A proteomic approach reveals transient association of reticulocalbin-3, a novel member of the CREC family, with the precursor of subtilisin-like proprotein convertase, PACE4

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SPCs (subtilisin-like proprotein convertases) are a family of seven structurally related serine endoproteases that are involved in the proteolytic activation of proproteins. In an effort to examine the substrate protein for PACE4 (paired basic amino-acid-cleaving enzyme-4), an SPC, a potent protein inhibitor of PACE4, an α1-antitrypsin RVRR (Arg-Val-Arg-Arg) variant, was expressed in GH4C1 cells. Ectopic expression of the RVRR variant caused accumulation of the 48 kDa protein in cells. Sequence analysis indicates that the 48 kDa protein is a putative Ca^{2+}-binding protein, RCN-3 (reticulocalbin-3), which had previously been predicted by bioinformatic analysis of cDNA from the human hypothalamus. RCN-3 is a member of the CREC (Cab45/reticulocalbin/ERC45/calumenin) family of multiple EF-hand Ca^{2+}-binding proteins localized to the secretory pathway. The most interesting feature of the RCN-3 sequence is the presence of five Arg-Xaa-Xaa-Arg motifs, which represents the target sequence of SPCs. Biosynthetic studies showed that RCN-3 is transiently associated with proPACE4, but not with mature PACE4. Inhibition of PACE4 maturation by a Ca^{2+} ionophore resulted in accumulation of the proPACE4–RCN-3 complex in cells. Furthermore, autoactivation and secretion of PACE4 was increased upon co-expression with RCN-3. Our findings suggest that selective and transient association of RCN-3 with the precursor of PACE4 plays an important role in the biosynthesis of PACE4.

Key words: Cab45/reticulocalbin/ERC45/calumenin (CREC), EF-hand, paired basic amino-acid-cleaving enzyme-4 (PACE4), reticulocalbin, subtilisin-like proprotein convertase (SPC).

INTRODUCTION

Members of the SPC (subtilisin-like proprotein convertase) family, so called because their catalytic domains resemble that of subtilisin, are involved in the proteolytic activation of precursor proteins at sites that possess paired basic amino acid residues. Seven members of the SPC family have been identified in mammals [1–3], including furin [4], PC1 (proprotein convertase 1) [5], PC2 [6], PACE4 (paired basic amino-acid-cleaving enzyme-4) [7], PC4 [8], PC6A [9] and PC8 [10,11].

The structural organization of the mammalian SPCs appears to be highly homologous. All possess a signal peptide, a propeptide, an SCD (subtilisin-like catalytic domain), and a homoB domain. SPCs are Ca^{2+}-dependent serine endoproteases and have been classified into the family S8, subfamily B. SPCs are synthesized as proenzymes in the ER (endoplasmic reticulum). The N-terminal propeptide of SPC plays multiple roles in guiding the activation of SPC *in vivo* [12]. Autocatalytic cleavage of the propeptide is essential for transport of SPC from the ER to the Golgi compartment. PACE4 is secreted as a mature active enzyme, whereas proPACE4 is retained in the ER [13,14].

In the present paper, we describe our attempts to identify the proteins that interact with PACE4 (EC 3.4.21.-) or its substrate proteins in GH4C1 cells by employing a proteomic approach using a specific SPC inhibitor. GH4C1 cells, a rat pituitary somatomammotroph tumour cell line, express PACE4 at high levels, the physiological significance of which remains unknown. The α1AT (α1-antitrypsin) RVRR variant is an engineered rat α1AT mutant that can inhibit PACE4, PC6 and furin specifically [15]. The RVRR variant carries three mutations ($Ala^{349} \rightarrow$ Arg, $Pro^{351} \rightarrow$ Arg and $Met^{352} \rightarrow$ Arg) in its RSL (reactive site loop) to provide the best sequence (Arg-Xaa-Arg-Arg) for efficient recognition and processing by PACE4 and furin. Following cleavage of the RSL of the RVRR variant by PACE4 or furin, the variant undergoes significant conformational rearrangement, resulting in the formation of an SDS-stable complex of the protease with the variant.

