer TGN localization, transfected cells were analyzed by immunofluorescent staining (post-fix). Both Tac-PC7cyt and Tac-PC7cyt-D⁷³⁰ were found to be concentrated in the TGN, but Tac-PC7cyt-A⁷¹³-D⁷³⁰ was not (Fig. 5). Instead a dispersed staining was observed. These results indicated that the region Ala⁷¹³–Asp⁷³⁰ is sufficient for endocytosis but not for TGN localization. TGN localization requires the presence of additional sequences, present in the proximal part of the cytoplasmic tail.

A Newly Defined PLC Motif Is Essential for Endocytosis—To determine the exact amino acids that are required for endocytosis, we substituted individual amino acids or groups of three amino acids with alanine(s) in Tac-PC7cyt-D⁷³⁰ and performed similar antibody uptake experiments. These AS constructs AS^{KEE716}, AS^{GTE719}, AS^{L720}, AS^{E721}, AS^{S722}, and AS^{V723} were internalized similar to Tac-PC7cyt-D⁷³⁰ (Fig. 6). In contrast, when amino acids Pro⁷²⁴, Leu⁷²⁵, or Cys⁷²⁶ were substituted with an alanine, the constructs remained mainly diffusely localized at the PM. Those results were confirmed with surface biotinylation experiments, as described above (Fig. 7). Indeed, biotinylated/unbiotinylated PC7 was significantly higher after transfection with the constructs AS^{P724}, AS^{L725}, and AS^{C726} as compared with TAC-PC7cyt. The other constructs showed

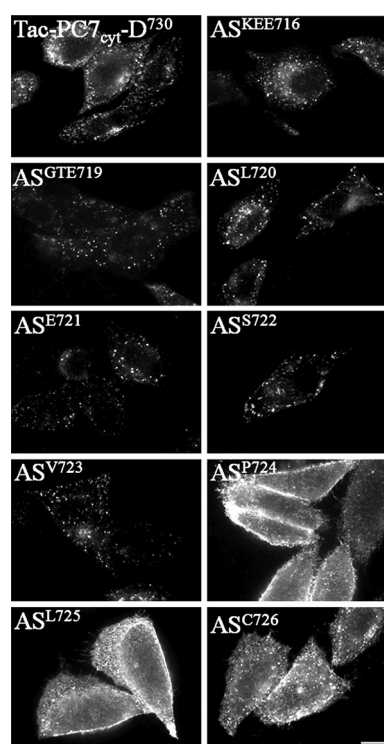


FIGURE 6. A newly defined PLC motif is essential and sufficient for endocytosis. Chimeric proteins were constructed consisting of the luminal and transmembrane domains of Tac (CD25) and part of the cytoplasmic domain of PC7 and in which individual amino acids or groups of three amino acids were substituted with alanine to determine the exact TGN sorting motif. Antibody uptake experiments (30 min 4 °C, 15 min 37 °C) using an anti-Tac antibody were performed on CHO-K1 cells transfected with those constructs. Three amino acids (PLC) were essential for the internalization of the chimeric construct. Scale bar = 10 μm.

surface biotinylation, comparable with TAC-PC7cyt. Even slightly less surface biotinylation was detected for constructs AS^{KEE716} and AS^{L720}. Altogether, those results demonstrate that Pro⁷²⁴, Leu⁷²⁵, and Cys⁷²⁶ are the only three amino acids essential for endocytosis.

DISCUSSION

In this study, we show that PC7 cycles between the TGN and the PM using motifs in the cytoplasmic tail. Internalization is mediated by CCVs and depends on a short cytoplasmic region containing the crucial amino acid sequence PLC. This short region is essential and sufficient for endocytosis but not sufficient to confer TGN localization.

