Mutations within the propeptide, the primary cleavage site or the catalytic site, or deletion of C-terminal sequences, prevents secretion of proPC2 from transfected COS-7 cells

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PC2 is a neuroendocrine endoprotease involved in the processing of prohormones and proneuropeptides. PC2 is synthesized as a proenzyme which undergoes proteolytic maturation within the cellular secretory apparatus. Cleavage occurs at specific sites to remove the N-terminal propeptide. The aim of the present study was to investigate structural requirements for the transfer of proPC2 through the secretory pathway. A series of mutant proPC2 constructs were transfected into COS-7 cells and the fate of the expressed proteins followed by pulse–chase analysis and immunocytochemistry. Human PC2 was secreted relatively slowly, and appeared in the medium primarily as proPC2 (75 kDa), together with much lower amounts of a processed intermediate (71 kDa) and mature PC2 (68 kDa). Mutations within the primary processing site or the catalytic triad caused the protein to accumulate intracellularly, whereas deletion of part of the propeptide, the P-domain or the C-terminal regions also prevented secretion. Immunocytochemistry showed that wild-type hPC2 was localized mainly in the Golgi, whereas two representative mutants showed a distribution typical of proteins resident in the endoplasmic reticulum. The results suggest that proenzyme processing is not essential for secretion of PC2, but peptides containing mutations that affect the ability of the propeptide (and cleavage sites) to fold within the catalytic pocket are not transferred beyond the early stages of the secretory pathway. C-terminal sequences may be involved in stabilizing such conformations.

INTRODUCTION

PC2 [1,2] is a member of the eukaryotic subtilisin-like endopeptidase family. Other members of this family include the yeast enzymes Kex2 [3,4] and Krp [5], as well as furin [6], PACE4 [7], PC3 (also known as PC1) [8,9], PC4 [10], PC5/PC6 [11] and PC7 (also known as LPC or PC8) [12–14]. These proteases are involved in the proteolytic cleavage of precursor proteins at specific sites to generate mature proteins during transfer through the secretory pathway.

All members of the eukaryotic subtilisin-like endopeptidase family share certain structural features. They contain an N-terminal signal peptide, a propeptide, a subtilisin-like catalytic domain, a large conserved domain named the P-domain or HomoB domain [15], and a C-terminal tail which varies in length and may contain cysteine- or threonine-rich regions (furin, PACE4, PC5), an amphipathic helix (PC2, PC3) or a transmembrane domain (Kex2, Krp, furin, and an alternatively spliced form of PC5/6 called PC6B). The role of the P-domain is unknown, but it has been shown to be essential for production of active protein, since small deletions in this region completely abolish Kex2 and furin enzyme activity [16,17].

During transit through the secretory pathway, the subtilisin-like endoproteases are converted into the mature active form by proteolytic removal of the N-terminal propeptide. In the case of human PC2 (hPC2), the propeptide contains two processing sites, cleavage at either of which can occur independently. Processing at the sequence Arg-Lys-Lys-Arg^{84} is autocatalytic [18,19], with a pH optimum and calcium requirement similar to those for substrate processing. Processing at the sequence Lys-Arg-Arg-Arg^{56} can be catalysed by another enzyme, possibly furin or PACE4. Proteolytic cleavage of the propeptide of PC2 is a relatively slow process (half-life approx. 8 h), and the conditions under which maturation occurs *in vitro* suggest that *in vivo* the propeptide is removed in a late secretory compartment [19].

The role of the N-terminal propeptide in PC2 is unknown. Possible functions include: (i) acting as an intramolecular chaperone in a manner similar to the propeptide of subtilisin BPN′ [20]; (ii) acting as an inhibitor that prevents premature activation of the endopeptidase; and (iii) involvement in sorting to the regulated secretory pathway. We have previously shown that proPC2 undergoes calcium- and pH-dependent aggregation and membrane association, and that a peptide corresponding to amino acids 57–85 of the propeptide can compete with proPC2 for membrane association [21]. The aim of the present study was to investigate the role of the propeptide of proPC2 in the ability of the latter to traverse the secretory pathway.

