Processing of proendothelin-1 by members of the subtilisin-like pro-protein convertase family

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Abstract Endothelial cells (ECs) secrete numerous bioactive peptides that are initially synthesized as inactive precursor proteins. One of these, proendothelin-1 (proET-1), undergoes proteolysis at specific pairs of basic amino acids. Here, we wished to examine the role of mammalian convertases in this event. Northern blot analysis shows that only furin and PC7 are expressed in ECs. In vitro cleavage of proET-1 by furin or PC7 demonstrated that both enzymes efficiently and specifically process proET-1. These data reveal that furin and PC7 have similar specificities towards proET-1 and suggest that both enzymes may participate in the maturation of proET-1 in ECs.

Key words: Endothelin-1; Precursor protein; Proteolytic cleavage; Furin; PC7; Endothelial cell

1. Introduction

Endothelial cells (ECs) secrete many proteins and peptides that are necessary for blood pressure homeostasis, coagulation and immune response. Many of these factors are initially synthesized as precursors and are matured in the constitutive secretion pathway. One of these, endothelin-1 (ET-1), originates from a protein of 212 amino acids (human), prepro-ET-1, which, after removal of the signal peptide, is further processed at specific basic residues leading to the production of bigET-1 [1]. This is followed by carboxy-terminal trimming of basic residues by carboxypeptidases. For the complete conversion of bigET-1 into biologically active ET-1, cleavage occurs at an unusual motif, $Trp^{73}\downarrow Val^{74}$, by the endothelin-converting enzymes (ECEs) [2]. Thus, processing of proET-1 is a multi-step process that requires the participation of at least three proteases in an organized manner.

Over the last 10 years, evidence has revealed that processing of precursor proteins at motifs such as Lys-Arg, Arg-Arg or Arg-X-X-Arg is performed by a family of calcium-dependent serine proteases called subtilisin-like pro-protein convertases (SPCs) [3–5]. There are presently seven members belonging to this family: SPC1 (furin/PACE), SPC2 (PC2), SPC3 (PC1/PC3), SPC4 (PACE4), SPC5 (PC4), SPC6 (PC5/PC6) and SPC7 (PC7/PC8/LPC). During its processing, a precursor protein will follow one of two secretory pathways, the constitutive or the regulated pathway and thus could encounter different subsets of SPCs depending on the cell type and the tissue in which the precursor is expressed. In the regulated pathway, SPC2 and SPC3, which are present primarily in neuroendocrine and endocrine cells, are thought to be responsible for processing of numerous peptide precursors [6]. The second possible route of secretion is the constitutive pathway, in which a pro-protein could be recognized and cleaved by furin, SPC4, SPC6 and PC7. The number of mammalian SPCs and their similar enzymatic specificity raise several intriguing questions as to their potential overlapping or distinct functions in precursor processing. Functional specificity could be determined by a high degree of substrate specificity by each enzyme and a distinct cellular expression or specific intracellular localization of each SPC. Numerous studies have been carried out to examine the detailed tissue and cellular distribution of each SPC in order to co-localize them with various precursor proteins [7–9].

As depicted in Fig. 1, six Arg-X-X-Arg cleavage sites (identified as R1a, R1b, R2–R5) within proET-1 are putative recognition sequences for members of the SPC family. Here, we show that furin and PC7 are expressed in ECs and that these enzymes are able to efficiently generate the proper intermediate peptides necessary for ET-1 production.

2. Materials and methods

2.1. Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were from Clonetics (San Diego, CA, USA) and were cultured according to the manufacturer's protocol. ECV304 cells (human endothelial umbilical vein cell line) were grown in M199 medium. BS-C-1 cells (African green monkey epithelial-like cell line) and CHO(dhFr$^-$) (Chinese hamster ovarian cell line deficient in dihydrofolate reductase) were grown in minimal essential medium; COS-7 (African green monkey fibroblast cell line) and AtT-20 (mouse pituitary adenocarcinoma cell line) were grown in Dulbecco's modified Eagle's medium; RINm5F cells (rat pancreatic insulinoma cell line) were cultured in RPMI 1640 medium. All media were supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin. BS-C-1 cell medium was also supplemented with 2 mM L-glutamine and 0.1 mM non-essential amino acids.