PC7 is localized, at least in part, in the TGN. Overexpression studies have shown partial co-localization with TGN markers, but a less confined expression pattern (10, 11). Ultrastructural analysis showed immunoreactivity associated with coated buds and vesicles adjacent to the Golgi stack. These overexpression studies are hampered by the large relative proportion of pro-PC7 in the endoplasmic reticulum, which obfuscates the juxtanuclear TGN staining. The high proportion of pro-PC7 is caused by the slow maturation and tendency to aggregate (9). The subcellular localization of endogenous PC7 has been studied in rat liver and the lymphoblastoid cell line T2, and these studies confirm the partial TGN localization and suggest that the remainder is localized in post-TGN structures (28, 35). In

Internalization of PC7 Is Mediated by a Novel Motif

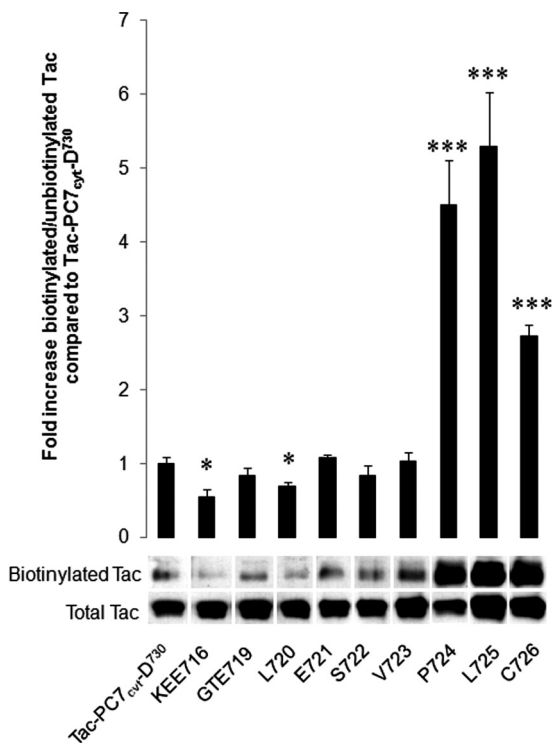


FIGURE 7. Surface biotinylation assay. CHO-K1 cells transfected with the same constructs as in Fig. 6. Surface molecules were biotinylated with sulfo-NHS-LC-biotin, and the ratio of biotinylated/unbiotinylated Tac chimeras was determined by Western blot analysis. A representative figure of this Western blot analysis is shown. Quantification of biotinylated and unbiotinylated Tac chimeras was performed using the Image Station 440 (Kodak Digital Science). Statistical analysis was performed using the Student's *t* test. Values were considered to be significantly different from Tac-PC7^{cyt}-D⁷³⁰ when *p* value < 0.05. *, *p* value < 0.05, ***, *p* value < 0.001. Error bars indicate S.D.

this study, we show that recombinant PC7 recycles in Chinese hamster ovary CHO cells to a juxtanuclear region that co-localizes to a large extent with the TGN marker TGN38. It has been shown that the TGN localization of furin is only partly overlapping with PC7 and PC5/6B (27, 28, with part of PC7 and PC5/6B in a post-TGN compartment. To what extent PC7 and PC5/6B co-localize has not been studied yet.

The involvement of CCVs in the endocytosis of PC7 is supported by the presence of PC7 in fractions enriched for CCVs and containing the markers α - and γ -adaptin. Furthermore, after hypertonic treatment of the cells or incubation with Pit-stop 2, which both interfere with clathrin-mediated endocytosis, PC7 accumulates at the PM. After furin (23) and PC5/6B (27), PC7 is now the third member of the PC family that has been shown to be internalized in CCVs.

The information that is required for internalization and TGN localization of PC7 is contained within the cytoplasmic tail and is transferable to a reporter construct. Sequence analysis showed that no tyrosine is present in the cytoplasmic tail, and therefore, no YXX(Y = Tyr, X = any amino acid and \emptyset = hydrophobic amino acid) motif, which is found in both furin and PC5/6B. On the other hand, the commonly found dileucine trafficking signal is present twice (Leu⁷⁴⁷-Leu⁷⁴⁸ and Leu⁷⁵²-Leu⁷⁵³), albeit not in the context of the extended motif (ED)XXXL(LI) or DXXLL (36). Deletion of the region containing these dileucine sequences did not appear to affect internal-