We reported previously that the biosynthesis of SPC can be specifically suppressed by expression of the corresponding antisense RNAs [16]. Using this method, expression of SPC was reduced by approx. 40–50 % compared with control cells. Although decanoyl-RVKR-chloromethane has been used as a general SPC inhibitor [17], inhibition is not specific for SPC as it can also inhibit trypsin-like serine proteases. In contrast, ectopic expression of the RVRR variant causes complete and specific inactivation of SPCs, including PACE4, PC6A and furin [15]. The proteomic profile of GH4C1 cells stably expressing the RVRR variant was analysed using 2D-PAGE (two-dimensional PAGE) in an effort to identify proteins that interact with PACE4. It was found that RCN-3 (reticulocalbin-3), a novel member of the CREC (Cab45/reticulocalbin/ERC45/calumenin) family [18], accumulated in the membrane fraction in significant amounts. Furthermore, we found that RCN-3 specifically bound to the PACE4 precursor protein and that secretion of mature PACE4 was enhanced by co-expression with RCN-3.

Abbreviations used: 2D-PAGE, two-dimensional PAGE; α1AT, α1-antitrypsin; CREC, Cab45/reticulocalbin/ERC45/calumenin; DTT, dithiothreitol; ER, endoplasmic reticulum; ERP29, ER protein, 29 kDa; HEK-293, human embryonic kidney; HSA, human serum albumin; PACE4, paired basic amino-acid-cleaving enzyme-4; PC, proprotein convertase; RCN-3, reticulocalbin-3; RSL, reactive site loop; RT, reverse transcription; SCD, subtilisin-like catalytic domain; SPC, subtilisin-like proprotein convertase.

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EXPERIMENTAL

Materials

Goat anti-rabbit IgG conjugated with FITC and goat anti-mouse IgG conjugated with Texas Red were purchased from Vector Laboratories. Anti-α1AT IgG was from Zymed Laboratories. Antibody against BiP (immunoglobulin heavy-chain binding protein) (anti-KDEL antibody) was from StressGen. Protein A–Sepharose used for immunoprecipitation was from Amersham Biosciences.