MATERIALS AND METHODS

DNA manipulations

DNA mutagenesis was carried out on an hPC2 cDNA (kindly supplied by Dr. D. F. Steiner, Howard Hughes Medical Centre, University of Chicago, IL, U.S.A.). (Δ587–613)hPC2, V54R,L56R-hPC2, (Δ81–84)hPC2, D142N-hPC2 and KDEL-hPC2 were all generated as described previously [19]. (Δ5–49)hPC2 was generated by digesting the wild-type cDNA with *Age*1 and *Eco*N1, making the DNA ends flush with T4 DNA polymerase and re-ligating. (Δ54-77)hPC2 was generated by loopout mutagenesis using an oligonucleotide with the sequence 5′ GTAACCTGCCTTTTTTCGGTCAAATCCCTTTG-CAAGGCCATTGTGATAAAA 3′. 414-hPC2, 569-hPC2 and 573-hPC2 were generated by amplifying hPC2 cDNA using a 3′

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; Endo H, endoglycosidase H; ER, endoplasmic reticulum; hPC2, human PC2; P,N,Gase F, P,N-glycanase F.

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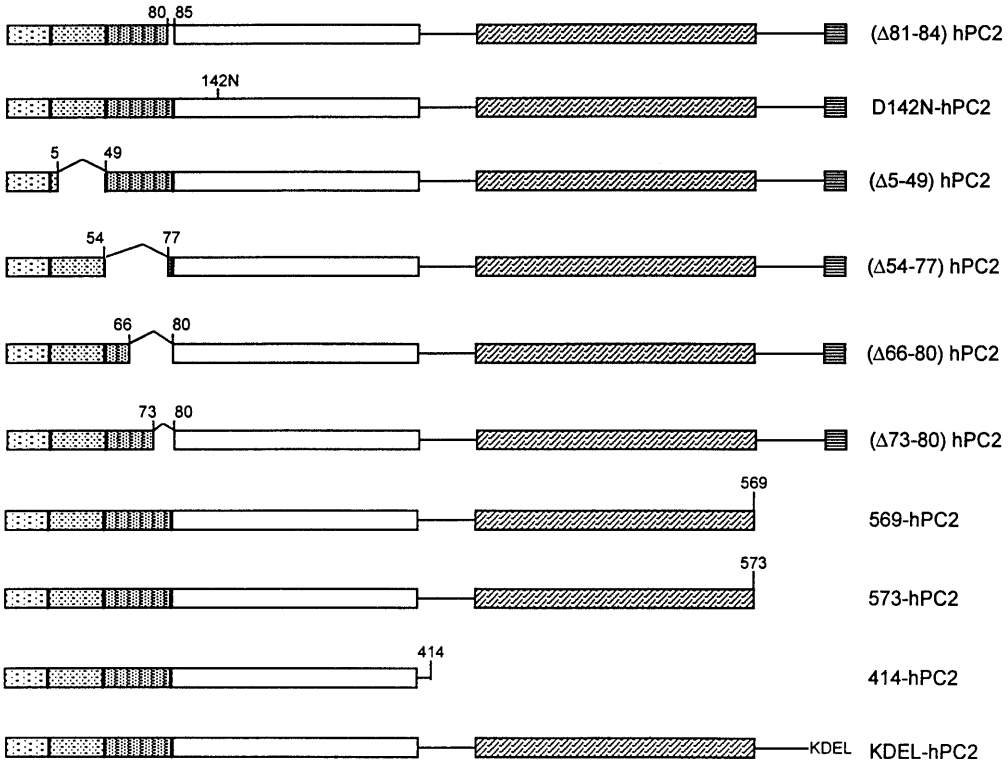

Figure 1 Structures of normal and mutant hPC2 peptides

Diagram showing the structures of hPC2 and the mutant constructs used in this study. The domains of PC2 are indicated, along with the catalytically important residues and the primary and secondary cleavage sites. Constructs were generated as described in the Materials and methods section.

oligonucleotide containing a stop codon after the codon for the residue indicated and a 5′ oligonucleotide encoding the N-terminus of the preproprotein. The PCR products were cloned and the region of interest sequenced and subcloned back into the wild-type cDNA. (Δ66–80)hPC2 and (Δ73–80)hPC2 were generated using the Excite® kit (Stratagene).

Cell culture and lipofection

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum in an atmosphere of 5% CO_2. Cells at about 40% confluence in six-well plates were transfected by mixing 2 μg of DNA and 24 μl of a 1 nM lipid suspension containing a 2:1 (molar ratio) mixture of dioleoyl-L-α-phosphatidylethanolamine (Sigma, Poole, Dorset, U.K.) and dimethyldioctadecylammonium bromide (Fluka) in 0.5 ml of serum-free DMEM. The lipid–DNA complexes were allowed to form for 20 min and then added to the washed cells for 4 h before serum was replaced [22].

