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Proprotein processing activity and cleavage site selectivity of the Kex2-like endoprotease PACE4

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Proprotein processing activity of the Kex2-like mammalian endoprotease PACE4 and its cleavage selectivity for sites with basic amino acid residues were determined. Using a recombinant vaccinia virus-based expression system, PACE4 was expressed in pig kidney PK(15) cells and, like two other Kex2-like endoproteases furin and PC6A, shown to correctly process the precursor of von Willebrand factor (pro-vWF). Furthermore, characteristics of the cleavage site selectivity of PACE4 were compared to those of furin and PC6A using the vWF cleavage site mutants vWFR-1G, vWFK-2A, and vWFR-4A as substrates. Cleavage site selectivity of PACE4 and PC6A appeared to be similar but they differed from that of furin.

Proprotein cleavage; PACE4; von Willebrand factor

1. INTRODUCTION

Recently, a novel family of eukaryotic endoproteolytic processing enzymes has been identified whose members appear capable to release biologically active polypeptides from inactive precursor proteins by cleaving the latter at sites with paired basic amino acid residues (for review see [1–3]). Such precursors include those for a variety of polypeptide hormones, neuropeptides, growth factors, growth factor receptors, plasma proteins, and viral glycoproteins [4]. The first known enzyme of this novel family is the Kex2 protease of yeast *Saccharomyces cerevisiae*, a Ca^{2+}-dependent serine protease with a subtilisin-like catalytic domain and involved in processing of pro-α-mating factor and prokiller toxin [5,6]. The mammalian prototype is furin [7], a ubiquitously expressed [8,9] Kex2-like [10] enzyme with demonstrated cleavage specificity for sequences with multiple basic amino acid residues [11–14]. Other mammalian enzymes are PC1/PC3 and PC2 [15–17], expression of which has sofar been found only in neuroendocrine cells, PC4 [18,19], which is expressed in spermatogenic cells, the ubiquitously expressed PACE4 [20], and, finally, PC6 [21,22], which is also known as PC5 [23]. PC6 occurs in two isoforms; PC6A [21], which has been found in a variety of tissues and cell lines, and PC6B [22], which has a much larger cysteine-rich region than PC6A and has been found mainly in intestine. For most of the mammalian Kex2-like endoproteases, the cleavage specificity and the requirement of basic amino acid residues immediately amino-terminally to the cleavage site have been established in co-expression experiments. However, not yet for PACE4.

The structural organization of the mammalian processing enzymes of this novel family appears to be highly similar, although they also contain unique features. One common feature of all enzymes is that they seem to be synthesized initially as zymogens, all with multiple basic residues at the presumed maturation cleavage site. Only the mature enzyme is assumed to be capable of processing substrates, therefore, removal of the pro-domain seems to be essential for acquiring substrate cleavage activity [24,25]. For human furin, it has been demonstrated that removal of its pro-domain takes place at the predicted cleavage site (K-R-R-T-K-R^{107}↓) and occurs via an intramolecular autoproteolytic process [24,25]. Another common feature of these enzymes is that their subtilisin-like catalytic domains are highly similar [26]. However, positional requirements with respect to basic amino acid residues next to the cleavage site in a substrate seem to vary. The neuroendocrine-specific endoproteases PC1/PC3 and PC2 preferentially cleave carboxy-terminally of paired basic residues, Lys-Arg or Arg-Arg [27]. In contrast, the preferred site for cleavage by furin seems to be the consensus sequence Arg-X-Arg/Lys-Arg [28], although furin is also capable to process efficiently cleavage site mutants of the precursor of von Willebrand factor (pro-vWF) with basic residues at the P1 and P2 or at the P1 and P4 positions only [25]. From recent site-directed mutagenesis studies of furin, further insight was obtained in structural features that seem to control selectivity of the enzyme for the basic residues in a cleavage site [25]. It was demonstrated that particular negatively charged residues in or near the S1–S4

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subsites of the substrate-binding region of furin are critical. Other negative charges were predicted to contribute to substrate binding in the S3, S5 and S6 subsites ([25] and Siezen et al., in preparation).