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Abbreviations: amc, 7-amido-4-methylcoumarine; EC, endothelial cell; ET-1, endothelin-1; HUVEC, human umbilical vein endothelial cell; SPC, subtilisin-like pro-protein convertase

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2.2. Northern blot analysis

Total RNA extraction using the guanidine–lithium chloride method and Northern blotting was performed as previously described [7]. 8 μg of total RNA was fractionated on a 1.2% agarose–formaldehyde gel, transferred to Nytran N membrane (Schleicher&Schuell, Keene, NH, USA) and probed with ^{32}P-labeled cRNA for each SPC for 18 h, washed in 0.1% sodium dodecyl sulfate (SDS), 0.1×saline sodium citrate, 1 mM EDTA for 3.5 h and exposed on BioMax MS films (Eastman Kodak Co., Rochester, NY, USA) for 1–7 days.

2.3. Purification of recombinant furin and PC7

Purification of SPC1/714, a truncated form of furin, was performed as previously described [10] with the following modifications to the protocol. The first purification step was a high performance tangential flow ultrafiltration (Pellicon-II, Millipore, Bedford, MA, USA) against a 30 kDa molecular weight cutoff (mwco) BioMax membrane (Millipore) where 500 ml of conditioned serum-free medium was concentrated to 100–150 ml. The proteins were then reconcentrated by ultrafiltration with a Centricon (model 8200, Amicon Inc., Beverly, MA, USA) against a polyethersulfone membrane, 30 kDa mwco (Millipore) to 12 ml. This was immediately applied to a HR Sephacryl S-100 26/60 (Amersham-Pharmacia Biotech.) gel filtration column at a flow rate of 0.5 ml/min. The purification was then resumed according to the original protocol. PC7Δ, a truncated secreted form of PC7, was purified according to published procedures [11].

2.4. Enzymatic assays and titration

Enzymatic assays were carried out in 100 mM HEPES pH 7.5, 1 mM $CaCl_2$ and 1 mM β-mercaptoethanol with 25 μM of fluorogenic substrate *N*-terbutoxycarbonyl-Arg-Val-Arg-Arg-7-amido-4-methylcoumarine (boc-RVRR-amc) for furin or 20 mM Bis–Tris pH 6.5, 1 mM $CaCl_2$ and 100 μM of pyroglu-Arg-Tyr-Lys-Arg-amc (p-ERTKR-amc) for PC7. Assays were performed in 0.5 ml at 37°C for 30 min for detection of activity or 1 h for kinetic analysis. Reactions were stopped by addition of 0.5 ml of 10 mM EDTA. Reactions were calibrated with free AMC and fluorescence was measured (EX_λ = 380 nm, EM_λ = 480 nm). The amount of active enzyme in the purified SPC preparations was evaluated by active site titration with the irreversible decanoyl-Arg-Val-Lys-Arg-chloromethylketone (dec-RVKR-cmk) inhibitor. The enzyme was incubated with nanomolar concentrations (0–30 nM) of titrant in 20% dimethyl sulfoxide in the respective buffer of each enzyme in a total volume of 50 μl and incubated at 37°C in 30 min. 100 μM of respective fluorogenic substrate was then added to give a final volume of 1 ml and reactions were incubated for 30 min. The reactions were stopped by adding 10 μl of 0.5 M of EDTA. Residual fluorescence was measured as described above.

2.5. Expression and purification of proET-1 precursors

Histidine-tagged pro-endothelin proteins were produced and purified using the QiaExpress system (Qiagen) as previously reported [12]. Briefly, JM109 bacteria were grown in 2×YT medium and protein expression was induced by adding 1.5 mM isopropyl β-D-thiogalactopyranoside. Cells were harvested, centrifuged and lysed in 8 M urea, 0.1 M sodium phosphate, 0.01 M Tris–HCl, pH 8.0. The lysate was applied to a Ni-NTA column and elution was performed according to the manufacturer's instructions. The eluate was then injected into a high-performance liquid chromatography hydrophobic C4 column (Vydac, S.P.E. Ltd.) equilibrated with 0.1% v/v trifluoroacetic acid, 0.05% triethylamine. Proteins were eluted using a 0–80% acetonitrile gradient in 0.1% v/v trifluoroacetic acid, 0.05% triethylamine at a flow rate of 1 ml and 1%/min. Fractions containing the purified protein were dried and reconstituted in 200 mM HEPES pH 7.6.