Pulse–chase and immunoprecipitation

At 48 h post-transfection the cells were incubated in methionine- and serum-free DMEM (Sigma) for 30 min. The medium was then removed and the cells pulse-labelled in 0.5 ml of the same medium supplemented with 170 μCi of [^{35}S]methionine (> 1000 Ci/mmol; Amersham) per well. After the 30 min pulse, the labelling medium was removed and replaced by 1 ml of complete medium containing 30 mg/l unlabelled methionine. Medium was removed at the appropriate time and the cells harvested in NDET [1% (v/v) Nonidet P40, 0.4% (w/v) deoxycholate, 66 mM EDTA, 10 mM Tris/HCl, pH 7.4). Cell extracts were incubated on ice for 5 min, and then the nuclei were centrifuged (13000 ***g***, 1 min) and the post-nuclear supernatant saved. hPC2 immunoreactivity was immunoprecipitated in NDET/0.3% SDS using anti-PC2 antiserum raised to amino acids Met^{233}–Asn^{613} of hPC2 expressed in *Escherichia coli* using the pEt12 vector.

Endoglycosidase digestion

Immunoprecipitated material was denatured by heating to 100 °C in 0.5% SDS and 1% β-mercaptoethanol. Half of the material was incubated at 37 °C for 2 h with 100 units of endoglycosidase H (Endo H) in 50 mM sodium citrate, pH 5.5. The other half was incubated at 37 °C for 2 h with 100 units of P,N-glycanase F (P,N,Gase F) in 50 mM sodium phosphate, pH 7.5, and 1% Nonidet P40. P,N,Gase F and Endo H were supplied by New England Biolabs (Hitchin, Herts., U.K.).

Immunocytochemistry

Immunocytochemistry was carried out as detailed in Cramer and Cutler [23] using the rabbit PEP4 anti-PC2 antibody (kindly supplied by Dr. D. F. Steiner, Howard Hughes Medical Centre, University of Chicago, IL, U.S.A.).

RESULTS

The aim of this study was to investigate the structural requirements for the transfer of proPC2 through the secretory apparatus. The approach adopted was to use site-directed mutagenesis to

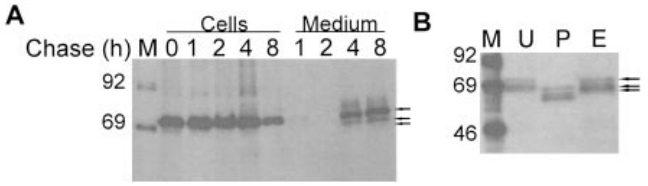

Figure 2 Expression of hPC2 in COS-7 cells

(**A**) Pulse–chase analysis of hPC2 immunoreactivity found in cell extracts and medium from cells transfected with pCR-PC2. At 48 h after lipofection, cells were labelled with [^{35}S]methionine for 30 min and chased in culture medium for the times indicated. Medium samples were removed and saved and the cells were harvested in immunoprecipitation buffer. Samples were immunoprecipitated with anti-PC2 antibody, analysed on an SDS/9%-PAGE gel and detected by fluorography. The track marked M shows molecular mass marker proteins (kDa). (**B**) Glycosidase digestion of secreted hPC2 immunoreactivity. Immunoprecipitated protein from medium after an 8 h chase was treated with P,N,Gase F (P), Endo H (E) or left untreated (U). Samples were analysed on an SDS/9%-PAGE gel and detected by fluorography. The track marked M shows molecular mass marker proteins (kDa).