Construction of expression vectors

Human *RCN-3* cDNA was amplified by PCR using a KpnI-linked N-terminal sense primer [5′-GGGGTACCATGATGTGGCGACCATCAGTTC-3′, corresponding to nucleotide numbers 33–54 of *RCN-3* cDNA (GenBank® accession number AY195859); initiation codon underlined] and an XbaI-linked C-terminal antisense primer [5′-GCTCTAGATCACAGCTCATCGTGGTGCC-3′, corresponding to nucleotide numbers 1000–1019 of *RCN-3* cDNA (GenBank® accession number AY195859); termination codon underlined]. Total RNA from HepG2 cells was reverse-transcribed using Superscript II (Gibco BRL), according to the manufacturer's instructions, and was used as a template for PCR. PCR was performed using Taq DNA polymerase (Promega) according to the manufacturer's protocol (30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, followed by an extension at 72 °C for 1 min). Following digestion with KpnI and XbaI, the PCR fragment was subcloned into the pcDNA3 vector (Invitrogen). RCN-3 mutants lacking the RXXR sequence (the RXXR sequence was replaced by KXXR) were prepared with two steps of PCR as follows. The 5′-terminal cDNA fragments (M1 and M2) containing the mutated RXXR SPC-recognition site was generated by PCR using KpnI-linked N-terminal sense and antisense mutagenic primers, 5′-GCGGTCCATCTTGTCCACG-3′, where Arg^{86} was replaced by lysine (underlined), or 5′-CCGTATGTGCTTCTGCTGCG-3′, where Arg^{112} was replaced by lysine (underlined). Wild-type *RCN-3* cDNA was used as the template. The full-length mutant *RCN-3* cDNAs were generated from the second PCR using the first PCR product as the sense primer and an XbaI-linked C-terminal antisense primer and cloned into the KpnI/XbaI sites of pcDNA3 vector (Invitrogen). Mutant *RCN-3* cDNA lacking the HDEL sequence located at the C-terminus was prepared by PCR using a KpnI-linked N-terminal sense primer and an XbaI-linked antisense primer, 5′-GCTCTAGATCAGTGCCGGGTCAGGTCCTC-3′, where His^{325} was replaced with a termination codon (underlined). *PACE4* (A-I isoform) cDNA was subcloned into the pALTERMAX vector as described previously [13]. Human *RCN-1* cDNA [19] was amplified by PCR using a KpnI-linked sense primer, 5′-GTAGGTACCCTCCTCGGGACGATGGC-3′, corresponding to nucleotides 40–57 of *RCN-1* cDNA (GenBank® accession number D42073) (initiation codon underlined), and an XbaI-linked antisense primer, 5′-CGCTCTAGAGGTGAGTGTCTATCAAAGCTC-3′, corresponding to nucleotide numbers 1039–1060 of *RCN-1* cDNA (GenBank® accession number D42073) (termination codon underlined). Total RNA from HT1080 cells was reverse-transcribed using Thermo-X reverse transcriptase (Invitrogen) according to the manufacturer's instructions and used as a template for PCR. The PCR fragment was digested with KpnI and XbaI and was subsequently cloned into the pcDNA3 expression vector. All constructs were verified by sequence analysis.

Mouse furin and PC6A expression plasmids were kindly provided by Professor K. Nakayama (Department of Physiological Chemistry, Kyoto University, Kyoto, Japan).

Establishment of GH4C1 cell line expressing α1AT RVRR variant

A GH4C1 cell line expressing the RVRR variant of α1AT was established as described previously [15]. Cells transfected with pcDNA3 (no insert) were used as a negative control.

Subcellular fractionation of GH4C1 cells

GH4C1 cells grown to confluence in 16 dishes (100 mm diameter) were harvested by scraping and washing with PBS, before storage at −30 °C. The frozen cell pellet was thawed and homogenized in 5 ml of ice-cold 0.25 M sucrose containing 5 mM Tris/HCl buffer (pH 7.2), 1 mM EDTA and a mixture of protease inhibitors {1 mM benzamidine/HCl, 5 μg/ml E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane], 10 μg/ml leupeptin and 5 μg/ml pepstatin} using a Polytron homogenizer for 5 s. The homogenate was centrifuged at 500 ***g*** for 10 min. The precipitate was rehomogenized in 3 ml of the same buffer and then centrifuged again. The supernatants were combined and used as the post-nuclear supernatant. The supernatant was centrifuged at 105 000 ***g*** for 1 h at 4 °C. The resultant precipitate and supernatant were used and designated as the membrane and soluble fractions respectively.

2D-PAGE of membrane proteins from GH4C1 cells

2D-PAGE was performed according to the method of O'Farrell [20] with minor modifications [21]. The membrane fraction from two or three 100-mm-diameter dishes was lysed with 0.3 ml of an 8 M urea solution containing 0.2 % Nonidet P40 and 10 mM DTT (dithiothreitol), centrifuged at 15 000 ***g*** for 15 min, and then subjected to electrophoresis. Following electrophoresis, proteins in the gel were electroblotted on to a PVDF membrane (0.45 μm pore-size) (Immobilon™; Millipore) and stained with Coomassie Brilliant Blue.