2.6. In vitro processing by SPCs

200 pmol (5 μg) of purified proET-1 was incubated with 1 pmol of furin or PC7 (final concentration of substrate 4 μM and enzyme 20 nM) in digestion buffer (100 mM HEPES pH 7.6, 1 mM $CaCl_2$, 1 mM β-mercaptoethanol and 0.5% Triton X-100 for digestion with furin and 20 mM Bis–Tris pH 6.5 and 1 mM $CaCl_2$ for PC7) for various periods of time. The digestion products were analyzed by 16.5% Tris-tricine SDS–PAGE. The gel was stained with GelCode blue (Pierce Chemical Co., Rockford, IL, USA) or transferred to a nitrocellulose Optitran membrane for immunoblotting with penta-His antibody (Qiagen) according to the manufacturer's protocol.

3. Results

3.1. Presence of furin and PC7 mRNA in ECs

To more fully comprehend the biosynthetic profile of pro-ET-1 in ECs, we initially focused on establishing the identity of those SPCs that are present in this cell type. Because of the exclusive tissue distribution of SPC2 and SPC3 in neuroendocrine cells and SPC5 in spermatid cells, we did not pursue their identification in ECs. Hence, only furin, SPC4, SPC6 and PC7 were investigated. To detect the presence of the mRNAs encoding SPCs in two EC lines, we used Northern blot analysis of total RNA from ECV304 cells and a primary EC line (HUVECs). As depicted in Fig. 2A, a band at 4.4 kb was detected after hybridization with a furin cRNA probe in HUVECs and ECV304 cells and in all of the control cell lines, consistent with its known ubiquitous distribution [8]. An hSPC4 cRNA probe revealed a band migrating at 4.4 kb in COS-7 and RINm5F cells, but not in the HUVECs or ECV304 cells (Fig. 2B). The 3.5 kb band detected in AtT-20 cells could be one of the multiple known isoforms of SPC4A [13]. The hSPC6 transcript was not detected by Northern blot analysis in HUVECs, ECV304, COS-7, RINm5F, AtT-20 and CHO(dhFr$^-$) cells (Fig. 2C). In Fig. 2D, we detected a specific 3.5 kb band corresponding to PC7 in both ECs. These results show that both furin and PC7 are expressed in HUVECs and ECV304 ECs.

3.2. In vitro digestion of proET-1 by furin and PC7

We then compared the efficiency of the two enzymes to process recombinant proET-1. In order to perform in vitro digestions of proET-1, sufficient quantities of the precursor as well as the purified enzymes were produced. To compare the efficiency of the two enzymes to cleave the ET-1 precursor, equivalent amounts of enzymes determined by titration with the irreversible inhibitor dec-RVKR-cmk (data not shown) were used. Fig. 3A shows the time course of the cleavage pattern observed after incubation of proET-1 with furin where four major forms were revealed. Intact recombinant proET-1 (identified as *a*) has a theoretical molecular mass of 23 791, which is in accordance with the molecular weight of 24 kDa obtained by SDS–PAGE and 23 800 obtained by mass spec-

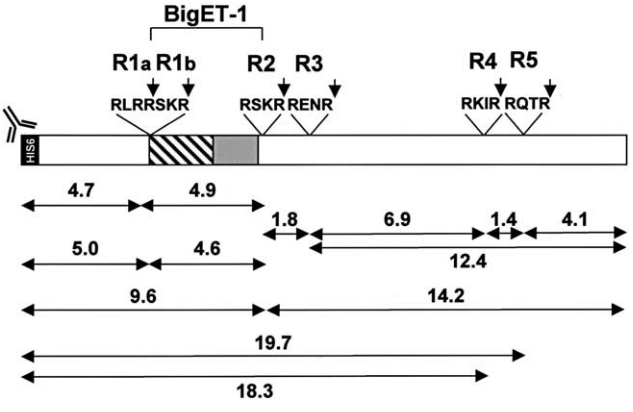

Fig. 1. Recombinant proET-1. Schematic representation of proET-1 and the potential fragments generated by the cleavage of the various Arg-X-X-Arg sites (R). Hatched bar indicates active ET-1 moiety whereas gray portion represents the bigET-1 region. Amino acid motifs constituting SPC recognition site are indicated. Vertical arrows indicate the putative convertase recognition sites. Black portion represents the His tag epitope. Size of fragments is in kDa.