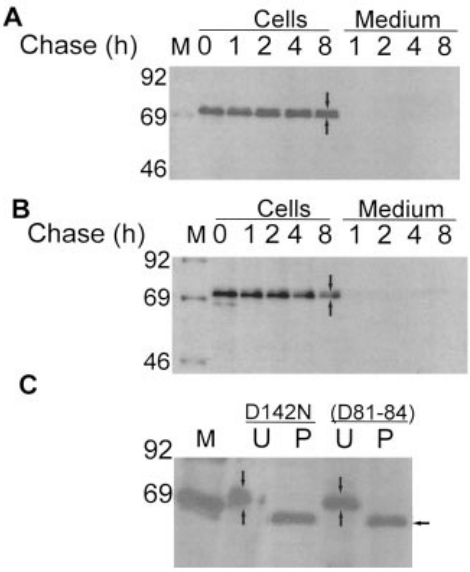

Figure 3 Expression of (Δ81–84)hPC2 and D142N-hPC2 in COS-7 cells

Pulse–chase analysis of hPC2 immunoreactivity found in cell extracts and medium from cells transfected with pCR-(Δ81–84)hPC2 (**A**) and pCR-D142N-hPC2 (**B**). At 48 h after lipofection, cells were labelled with [^{35}S]methionine for 30 min and chased in culture medium for the times indicated. Medium samples were removed and saved, and the cells were harvested in immunoprecipitation buffer. Samples were immunoprecipitated with anti-PC2 antibody, analysed on an SDS/9%-PAGE gel and detected by fluorography. The track marked M shows molecular mass marker proteins (kDa). (**C**) Glycosidase digestion of retained hPC2 immunoreactivity. Immunoprecipitated protein from extracts of cells transfected with either (Δ81–84)hPC2 or D142N-hPC2 after an 8 h chase was treated with P,N,Gase F (P) or left untreated (U). Samples were analysed on an SDS/9%-PAGE gel and detected by fluorography. The track marked M shows molecular mass marker proteins.

introduce specific point or deletion mutations within a human PC2 cDNA, and to transfect these constructs into the monkey kidney cell line COS-7. These cells were used because of the high-level expression that could be attained using the pCR expression vector.

The hPC2 constructs used in this study are shown in Figure 1. hPC2 is a 613-amino-acid protein containing: (1) a signal peptide; (2) a propeptide (amino acids 1–84) which contains two cleavage sites at Lys-Arg-Arg-Arg^{56} and Arg-Lys-Lys-Arg^{84}; (3) a catalytic domain containing the Asp-His-Ser catalytic triad which is characteristic of serine proteases; (4) a P-domain (amino acids 415–569) which is conserved among members of the family; and (5) a C-terminal hydrophobic sequence (amino acids 592–613). Two mutants were made which from previous studies [24] were known to prevent proPC2 processing, i.e. ΔRKKR^{84}-hPC2 and D142N-hPC2. Further constructs were designed to investigate the role of the propeptide in PC2 secretion. However, preliminary experiments indicated that complete deletion of the propeptide affected the ability of the nascent polypeptide to fold correctly, with the result that the expressed protein was degraded within the endoplasmic reticulum (ER) (K. I. J. Shennan and K. Docherty, unpublished work). We therefore made a series of smaller deletions, one of amino acids 5–49, a second of amino acids 54–77, a third of amino acids 66–80 and a fourth in which amino acids 73–80 were deleted. Note that the primary (Arg-Lys-Lys-Arg^{84}) and secondary (Lys-Arg-Arg-Arg^{56}) processing sites are retained within all these constructs. To investigate the role of C-terminal sequences, we generated several C-terminally truncated forms of hPC2: 414-hPC2, which lacks the P-domain; 569-hPC2, which terminates at the putative C-terminus of the P-domain; and 573-hPC2, which terminates at the end of the C-terminal region of identity between PC2 and PC1/3. We also generated KDEL-hPC2, which has an ER retention signal added to its C-terminus.

Expression of wild-type hPC2 in COS-7 cells resulted in the appearance of a single intracellular 75 kDa protein (Figure 2A) which was not present in mock-transfected cells (results not shown). This protein corresponds to proPC2. The amount of the 75 kDa proPC2 protein in the cell decreased after 2 h, coinciding with the appearance in the medium of three proteins of 75, 71 and 68 kDa. Proteins of similar sizes have been observed following expression of hPC2 in *Xenopus* oocytes [24] as well as in isolated rat islets of Langerhans [25], and have been shown to correspond to proPC2 (75 kDa), an intermediate generated following cleavage at the sequence Lys-Arg-Arg-Arg^{56} (71 kDa) and mature PC2 generated following cleavage at the sequence Arg-Lys-Lys-Arg^{84} (68 kDa). The 75, 71 and 68 kDa proteins appeared simultaneously in the medium, and there was no further processing in the medium during a subsequent 24 h chase period. Although the majority of the secreted material was unprocessed, these results suggest that a significant amount of processing was occurring within the COS-7 cells, and that the processed material was rapidly secreted with no detectable intracellular accumulation. Expression of hPC2 at lower levels resulted in the secretion of a higher proportion of processed forms, indicating that the processing activity is saturable and suggesting that it is due to an endogenous activity in COS-7 cells. It would not be expected that material should accumulate in the secretory pathway in this cell line, as COS-7 cells do not have a regulated secretory pathway.

Treatment with Endo H resulted in a small reduction of about 2–3 kDa in the molecular masses of all three of the secreted proteins (Figure 2B). Treatment with P,N,Gase F generated a larger decrease in molecular mass, of about 6–7 kDa. These results indicate that, although most of the asparagine-linked sugar moieties have been modified to acquire Endo H resistance, a significant fraction have not, or have undergone further modifications to re-acquire sensitivity. A 46 kDa immunoreactive protein secreted with PC2 is visible in Figure 2(B). This protein remains uncharacterized but, given the similar effects of Endo H and P,N,Gase F on this protein, it is possible that it is a C-terminal cleavage product of PC2, presumably containing the three potential glycosylation sites.