Lysyl endopeptidase digestion, peptide purification and sequencing

The Coomassie Blue-stained protein spots were excised from 10–20 filters, reduced with DTT, alkylated with iodoacetic acid in the presence of 8 M guanidinium chloride, and digested with lysyl endopeptidase (*Achromobacter* protease I; Wako Chemicals) according to the method of Iwamatsu and Yoshida-Kubomura [22]. Samples were then subjected to reverse-phase HPLC on a μRPC C_2/C_{18} pc3.2/3 column (Amersham Biosciences). Peptides were eluted at 0.24 ml/min using a linear gradient of acetonitrile (5–50 % in 35 min) in 0.1 % trifluoroacetic acid. The peptide sequence was analysed using an automated protein sequencer (Shimadzu PPSQ-10).

Preparation of the anti-(human RCN-3) antibody

In an effort to generate a polyclonal antibody against human RCN-3, recombinant His_6–RCN-3 was prepared as follows. Full-length *RCN-3* cDNA was cloned into the pQE30 vector containing an N-terminal His_6 tag (Qiagen) and then transformed into *Escherichia coli* strain JM109 cells. RCN-3 was expressed and purified by affinity chromatography using Ni^{2+}-nitrilotriacetate–agarose (Qiagen) according to the manufacturer's instructions. The final preparation yielded a single 36 kDa band on SDS/PAGE (10 % gel) [23] and was used for the immunization of rabbits.

RT (reverse transcription)–PCR analysis of the *RCN-3* transcript

The human *RCN-3* transcript containing the complete open reading frame was amplified by PCR using cDNA reverse-transcribed from total RNA derived from various human culture cells

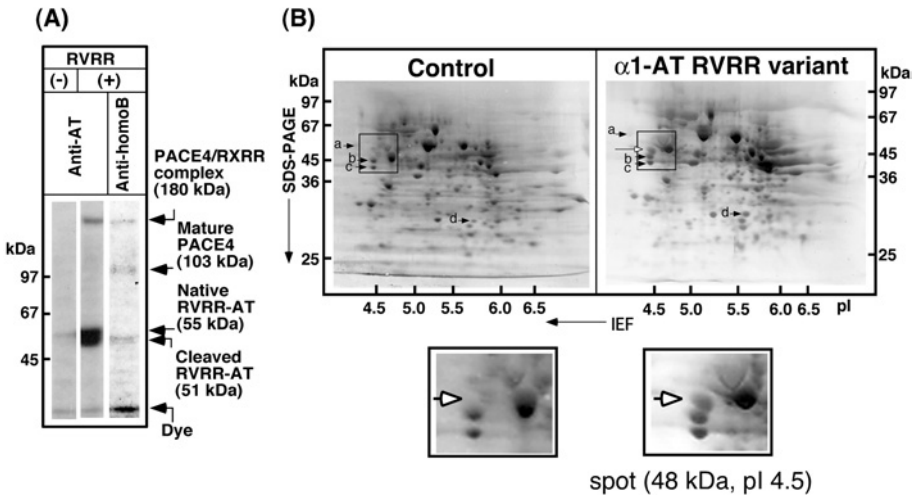

Figure 1 Accumulation of the 48 kDa protein in GH4C1 cells expressing the α1AT RVRR variant

(**A**) Expression of the RVRR variant in GH4C1 cells transfected with the expression vector for the RVRR variant. Cells were labelled with [^{35}S]Met/[^{35}S]Cys for 6 h. The conditioned medium from control and RVRR-expressing cells was subjected to immunoprecipitation with anti-α1AT (Anti-AT) and anti-PACE4 (anti-homoB) antibodies, and were then analysed by SDS/PAGE (10 % gel). (**B**) 2D-PAGE analysis of membrane fraction from control and RVRR-expressing GH4C1 cells. The 48 kDa protein spot is indicated by an open arrow and is enlarged below. Protein spots, indicated by a, b, c and d, were identified as calreticulin, reticulocalbin-1, reticulocalbin-1 and ERP29 respectively, as shown in Table 1. IEF, isoelectric focusing. Molecular-mass sizes are given in kDa.