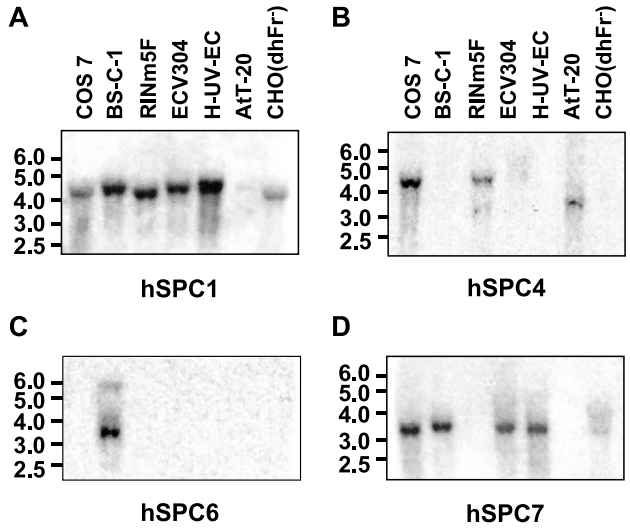

Fig. 2. SPC mRNA expression in cell lines. 8 μg of total RNA extracted from different cell lines was separated on a denaturing agarose gel and transferred to a nylon membrane. The membrane was then hybridized with human SPC specific probes for furin (A), SPC4 (B), SPC6 (C) and PC7 (D). The membrane was washed and exposed on film for a period of 1–7 days. Molecular weight markers are in kb.

trometry (data not shown). After 15 min incubation with furin, we observed a significant reduction of the undigested proET-1 and the appearance of shorter polypeptides. The larger of these (fragment *b*) corresponds to an N-terminal fragment when proET-1 is processed at one of the two sites, R4 or R5, yielding a 18.3 or 19.7 kDa fragment, respectively. Mass spectrometry of the digested sample confirmed cleavage at R4 due to the presence of a molecular ion peak at 18 547 (data not shown). Cleavage at the R2 site produced two fragments, 14.2 and 9.6 kDa (*c* and *d*, respectively), also confirmed by mass spectroscopy. We observed a proportional reduction of the 14.2 kDa fragment and an increase of the 12.4 kDa peptide (identified as *c*′) produced after 2 h of incubation, corresponding to the cleavage of the 14.2 kDa fragment at the R3 site. The 5.0 kDa fragment generated after 2 h corresponds to the N-terminal fragment (*e*) after the cleavage of the R1 site.

To further confirm the identity of the fragments generated, we used an anti-His tag antibody and immunoblotting. Since the His tag is placed at the N-terminal side of proET-1 (see Fig. 1) only the N-terminal fragments will be detected. As shown in Fig. 3B, the antibody recognizes the 23.8 (*a*), the 19.7–18.3 (*b*) and the 9.6 (*d*) kDa bands while the 14.2 kDa fragment (*c*) is not recognized, enabling us to confirm its identity even if its apparent molecular weight is higher than its real molecular weight. Since the 5.0 kDa fragment is also tagged with the His tag, we can confirm that it is the N-terminal fragment generated by the cleavage of the R1 site and not a fragment generated by the cleavage of a C-terminal site. Thus furin cleaves all furin recognition sites present in proET-1, albeit with different efficiencies. The R2 and R4 sites are cleaved preferentially while the R1a or R1b and R3 sites are less efficiently cleaved.

With an equal concentration of PC7 (20 nM) and identical experimental conditions, the processing of proET-1 yielded a similar cleavage pattern as for furin (Fig. 4A). Indeed, we can detect the 18.3, 14.2 and 9.6 kDa as well as the 5.0 kDa band. However, PC7's efficiency to cleave proET-1 was lower than furin's since the proET-1 band (*a*) was still detected up to 2 h after the start of the incubation, compared to 30 min with furin. Fig. 4B shows the immunoblot with the anti-His tag antibody which enabled us to confirm the bands generated with PC7. Again, the antibody recognized the 19.7 or 18.3 and the 9.6 kDa bands as well as the pro-protein. A longer exposure was necessary to detect the 5.0 kDa band (1 h lane). In Figs. 3A and 4A we were able to show that both SPCs cleaved each side of the bigET-1 intermediate (R1 and R2 sites) and therefore should produce a fragment corresponding to bigET-1 in vivo. However, no fragments with the same