Here, we report results of studies in which the proprotein processing activity of PACE4 is established and requirements with respect to the positioning of basic residues next to the cleavage site in a substrate are defined. As substrates, wild-type von Willebrand factor (vWF) and three cleavage site mutants of vWF were used; the latter included P1 mutant vWFR-1G, P2 mutant vWFK-2A, and P4 mutant vWFR-4A. Our studies indicate that cleavage site selectivity of PACE4 resembles that of PC6A and differs from that of furin. Cleavage by PACE4 and PC6A is apparently restricted to a limited variation in the positioning of the basic residues next to the substrate cleavage site. This in contrast to furin which appears to be a more multi-purpose processing enzyme.

2. MATERIALS AND METHODS

2.1. *cDNAs and molecular cloning*

A mouse *Not*I cDNA fragment containing all coding sequences of PC6A [21] was cloned into pGEM13Zf(+) (Promega) allowing expression under control of the T7 promoter. The resulting clone was named pGEMPC6A. pGEMPC6A DNA was then digested with restriction endonucleases *Acc*I and *Hin*dIII to remove all coding sequences of PC6A 3′ of the signal peptide sequence. Human PACE4 sequences were derived from a PACE4 cDNA cloned into the *Not*I site of pBluescript II KS(+) (Stratagene); this cDNA clone contained all PACE4 coding sequences except for 43 nucleotides at the 5′ end [20]. DNA of this PACE4 clone was digested with restriction endonuclease *Hin*dIII and partially digested with *Acc*I to obtain a 3.2 kbp PACE4 cDNA fragment, which contained all PACE4 coding sequences except those encoding the signal peptide. The 3.2 kbp *Acc*I–*Hin*dIII PACE4 cDNA was ligated to the *Acc*I–*Hin*dIII pGEMPC6A DNA fragment and the resulting construct was named pGEMPRE$_{PC6A}$PACE4. pGEMPRE$_{PC6A}$PACE4 DNA encodes a chimeric protein composed of the PC6A signal peptide linked to pro-PACE4 as outlined in Fig. 1. pGEMFUR contains the coding sequences for human furin.

2.2. *Infection and lipofection of PK(15) cells*

Pig kidney PK(15) cells were propagated in DMEM supplemented with fetal calf serum (10% v/v), glutamine (2 mM) and antibiotics (penicillin (100 U/ml) and streptomycin (100 μg/ml)). Cells were infected with recombinant vaccinia virus V.V.:T7 at an m.o.i. of 5 in medium containing bovine serum albumin (20 μg/ml). Recombinant vaccinia virus V.V.:T7 produces T7 RNA polymerase [29]. After infection for 1 h, medium was removed and cells were lipofected for 16–18 h with 4 μg DNA using DOTAP transfection-reagent (Boehringer Mannheim Biochemical), according to the suppliers instructions. Recombinant DNA constructs, which express human furin, wild type pro-vWF (cleavage site R-S-K-R^{763}↓) or pro-vWF cleavage site mutants vWFR-1G (P1 mutant, cleavage site R-S-K-G^{763}↓), vWFK-2A (P2 mutant, cleavage site R-S-A-R^{763}↓) or vWFR-4A (P4 mutant, cleavage site A-S-K-R^{763}↓) under control of the T7 promoter, have been described elsewhere [25].

2.3. *Radiolabelling of cells and analysis of biosynthesis of proprotein processing enzymes ans vWF substrates*

Biosynthesis of proprotein processing enzymes (furin, PC6A and PACE4) and pro-vWF substrates (wild-type pro-vWF and pro-vWF cleavage site mutants) were studied 16–18 h after lipofection. Prior to radiolabelling of PK(15) cells, medium was removed and cells were incubated for 1 h in RPMI medium that lacked methionine. Subsequently, cells were labelled for 30 min in the presence of [^{35}S]methionine (100 μCi/ml, spec. act. > 800 Ci/mmol), followed by a chase of 4 h with unlabelled methionine (final concentration 1 mM). After centrifugation for 5 min at 3,000 rpm, the medium samples were concentrated by TCA-precipitation in the presence of bovine serum albumin (50 μg/ml). All samples were analyzed under reducing conditions on a 5% SDS-polyacrylamide gel.