as described above. The rat *RCN-3* transcript in GH4C1 cells was analysed by RT–PCR using a mouse RCN-3 sense primer [5′-GAGGAGTTCCCTCACATGCG-3′, corresponding to the nucleotide region 647–666 of mouse *RCN-3* cDNA (GenBank® accession number AK077943)] and a human RCN-3 XbaI-linked antisense primer [5′-GCTCTAGATCACAGCTCATCCTGGTG-CC-3′, corresponding to nucleotide region 1032–1051 of mouse *RCN-3* cDNA (GenBank® accession number AK077943); underlined nucleotides were replaced by guanosine in the mouse cDNA].

Biochemical analysis

Protein was determined by the method of Bradford [24] using BSA as a standard. The Ca^{2+}-binding activity of recombinant RCN-3 was determined by $^{45}Ca^{2+}$ blot analysis [19]. Western blot analysis of RCN-3 was performed using anti-RCN-3 antibody as described previously [16].

Transfection, radiolabelling and immunoprecipitation

HEK-293 (human embryonic kidney) and Cos-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) foetal calf serum and antibiotics under 5 % CO_2. Transfection and radiolabelling of the cells, followed by immunoprecipitation of PACE4 with anti-SCD [25], anti-homoB [13] or anti-propeptide antibody [14] was performed as described previously. The immunoprecipitation of HSA (human serum albumin) and RCN-3 was performed as described previously [16].

Confocal immunofluorescence microscopy

Cells grown on glass coverslips were washed twice with PBS and fixed for 20 min in 4 % (w/v) formaldehyde. Following sequential washes with PBS and 50 mM NH_4Cl in PBS, cells were permeabilized for 5 min in 0.1 % (v/v) Triton X-100, washed three times with PBS, and then treated with 5 % (w/v) BSA in PBS for 30 min. Cells were subsequently incubated with the first antibody (rabbit anti-RCN-3 IgG 1:1000 or mouse anti-KDEL IgG 1:500) for 2 h. Following three washes with PBS, cells were incubated for 1 h with goat anti-rabbit IgG conjugated with FITC (1:100) or anti-mouse IgG conjugated with Texas Red (1:100). Coverslips were washed thoroughly with PBS and mounted in fluorescent mounting medium (Vectashield H-1000; Vector Laboratories). A confocal laser-scanning microscope (Radiance; Bio-Rad Laboratories) was used to obtain staining profiles.

RESULTS

RCN-3 accumulation in GH4C1 cells expressing the α1AT RVRR variant

Immunoprecipitation confirmed expression of the α1AT RVRR variant in GH4C1 cells transfected with the RVRR expression vector. The variant secreted into the culture medium was detected as intact (55 kDa) and cleaved (51 kDa) forms at high levels in the RVRR variant-expressing cells (RVRR cells) (Figure 1A). The 180 kDa band was detected using both anti-α1AT and anti-PACE4 (anti-homoB) antibodies. These results indicate that the 180 kDa band corresponds to the acyl complex of SPC (PACE4, furin and PC6) with the variant. These bands were not detected in control cells. Membrane fractions from control and RVRR cells were analysed by 2D-PAGE. Typical protein maps are shown in Figure 1(B). To evaluate the reproducibility of protein maps, 30 gels separated on different days were compared. The significant difference of spot intensity between control and the RVRR cells were found in the 48 kDa spot with the highest reproducibility. The 48 kDa acidic protein (pI 4.5) accumulated markedly in cells expressing the RVRR variant. Only small amounts of this protein were detected in control cells.