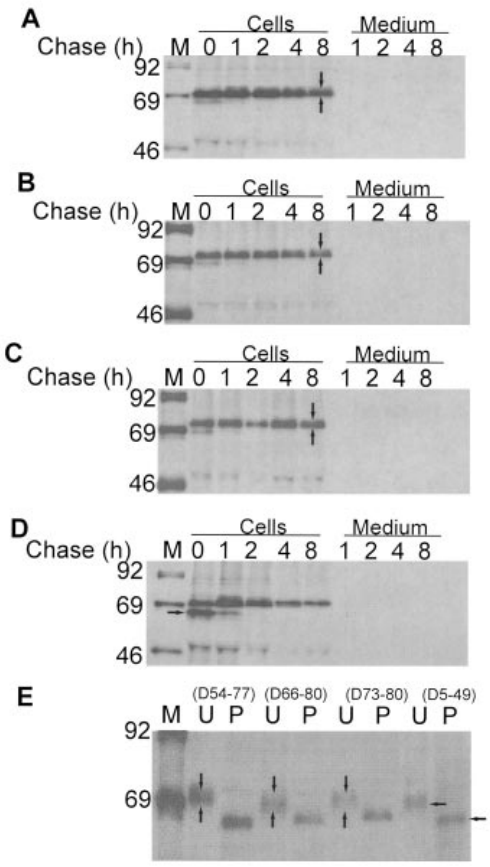

Figure 4 Expression of (Δ54–77)hPC2, (Δ66–80)hPC2, (Δ73–80)hPC2 and (Δ5–49)hPC2 in COS-7 cells

Pulse–chase analysis of hPC2 immunoreactivity found in cell extracts and medium from cells transfected with pCR-(Δ54–77)hPC2 (**A**), pCR-(Δ66–80)hPC2 (**B**), pCR-(Δ73–80)hPC2 (**C**) and pCR-(Δ5–49)hPC2 (**D**). At 48 h after lipofection, cells were labelled with [^{35}S]methionine for 30 min and chased in culture medium for the times indicated. Medium samples were removed and saved, and the cells were harvested in immunoprecipitation buffer. Samples were immunoprecipitated with anti-PC2 antibody, and immunoprecipitates were analysed on an SDS/9%-PAGE gel and detected by fluorography. The track marked M shows molecular mass marker proteins (kDa). (**E**) Glycosidase digestion of retained hPC2 immunoreactivity. Immunoprecipitated protein from extracts of cells transfected with (Δ54–77)hPC2, (Δ66–80)hPC2, (Δ73–80)hPC2 or (Δ5–49)hPC2 after an 8 h chase was treated with P,N,Gase F (P) or left untreated (U). Samples were analysed on an SDS/9%-PAGE gel and detected by fluorography. The track marked M shows molecular mass marker proteins (kDa).

Expression of the (Δ81–84)hPC2 (Figure 3A) and D142N-hPC2 (Figure 3B) mutants resulted in the appearance of 75 kDa proteins that were retained within the cell. Both proteins appeared to be relatively stable; however the 75 kDa proteins increased in size to 78 kDa over an 8 h chase period. Treatment of the material immunoprecipitated from cell extracts after an 8 h chase with P,N,Gase F resulted in the reduction of both bands to a single band of approx. 67 kDa (Figure 3C), indicating that the increase in mass was due to additional glycosylation. This suggests that these mutant peptides undergo initial glycosylation, followed by slow subsequent processing/modification. The glycosylation can also be completely removed with Endo H (results not shown), in contrast to the pattern of partial Endo H sensitivity found with secreted wild-type hPC2, suggesting that these mutants do not progress beyond the cis-Golgi and accumulate either in the ER or in the early Golgi.