3. RESULTS AND DISCUSSION

To establish that PACE4 is indeed a genuine member of the Kex2 family of endoproteases, proprotein processing studies were performed, first with wild-type pro-vWF as a substrate. Since the structures of PACE4 and PC6A were reported to be strikingly similar [21], mouse PC6A was included in the study to allow comparison of the biological activities of the two proteins. Furin, the mammalian prototype of this novel family of processing enzymes, was also included. To efficiently express the processing enzymes and the pro-vWF substrate, we decided to clone the corresponding cDNAs into pGEM13Zf(+), allowing expression under control of the T7 promoter, and to use pig kidney PK(15) cells that were infected with recombinant vaccinia virus V.V.:T7 as host cells; upon V.V.:T7 infection, the PK(15) cells were previously shown to produce relatively large quantities of T7 RNA polymerase [25]. It should be noted that in this expression system, endogenous protein synthesis is highly suppressed allowing direct detection of secreted proteins without additional purification steps and thus monitoring the biosynthesis of PACE4 and PC6A. This was important since specific anti-PACE4 and anti-PC6A antibodies to detect these proteins in immunoprecipitation or Western blot analysis were not

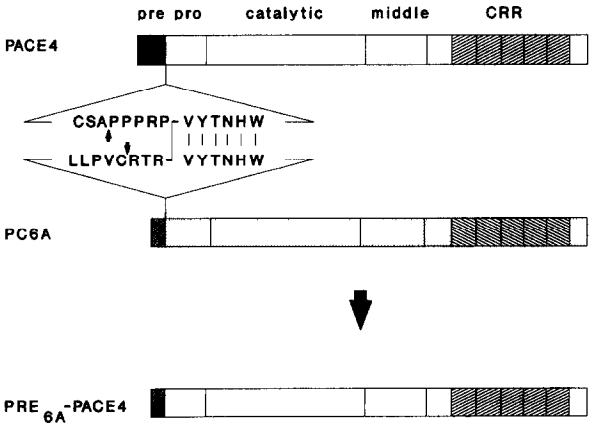

Fig. 1. Construction of chimeric PRE$_{PC6A}$-PACE4 which encodes a precursor protein that consists of the PC6A signal peptide and pro-PACE4. The predicted mature product encoded by PRE$_{PC6A}$-PACE4 DNA is assumed to be identical to wild-type PACE4. Domain swapping was performed just upstream of identical regions of the PC6A and PACE4 proteins, as is indicated by a connecting line. Predicted signal cleavage sites are indicated with arrows. The resulting chimeric PRE$_{PC6A}$-PACE4 protein is shown in the lower part of the figure.

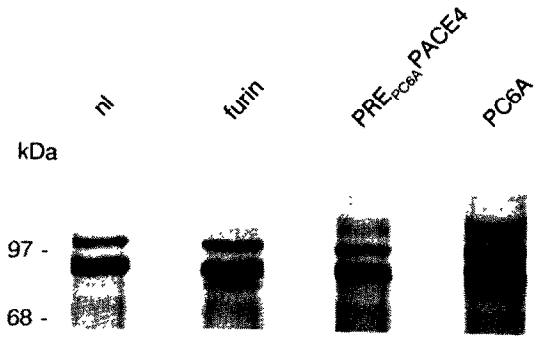

Fig. 2. Biosynthesis of furin, PACE4 and PC6A. After infection of PK(15) cells with V.V.:T7 virus, cells were lipofected with 4 μg of pGEMFUR, pGEMPRE$_{PC6A}$PACE4 or pGEMPC6A DNA, as indicated. Analysis of the newly synthesized proteins was performed as described in Materials and Methods. In the first lane, proteins produced by cells that were not lipofected (nl) are shown. Relative positions of furin, PACE4 and PC6A are indicated with arrows.