In an effort to identify the 48 kDa protein by amino acid sequencing, the protein was transferred on to a PVDF membrane and then digested with *Achromobacter* protease I (lysyl endopeptidase). Three peptides (peptides 1–3) were purified by HPLC equipped with a reverse-phase column and sequenced, yielding 45 residues in total (Figure 2A). The sequences determined for peptides 1, 2 and 3 were DGYVQVEEYIADLYS, GRLDGSEVGYWVLPPS and EILSNTNMFVGSQA respectively. Extensive sequence similarity was found between these

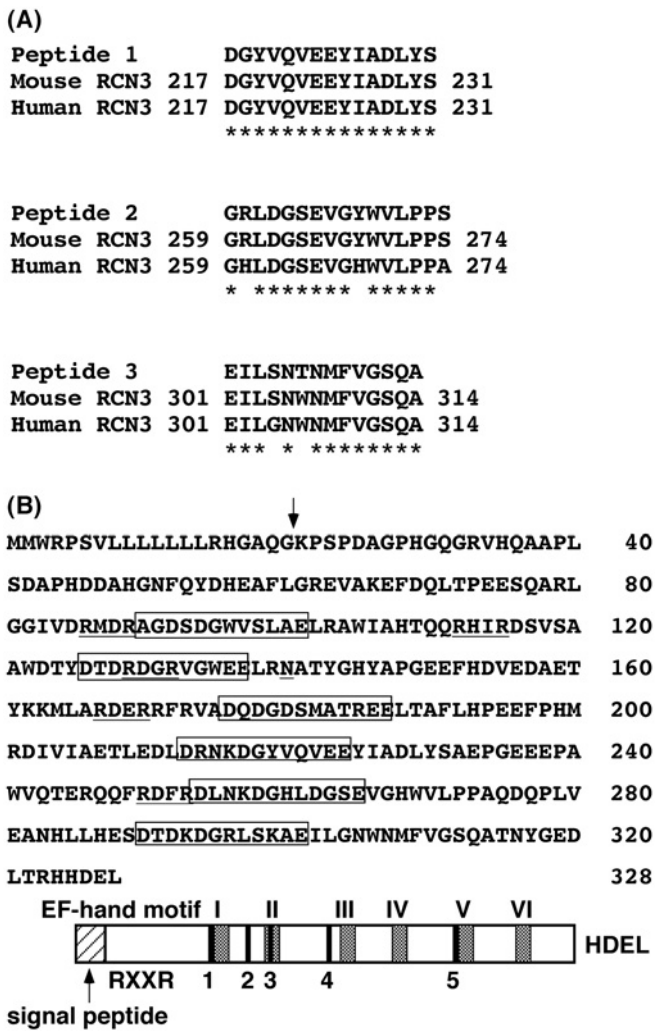

Figure 2 Identification of the 48 kDa protein by sequence analysis

(**A**) Alignment of peptide sequences 1, 2 and 3 derived from the 48 kDa protein with human (GenBank® accession number AF183423) and mouse (GenBank® accession number AK077943) RCN-3. (**B**) Predicted amino acid sequence and schematic domain structure of human RCN-3. The arrow indicates the possible cleavage site of the signal peptide. Residues corresponding to the calcium-binding loop of the EF-hands predicted from homology with the CREC family are boxed. The SPC-recognition sequence RXXR is underlined. A possible glycosylation site (Asn^{140}) is also underlined.

sequences and the translated sequence of mouse (GenBank® accession number AK077943) and human (GenBank® accession number AF183423) cDNA encoding RCN-3. Thus the sequence data indicates that the 48 kDa protein that had accumulated in GH4C1 cells expressing the RVRR variant represents a rat RCN-3. Slight differences in the sequence between the 48 kDa protein and those from mouse and human might reflect possible species-related differences. Figure 2(B) shows the complete amino acid sequence of human RCN-3 predicted from its cDNA nucleotide sequence. The predicted molecular mass of RCN-3 (35 kDa) is smaller than the size of the protein (48 kDa) detected in GH4C1 cells, whereas it was similar in size to recombinant RCN-3 (36 kDa) purified from *E. coli*.