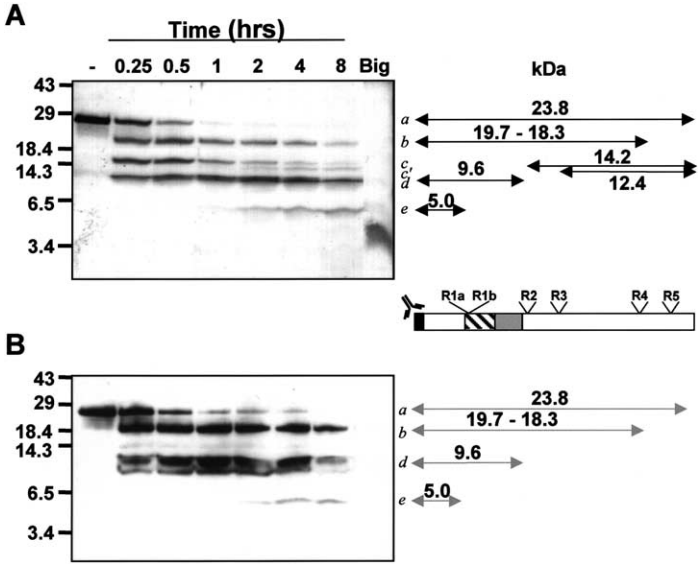

Fig. 3. Cleavage of proET-1 by furin. A: 5 μg of proET-1 (4 μM final) was incubated with 1 pmol of purified furin (20 nM final) for different periods of time (0.25, 0.5, 1, 2, 4, 8 h). Ensuing samples were analyzed by Tris-tricine 16.5% SDS–PAGE and the gel was stained with GelCode blue. The commercial bigET-1 and the undigested proET-1 (–) were loaded as control. Molecular weight markers are identified on the left. B: Identification of the N-terminal fragments generated by PC7. Duplicate samples as in (A) were analyzed by immunoblot with anti-His tag antibody.

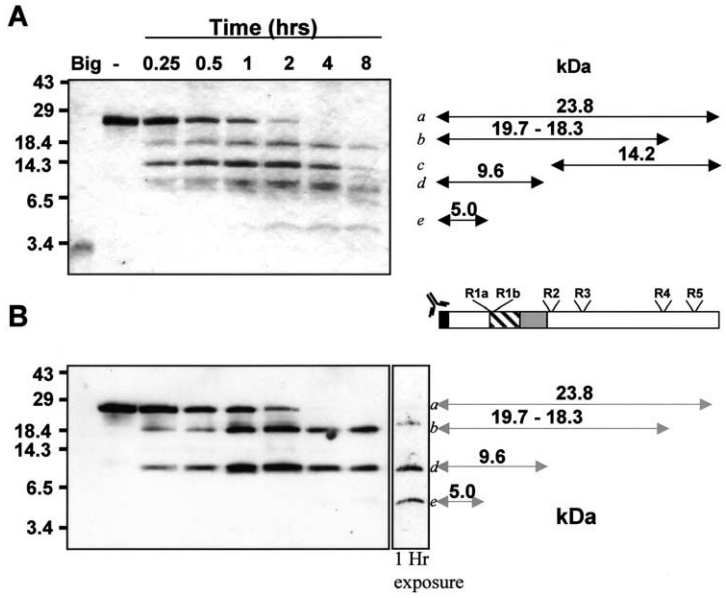

Fig. 4. Cleavage of proET-1 by PC7. A: 5 μg of proET-1 (4 μM final) was incubated in the presence of 1 pmol of purified PC7 (20 nM final) in a total volume of 50 μl for different periods of time (0.25, 0.5, 1, 2, 4, 8 h). Ensuing samples were analyzed as in Fig. 3A. The commercial bigET-1 and the undigested proET-1 (–) were loaded as control. B: Identification of the N-terminal fragments generated by PC7. Duplicate samples as in (A) were analyzed by immunoblot with anti-His tag antibody.

molecular size as bigET-1 were detected. We prolonged the incubation period of proET-1 with each SPC to 16 h to allow for the complete digestion and the accumulation of smaller fragments (Fig. 5). We detected a 4.6 kDa band (identified by the arrow, *f*), possibly corresponding to bigET-1-Lys-Arg. Western blotting revealed that the anti-His antibody did not recognize the 4.6 kDa band, ruling out the possibility that it is an N-terminal fragment (result not shown).