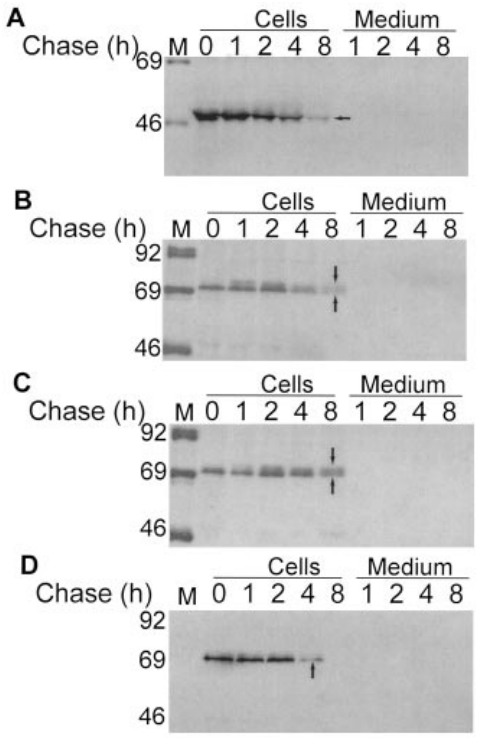

Figure 5 Expression of 414-hPC2, 569-hPC2, 573-hPC2 and KDEL-hPC2 in COS-7 cells

Pulse–chase analysis of hPC2 immunoreactivity found in cell extracts and medium from cells transfected with pCR-414-hPC2 (**A**), pCR-569-hPC2 (**B**), pCR-573-hPC2 (**C**) or pCR-KDEL-hPC2 (**D**). At 48 h after lipofection, cells were labelled with [^{35}S]methionine for 30 min and chased in culture medium for the times indicated. Medium samples were removed and saved, and the cells were harvested in immunoprecipitation buffer. Samples were immunoprecipitated with anti-PC2 antibody, and immunoprecipitates were analysed on an SDS/9%-PAGE gel and detected by fluorography. The track marked M shows molecular mass marker proteins (kDa).

In order to investigate whether the propeptide contains a signal which must be removed before PC2 can pass through the secretory compartment, we next expressed a series of constructs containing deletions of regions in the propeptide. Expression of any of the mutants containing deletions between the primary and the secondary processing sites [(Δ54–77)hPC2 (Figure 4A), (Δ66–80)hPC2 (Figure 4B) and (Δ73–80)hPC2 (Figure 4C)] resulted in a similar pattern of intracellular retention to that found with D142N-hPC2 and (Δ81–84)hPC2, i.e. no immunoreactivity was secreted, while the retained protein was not degraded appreciably over the chase period and underwent a slow, inefficient, increase in mass that could be reversed by treatment with P,N,Gase F (Figure 4E). In contrast, deletion of the region N-terminal to the secondary processing site [(Δ5–49)hPC2 (Figure 4D)] had a different effect. Within the labelling period (0 h chase) there were two principal products of 66 and 70 kDa. The 66 kDa protein was unglycosylated proPC2 (results not shown) which, during the subsequent chase period, was converted into the 70 kDa form. It should be noted that there was no detectable accumulation of unglycosylated proPC2 protein when wild-type hPC2 was expressed in COS-7 cells. The (Δ5–49)hPC2 70 kDa proPc2 protein was not secreted; however, there was no further glycosylation as seen with the other propeptide mutants.

Deletion of the P-domain (414-hPC2; Figure 5A) prevented the secretion of PC2. In this mutant all three glycosylation sites