RCN-3 possesses one potential N-glycosylation site at Asn^{140}. Treatment with endoglycosidase H or endoglycosidase F reduced the size of RCN-3 to 45 kDa (results not shown). Although these results suggest that RCN-3 possesses N-linked high-mannose-type oligosaccharides, the difference in molecular mass between

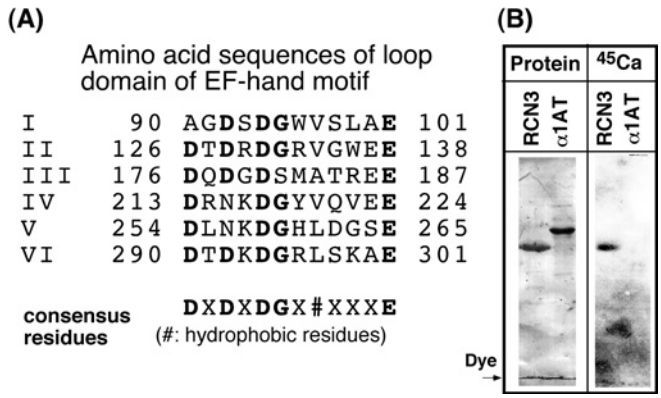

Figure 3 RCN-3 possesses Ca^{2+}-binding activity

(**A**) Alignment of the different EF-hand loops (I–VI) of RCN-3. Consensus amino acid residues in the loop domain are shown. (**B**) $^{45}Ca^{2+}$ overlay assay of recombinant RCN-3. Recombinant RCN-3 (1 μg) and α1AT (1 μg) were electrophoresed and transferred on to a nitrocellulose membrane. Following incubation with $^{45}Ca^{2+}$, the membrane was rinsed with water and then exposed to X-ray film. The same membrane was stained with Amido Black to detect proteins.

RCN-3 from GH4C1 cells and recombinant RCN-3 can not be fully explained by N-glycosylation. Further analysis is necessary to delineate any possible post-translational modification of RCN-3. RCN-3 possesses six EF-hand motif repeats and a C-terminal ER retrieval signal (HDEL), common features of CREC family members [18]. Each repeat possesses the general feature of a Ca^{2+}-binding EF-hand domain according to Kretsinger's rule [26], as shown in Figure 3(A). The central glycine residue is conserved in these motifs except at site III, where glycine is replaced by serine. To determine whether the six EF-hand motifs in RCN-3 actually possess Ca^{2+}-binding activity, a $^{45}Ca^{2+}$ blot assay was performed using recombinant RCN-3 (Figure 3B). A strong signal was detected with His_6-tagged RCN-3, but no signal was observed for His_6-tagged α1AT. The positive band was 36 kDa in size, equivalent to the size of recombinant RCN-3 protein detected by Amido Black staining, thus confirming that RCN-3 possesses Ca^{2+}-binding ability.

Intracellular location of RCN-3

In an effort to determine the subcellular location of RCN-3, a polyclonal antibody against recombinant RCN-3 was raised. As shown in Figure 4(A), the specificity of RCN-3 antiserum was demonstrated by immunoblotting. A single band corresponding to the size of RCN-3 (48 kDa) in the cell extract derived from cells transfected with RCN-3 expression vector was detected. The intracellular location of RCN-3 was determined by cell fractionation and indirect immunofluorescence microscopy. GH4C1 cells (control and RVRR cells) were fractionated and analysed by immunoblotting (Figure 4B). RCN-3 was detected as a single 48 kDa band in the membrane fraction of RVRR cells, although RCN-3 was not detected in control cells. The intracellular location was confirmed by double-immunostaining of HepG2 cells. RCN-3 was detected in HepG2 cells. As shown in Figure 4(C), RCN-3 was present in a diffuse ER-like network, in small vesicles clustered around the nucleus. Double-staining using anti-KDEL antibody revealed overlapping patterns. These results indicates that RCN-3 is an ER-resident protein. Some of immunofluorescent staining with anti-RCN-3 antibody was observed within nucleus. This faint staining was also seen when control antibody was used (results not shown), suggesting that the staining of nucleus was non-specific.