| | |
|------------------|---|
| Human | K ⁷¹⁴ E E G T E L E S V P L C S S K D ⁷³⁰ |
| Mouse | K ⁷¹³ D A G T A L E S M P L C S S K D ⁷²⁹ |
| Rat | K ⁷¹³ E V G T A L E S M P L C S S K D ⁷²⁹ |
| Canis familiaris | R ⁷¹⁴ E E G T E L E S V P L C N S K D ⁷³⁰ |
| Xenopus | Q ⁷⁰³ E L E A G S E S V P L F G V E Q ⁷²¹ |
| Cat | R ⁷⁰⁹ E E G T E L E S V P L C N S K D ⁷²⁵ |
| Rabbit | K ⁷¹⁶ E E G T E L E S V P L C H S K D ⁷³² |
| Guinea pig | R ⁷¹⁴ E E G T E L E S V P L C N S K D ⁷³⁰ |

FIGURE 8. Sequence alignment of part of cytoplasmic tail of PC7, containing internalization motif, from different organisms. Alignment of the amino acid sequence corresponding to the region Lys⁷¹⁴-Asp⁷³⁰ of the cytoplasmic tail of human PC7 in mouse, rat, dog, *Xenopus*, cat, rabbit, and guinea pig is shown.

ization, confirming that they do not play a major role in this trafficking step.

Our results show that internalization of PC7 depends on a short region containing the essential contiguous amino acid motif proline, leucine, and cysteine. The PLC motif was found to be the dominant, if not only, motif for internalization. Mutation of the leucine into an alanine in full-length PC7 blocked internalization to the same extent as deletion of the entire cytoplasmic tail. This motif is different from all previously identified internalization motifs. The amino acids are also found in the KCPL motif of P-selectin, which mediates sorting from endosomes to lysosomes (37). However, because the order of the amino acids is not conserved and no interacting proteins are currently known, the possible relevance of this amino acid similarity remains to be determined. Comparison of the PLC motif in other species shows that the sequence ESVPLC is highly conserved (Fig. 8). Nevertheless, the cysteine residue is replaced by a phenylalanine in *Xenopus laevis*, and the valine residue is replaced by methionine in mouse and rat. Single amino acid replacement of the first three amino acids of this sequence did not show a deleterious effect on internalization, so it is remarkable that these amino acids are highly conserved as well. The cysteine to phenylalanine substitution is nonconservative and suggests that this residue might be somewhat less important. This is consistent with the cell surface biotinylation experiment, which detected slightly less PC7 at the PM after mutation of the cysteine as compared with mutations of the proline or leucine.

Although the short region containing the PLC motif is sufficient for internalization, additional sequences in the cytoplasmic tail, proximal to the TM domain, are needed for TGN localization. This region of ~25 amino acids contains the two cysteines that are reversibly palmitoylated (10). However, these cysteines have been shown to affect the half-life of PC7 but not its TGN localization (11). Another remarkable feature is the presence of seven basic amino acids near these cysteines, of which five are clustered in the sequence HRSRKAK. Furin and PC5/6B contain clusters of acidic amino acids that are necessary for TGN localization and interact with the connector protein PACS-1 (23, 26, 27). No acidic cluster is present in PC7, but the basic cluster might function as the binding site for a TGN-directing protein.

The need for seven PCs is not fully understood, given the similar substrate specificity and overlapping expression patterns. However, the severe phenotypes in most PC KO (14) indicate that there is limited or no redundancy for the process-

ing of some substrates. This may be related to the specific cleavage site of a substrate or the unique temporospatial coexpression of the substrate and the PC. Characterizing the intracellular trafficking of PCs may contribute to understanding the specific PC-substrate pairs and hence the identification of a PC for possible therapeutic intervention. In this study, we have demonstrated that PC7 cycles between the PM to the TGN in CCVs using a novel transferable PLC motif in its cytoplasmic tail. This transferable motif is essential for internalization but not sufficient for TGN localization.

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