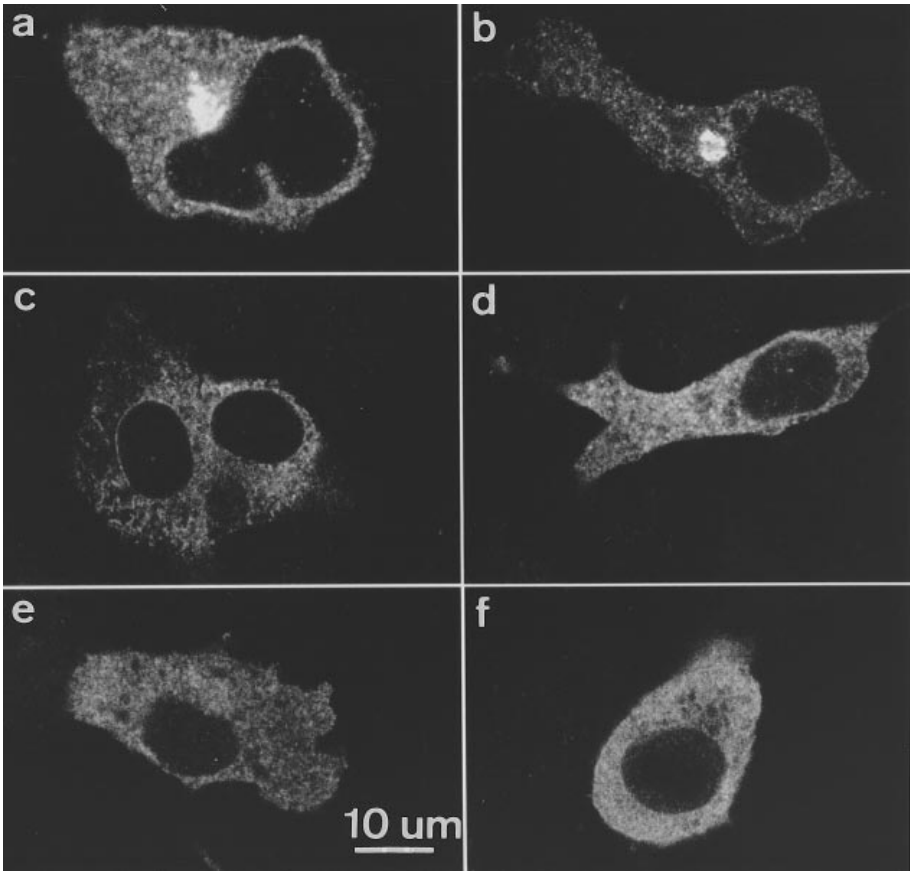

Figure 6 Immunocytochemistry of hPC2, D142N-hPC2 and KDEL-hPC2 in COS-7 cells

Cells were transfected with pCR-PC2 (**a**, **b**), pCR-D142N-PC2 (**c**, **d**) or pCR-PC2-KDEL (**e**, **f**), and plated out on to glass coverslips 48 h after lipofection and stained using the PEP4 anti-PC2 antibody and a fluorescently labelled goat anti-rabbit antibody. Staining for hPC2 is particularly concentrated in a perinuclear region typical of Golgi proteins, whereas staining for D142N-hPC2 and KDEL-hPC2 is reticular, suggesting an ER localization.

had been deleted, and so the protein could not be glycosylated. However, unlike the other mutants, 414-hPC2 appeared to be degraded in the cell, albeit slowly. Two smaller C-terminal deletions, 569-hPC2 (Figure 5B) and 573-hPC2 (Figure 5C), both of which leave the P-domain intact, prevented secretion. In both of these cases the retained protein was not degraded and underwent slow additional glycosylation.

With mutant KDEL-hPC2 (Figure 5D), a single protein of 75 kDa accumulated in the cell, with no increase in the molecular mass of the retained material. However, KDEL-hPC2 appears to be less stable in the cell, with most of the labelled protein being degraded after a 4 h chase and no labelled intracellular immunoreactivity evident after an 8 h chase.

The pulse–chase experiments identified two types of mutation, both of which resulted in the intracellular retention of the expressed protein. In one type the expressed protein was not proteolytically cleaved and the resultant propolypeptide was stably retained and increased in size by about 3 kDa due to additional glycosylation. In the second type the expressed protein (i.e. 414-hPC2 and KDEL-hPC2) was also not cleaved, but did not increase in mass and was degraded over the chase period. To determine whether the two types of mutation affected the intracellular location of the expressed proteins, immuncytochemistry was performed on selected constructs. Cells expressing wild-type hPC2 exhibited the reticular pattern characteristic of the ER plus perinuclear concentration typical of Golgi proteins (Figures 6a and 6b). Cells expressing either type of mutation, i.e. D142N-hPC2 (Figures 6c and 6d) and KDEL-hPC2 (Figures 6e and 6f) exhibited reticular staining, but with no perinuclear concentration. No significant difference in localization between the two classes of mutation could be seen at the resolution afforded by light microscopy.

DISCUSSION

Expression of hPC2 in COS-7 cells resulted predominantly in the secretion of 75 kDa proPC2, with lesser amounts of the 71 and 68 kDa processed forms. We have previously shown that *Xenopus* oocytes secrete the 75 and 71 kDa molecules and that subsequent cleavage to the 71 and 68 kDa forms occurs within the medium [24]. The 75 to 71 kDa cleavage is probably mediated by a *Xenopus* furin-like protease(s), whereas the 75/71 to 68 kDa cleavage occurs by way of an autocatalytic mechanism which is activated in the medium as it becomes slightly acidified during extended culture of the oocytes [24]. In COS-7 cells the degree of processing is not proportional to the level of expression, and in the culture medium there is no further cleavage of the 75/71 kDa species to the 68 kDa mature enzyme, suggesting that cleavage of the 75 kDa proPC2 molecule to the 71 kDa intermediate and the

68 kDa mature enzyme occurs within the cell and is catalysed, at low efficiency, by an endogenous protease.