available. As far as PC6A was concerned, relatively high levels of PC6A were obtained in PK(15) cells (data not shown). For PACE4, however, the available cDNA appeared to be incomplete and to lack 5′-end coding sequences. Probably because of the fact that the missing 5′ region was very GC-rich, it appeared difficult to clone the missing sequences by using the polymerase chain reaction (PCR). As an alternative approach, we constructed chimeric DNA PRE$_{PC6A}$-PACE4, which consisted of the 5′-end sequences of PC6A, including the signal peptide cleavage site, and the PACE4 sequences encoding pro-PACE4 (Fig. 1). The fusion site of the PC6A and PACE4 sequences was selected close to the sites where the signal peptides were assumed to be cleaved off. Within these regions, the deduced amino acid sequences of PACE4 and PC6A appeared to be identical over a stretch of eight amino acids and highly similar throughout the entire pro-region. This facilitated the planned exchange and reduced the possibility of disturbing secondary structures. In fact, of all dibasic processing enzymes presently known, PC6A appeared to structurally resemble PACE4 the most. It should be noted that the predicted signal peptide of PACE4 is larger than that of PC6A [20–22]. Since the site for cleaving off the PC6A signal peptide was preserved in PRE$_{PC6A}$-PACE4, it was expected that this would occur normally. The predicted mature product encoded by PRE$_{PC6A}$-PACE4 DNA was assumed to be identical to wild-type PACE4. Finally, it appeared that the sequences around the putative initiation codon for PC6A were in better agreement with Kozak's rules [30] than those around the predicted start codon for PACE4. In light of this all, similar high expression levels of PACE4 encoded by PRE$_{PC6A}$-PACE4 DNA were expected as observed in pilot experiments for PC6A. In Fig. 2 the secreted levels of human furin, human PACE4, and mouse PC6A found in the medium of PK(15) cells are shown. The molecular weight of the secreted form of human furin was estimated to be about 85 kDa, as has been reported before [31,32]; it presumably corresponds to a processed form of furin lacking the carboxy-terminal transmembrane domain. The electrophoretic mobilities of secreted PACE4 and PC6A appeared to be similar, with that of PC6A slightly higher. Their observed molecular weights were estimated to be about 115 kDa which is somewhat higher than their calculated molecular weights and this might be due to glycosylation [20–22]. The appearance of rather broad bands in SDS-PAGE might also reflect this or might point towards the presence of double bands as observed with the *Drosophila* furins [33]. The results of the expression experiments clearly established that PACE4 and PC6A were synthesized at relatively high levels in PK(15) cells under the selected expression conditions. This opened the way to assay the predicted proprotein processing activity of PACE4.

To establish that PACE4 indeed possesses proprotein processing activity with specificity for cleavage sites with paired basic amino acids, we studied its ability to process wild-type pro-vWF in similar co-expression experiments as performed before for furin [11,14,25]. As can be seen in Fig. 3 (row 1), transfection into PK(15) cells of pGEMvWF DNA, which encodes wild-type pro-vWF, resulted in the production of pro-vWF and a minute amount of mature vWF. The production of some mature vWF is assumed to be the result of processing by endogenous furin or a furin-like processing enzyme. Due to the viral infection, however, this endogenous activity appeared to be suppressed almost completely, thus making the assay rather sensitive with

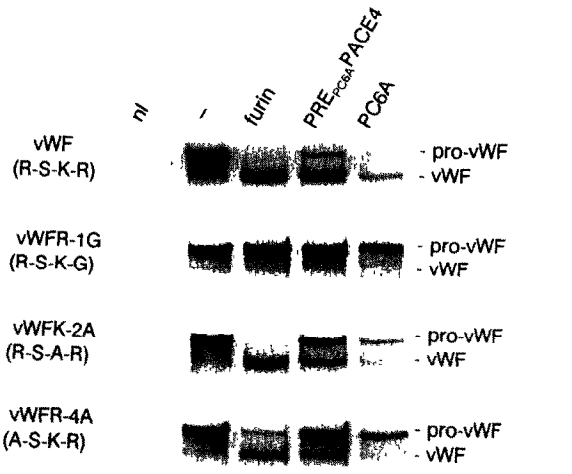

Fig. 3. Analysis of endoproteolytic processing of pro-vWF, pro-vWFR-1G, pro-vWFK-2A and pro-vWFR-4A. PK(15) cells were first infected with recombinant vaccinia virus V.V.:T7 and subsequently lipofected with 2 μg of pGEMvWF DNA (first row, except the first lane), pGEMvWFR-1G DNA (second row), pGEMvWFK-2A DNA (third row) or pGEMvWFR-4A DNA (fourth row) in combination with 2 μg of either pGEM vector DNA (indicated with −), pGEMFUR DNA, pGEMPRE$_{PC6A}$PACE4 or pGEMPC6A DNA, as is indicated above the lanes. In the first lane of the upper row, V.V.:T7-encoded proteins secreted from cells that were not lipofected (nl) are shown.

respect to detecting exogenous processing activity. Cotransfection of pGEMvWF and pGEMFUR DNA resulted in the production of only mature vWF. Apparently, pro-vWF is processed into mature vWF to completion by exogenous furin. Almost complete processing of pro-vWF was observed when PC6A was coexpressed. In case of PACE4, enhanced processing of pro-vWF was observed, however, not all pro-vWF could be converted into mature vWF. The results of these co-expression studies indicate that PACE4 can process pro-vWF into mature vWF in a similar way as the enzymes furin and PC6A do. This establishes the membership of PACE4 of the Kex2 family of proprotein processing enzymes.