3.3. Processing of proET-1 convertase recognition site mutants

Another approach was used to confirm the identity of the different bands generated. Production of proET-1 cleavage site mutants allowed us to further ascertain the identity of each band. Fig. 6A shows a schematic representation of the mutants generated. A first mutant, identified as R1G, was constructed by changing Arg^{49} to Gly (numbering according to the native preproET-1 protein) in position P4 of the R1b site. This mutation abolishes both furin recognition sites at the N-terminus of bigET-1. A second mutant, identified as R1GR2G, was constructed by changing Arg^{89} to Gly of the R2 site in addition to the R1G mutation. Fig. 6B,C shows

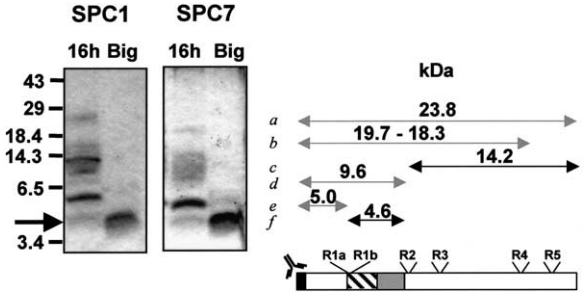

Fig. 5. Production of bigET-1-Lys-Arg by furin and PC7. 5 μg of proET-1 was incubated in a volume of 50 μl (4 μM final) in the presence of 1 pmol of furin or PC7 (20 nM final) for 16 h and analyzed on SDS–PAGE as in Fig. 3A. Commercial bigET-1 (Big) was loaded as a control.

both mutants (R1G and R1GR2G) and the proET-1 WT incubated without or with furin (Fig. 6B) or PC7 (Fig. 6C). Cleavage of the proET-1/R1G mutant yields the same profile of cleavage as the proET-1 WT (lane WT, 1 h) except that the 5.0 kDa band (fragment *e*) is now absent because the R1a/b site is abolished. The intermediate 14.2 and 9.6 kDa fragments (fragments *c* and *d*) are still generated, demonstrating that the R1G mutation does not alter the processing of the C-terminal of bigET-1 (R2 site). When both sites were mutated (R1GR2G), only the 18.3 kDa fragment (fragment *b*) is produced. The identification of the bands from the mutant proET-1 was also confirmed by Western blotting (results not shown).

4. Discussion

ECs synthesize precursor proteins, many of which undergo endoproteolysis, an obligatory step for the production of the biologically active moieties. The proET-1 precursor contains many potential recognition sequences for members of the SPCs. We had previously reported how proET-1 is processed by furin both in vitro and in vivo at a time when PC7 had not been identified [12,14]. In the present work, we have pursued our characterization of the mammalian convertases found in ECs and involved in the processing of proET-1. We first determined by Northern blot analysis which of the convertases are present in ECs. We detected the presence of two convertases in ECs, namely furin and PC7. The mRNAs were detected in primary HUVECs and in ECV304 cells, an immortalized EC line. Both convertases are considered to possess an ubiquitous tissue distribution. The transcripts encoding SPC4, SPC6A and SPC6B were not detected in our EC lines. Since SPC6 had previously been detected in ECs of the aorta by in situ hybridization [15], it is possible that some ECs found within the cardiovascular system express SPC6 while others do not.