The study with PNGase F indicated that all three potential glycosylation sites are utilized. Approx. 30% of the glycosyl residues on the secreted proteins are sensitive to Endo H, suggesting either that they have undergone further unusual modifications or that, for some reason, the high-molecular-mass mannose chains have not been removed. This pattern of partial Endo H sensitivity is similar to that found with PC2 immunoprecipitated from rat islets of Langerhans [25], but different from that found in transfected CHO cells or RIN msf cells, where none of the sugar residues are resistant to Endo H [26].

Mutagenesis was undertaken to investigate the structural requirements for the transfer of PC2 through the secretory pathway. Previous studies on furin had shown that cleavage of the propeptide may be a prerequisite for exit from the ER [27–29]. Dissociation of the propeptide and activation of the enzyme may occur in a later compartment. Unexpectedly, in the present study all the mutant PC2 molecules were retained within the cell as a proenzyme. Thus, whereas wild-type proPC2 (75 kDa) was efficiently secreted from COS-7 cells, mutations within the catalytic pocket or cleavage site, or deletion of propeptide sequences flanking the cleavage site or of sequences at the C-terminus of proPC2, resulted in the retention of proPC2 within the cell. These results suggest that the efficient transfer of proPC2 through the secretory pathway is dependent on a structural conformation or post-translational modification that is disrupted by disparate mutations spanning the length of the protein.

Homology modelling of furin suggests that residues $+1$ to -6 are important in interactions with the substrate-binding region [30]. As PC2 shows substantial sequence identity with furin, it seems likely that these interactions are also important for substrate recognition in PC2, and it is possible that some structural motif between residues 73 and 77 (i.e. -8 to -12) is required. Recent molecular modelling studies have suggested that the primary cleavage site folds into the active site of the prohormone, and it may be that the deletions in the propeptide [i.e. (Δ54–77)hPC2, (Δ66–80)hPC2 and (Δ73–80)hPC2], or deletion of the Arg-Lys-Lys-Arg^{84} cleavage site, or mutagenesis of the catalytic pocket (D142N), may affect this interaction, and that correct insertion of the propeptide into the active site is required before exit from the ER can occur.

There may be a different explanation for the retention of the (Δ5–49)hPC2 mutant. This deletion causes an approx. 3-fold reduction in the rate of initial glycosylation, and this mutant does not acquire the additional glycosylation found with the other propeptide deletion mutants. It is likely that the decrease in the glycosylation rate is a result of misfolding of the molecule, since it is assumed that this region of the protein acts as an intramolecular chaperone, as is the case with the homologous bacterial protease subtilisin BPN′ [31]. In subtilisin a portion of the propeptide is required for formation of the correct tertiary structure, and denatured protein will only refold if the propeptide is present, whether in *cis* or *trans* conformation.

The C-terminally truncated mutant 414-hPC2 is turned over in the cell relatively rapidly. In the case of Kex2 it was found that mutant proteins containing deletions in the P-domain were catalytically inactive and were not secreted. It was suggested that they may be acting by disrupting recognition of the processing site, and that the mutants accumulate in the cell because of their inability to undergo processing to remove the N-terminal propeptide [17]. However, in the case of PC2 it appears that deletion of the P-domain causes a structural alteration that results in degradation of the resultant proenzyme in the ER. In contrast, the other two C-terminally truncated mutants, 569-hPC2 and 573-hPC2, behaved in an identical manner to D142N-hPC2 and the four peptides containing deletions adjacent to and including the primary processing site. This is puzzling, as neither of these deletions stretch into the P-domain, as defined by identity with Kex2, and we have seen that a smaller deletion in the C-terminus (PC2 M1) is secreted in a similar manner to the wild type [24]. The structures of the P-domain and the C-terminus are unknown at present, and it may be that these regions interact with the propeptide in some way.

Pulse–chase analysis of cells expressing KDEL-hPC2 showed that, as expected, this mutant is not secreted. The protein does not undergo the additional glycosylation found with most of the mutants and is degraded relatively rapidly. The similarity of the fate of this mutant to that of 414-hPC2 suggests that both of these proteins are retained in the same compartment.

The similarity in the immunocytochemistry for KDEL-hPC2 and D142N-hPC2 suggests that all the mutants are retained in the ER. However, the differences in additional glycosylation suggest that the majority of the mutants are retained less efficiently (and possibly therefore by a different mechanism) than KDEL-hPC2. It does not appear that these mutants are simply trapped in the ER, or are retained by the same retrieval pathway as KDEL-hPC2. It is possible that these mutants are blocked in a very early Golgi compartment and 'back up' into the ER.

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