The fact that PACE4 seemed to be less efficient in processing pro-vWF as compared to human furin and mouse PC6A points towards potential differences between these enzymes, possibly differences in cleavage site specificity. To address this issue, we have studied the capabilities of PACE4 and PC6A to process cleavage site mutants of pro-vWF in which basic residues next to the processing site had been submitted. Experiments with P1 mutant cWFR-1G in which the arginine residue (Arg^{763} in the P1 position) had been substituted by a glycine revealed that none of the three processing enzymes, furin, PACE4 or PC6A, could process its precursor, pro-vWFR-1G (Fig. 3, row 2). From observations of earlier studies of furin [25,34], it had become clear already that the Arg^{763} residue in the P1 position is very critical. The S1 binding pocket is predicted to be a large, elongated pocket with residue D199 dominantly involved in electrostatic interaction with the P1 basic residue (Arg^{763}). On the other hand, substitution of the arginine into glycine might also be very unfavourable since glycine is much smaller than arginine and in fact has no side chain that could interact with either a negatively charged residue or a hydrophobic residue of the S1 binding pocket. Experiments with P2 mutant vWFK-2A in which Lys^{762} was replaced by an alanine indicated that both PACE4 and PC6A were capable to process to some extent the corresponding precursor pro-vWFK-2A but apparently not as completely as achieved with furin. PACE4 and PC6A were found to process only minute amounts, if any at all, of the precursor of P4 mutant vWFR-4A in which Arg^{760} was replaced by an alanine; in contrast, human furin could process pro-vWFR-4A, to a large extent. With respect to their ability to process the three cleavage site mutants of the von Willebrand factor, the proprotein processing enzymes PACE4 and PC6A seem to resemble each other but to differ from the widely expressed enzyme furin.

Processing characteristics of PC6A have been described before [21]. They were obtained in co-expression studies using rat pituitary GH_4C_1 cells and native or mutant prorenin of mouse as substrates. In these studies, only very limited processing was observed of native mouse *Ren-2* prorenin which possesses the following cleavage site E-W-D-V-F-T-K-R^{63}↓-S-S-L-T. Introduction of an arginine residue at the P4 position (replacement of Phe^{60} by Arg) resulted in enhanced cleavage of this prorenin mutant. Prorenin mutants with arginine residues in the P1 (Arg^{63}) and P4 (Arg^{60}), or only in the P1 (Arg^{63}) position were not processed. In general, the results of our studies are in good agreement with these observations. The only apparent discrepancy seems to be the processing of the substrate with basic residues at the P1 (residue 63) and P4 (residue 60) position. It is possible that the assay system that we used in our studies is more sensitive than the one used by Nakagawa et al. [21]. Furthermore, the fact that different cell types, substrates, and expression systems were used might also explain the observed difference.

Comparison of nine negatively charged and presumably critical amino acid residues in the S1–S6 subsites of the substrate binding regions of human furin, human PACE4, and mouse PC6A revealed no differences between those of PACE4 and PC6A. Compared to furin, however, two residues appeared to be different. In light of the fact they were found in the S3 and S5 binding pockets, it is difficult to imagine that they constitute an important factor for the observed differences in cleavage site selectivity between PACE4 and PC6A on the one hand and furin on the other hand. One should also bear in mind that proprotein conversion is also determined by other structural features of the substrate and, furthermore, by the intracellular microenvironment where the actual processing step takes place.

In conclusion, our studies establish that PACE4 is a member of the Kex2 family of proprotein processing enzymes and that, as far as positional requirements for basic amino acid residues next to the cleavage site in the substrate are concerned, there seem to be more constraints for proper functioning of PACE4 and PC6A as compared to furin. Furin, more or less, seems to be a general or multi-purpose processing enzyme. The differences in cleavage site requirements observed here between PACE4 and PC6A on one side and furin on the other side seem to further support the increasingly more clear functional diversity among the members of this novel family of mammalian subtilisin-like proprotein processing enzymes.

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