Using recombinant enzyme and purified precursor proteins,

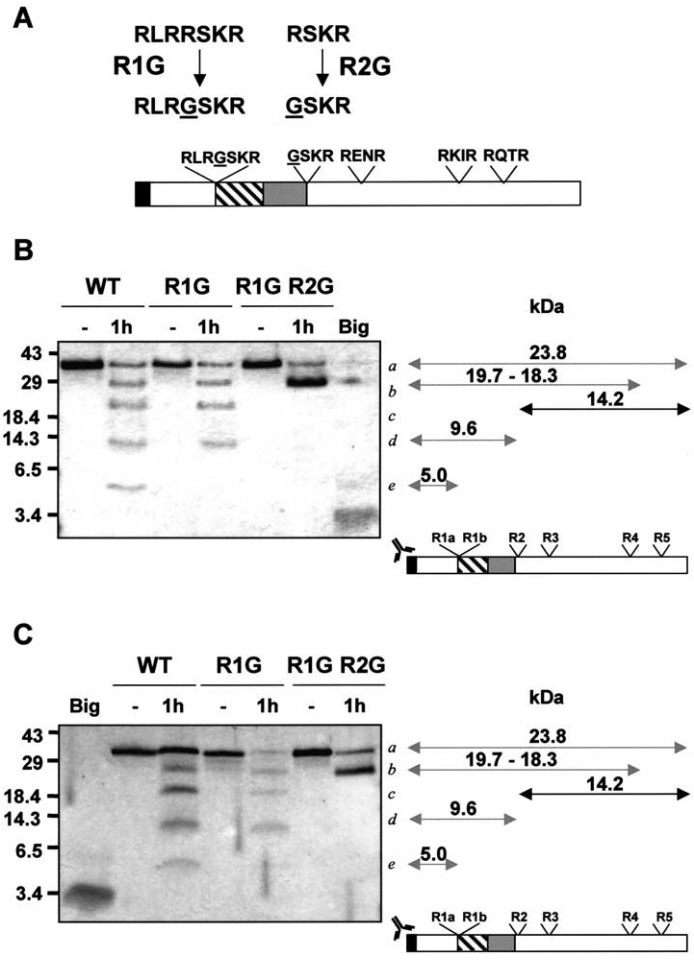

Fig. 6. Cleavage of mutant proET-1 by furin or PC7. A: Schematic representation of mutant proET-1. The same legend as in Fig. 1 is used. Substituted amino acids that generated R1G and R1GR2G mutants are underlined. 5 μg of proET-1 WT (4 μM final), mutant R1G or mutant R1GR2G was incubated for 1 h in a total volume of 50 μl in the absence (−) or presence of 1 pmol of furin (20 nM final) (B) or PC7 (C) and analyzed as in Fig. 3A. Commercial bigET-1 (Big) was loaded as a control.

we established in vitro the ability of each convertase to generate the appropriate cleavage fragments leading to the production of bigET-1-Lys-Arg, an obligatory intermediate in the biosynthesis of the active ET-1 peptide. The results indicate that when equal quantities of furin or PC7 are incubated with proET-1, furin cleaves the precursor with better efficiency when compared to PC7. Conversely, the pattern of processing products, as observed by SDS–PAGE, is identical, indicating that the specificity of both enzymes is similar. The order and time of appearance of the different peptides shows that the C-terminus site of the bigET-1 (R2) and the R4 sequence are preferred recognition sites for both convertases. As previously noted, cleavage of the R2 site is an important prerequisite for cleavage by ECE-1 [14]. It is at present unknown whether cleavage at R4 would yield a biologically active peptide or whether further cleavage at R5 occurs. Both sites, R4 and R5, contain optimal convertase recognition sequences with P4 Arg residues and Ser residues in P1′, signatures often found in precursors cleaved by furin.

At the N-terminus of the bigET-1, there is a double convertase recognition sequence (R1a/R1b). It is likely that cleavage occurs at Arg^{52} (R1b) instead of Arg^{49} (R1a) because cleavage of the latter would yield Ser-Lys-Arg-ET-1 following processing by ECE. It has been shown that the integrity of the amino-terminal end of ET-1 is necessary to the biological activity of the peptide [16]. Furthermore, it is unlikely that cleavage partitioned between cleavage at R1a and R1b because digestion of the precursor by furin and chymosin was shown to result in the generation of an identical pressure activity on a pharmacological preparation as the one obtained with the same concentration of synthetic ET-1 [12]. This suggests that furin selectively cleaves the second site (R1b) despite a P1′ Cys residue involved in disulfide bonding within the ET-1 peptide. Taken together, the results illustrate how furin and PC7 possess very similar specificities towards proET-1, supporting specificity studies performed on short fluorogenic peptides and on precursor proteins [17,18]. Indeed, both enzymes show an absolute requirement for arginine in the P1 position. Furin necessitates P4 or P6 arginines while, in addition, an essential P2 basic residue is needed for PC7. These requirements may explain the less efficient processing of proET-1 by PC7 but reconciles the similar efficacy to furin at cleaving the R2 site that possesses a Lys residue in P2.

ProET-1 is the only member of the endothelin/sarafotoxin family that contains six potential convertase cleavage sites, three being located in the C-terminal fragment. ProET-2 contains one cleavage site in its COOH-terminal whereas ET-3 precursor does not. It would be interesting to extend our

findings on the processing of proET-1 to proET-2 and proET-3 since these two precursors have a different tissue expression profile and yield ET-related peptides involved in other physiological effects than those associated with ET-1 [19]. They could be co-localized with other SPCs, especially SPC4, which is abundantly expressed in the nervous system. SPC4 and SPC6 might be implicated in the maturation of ET-2 and ET-3 precursors or even ET-1 outside of the vascular system.

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