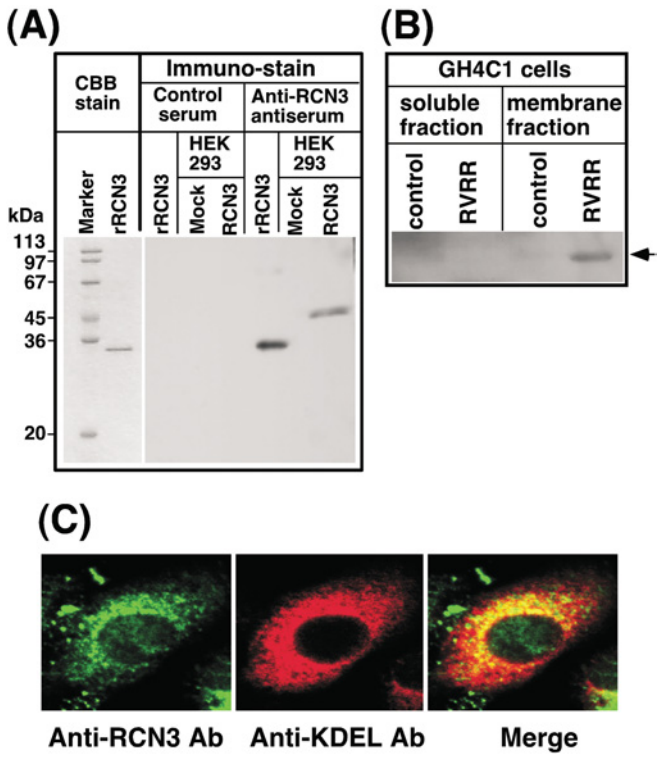

Figure 4 Intracellular location of RCN-3 protein

(**A**) Specificity of anti-RCN-3 antibody. Purified recombinant RCN-3 (rRCN-3, 20 ng) from *E. coli* and cell extract (16 μg of protein) derived from HEK-293 cells transiently expressing RCN-3 were subjected to SDS/PAGE (10% gel) and then analysed by Western blotting using RCN-3 antiserum. Molecular-mass sizes are given in kDa. (**B**) Western blot analysis of RCN-3 protein in the soluble and membrane fractions derived from control GH4C1 cells and cells expressing α1AT RVRR variant (RVRR). (**C**) Localization of RCN-3 by immunofluorescent staining. HepG2 cells were double-stained with anti-RCN-3 (visualized using a secondary FITC-conjugated antibody) or anti-KDEL antibody (visualized using a secondary Texas-Red-conjugated antibody). Staining profiles were observed using a confocal laser-scanning microscope. Ab, antibody.

Cell distribution of RCN-3

The expression profile of the *RCN-3* transcript in various human culture cells was compared using RT–PCR analysis. Figure 5(A) shows the ethidium-bromide-stained agarose gel. When PCR was performed using either the sense or antisense primer alone, no amplified band was detected (results not shown). A major band corresponding to the expected size (980 bp) was detected in all cell lines, although several minor bands also appeared. The identity of the major PCR product was confirmed by sequencing. The major *RCN-3* transcript (980 bp) was expressed in BeWo cells (choriocarcinoma cell line), Dami cells (megakaryocyte cell line), and SHSY5Y and SAN cells (neuroblastoma cell lines) at high levels. It was also expressed in HepG2, HEK-293, T3M-3 (choriocarcinoma), NBL-S (neuroblastoma) and SKNDZ (neuroblastoma) cells. These results indicate that RCN-3 is ubiquitously expressed in a variety of cell types. The smaller PCR product (880 bp) was detected in all cell lines examined, although the expression level differed. The nucleotide sequence (nucleotides 355–448) coding for Ser^{119}–Glu^{150} was deleted in the 880 bp transcript, suggesting that the smaller PCR product (880 bp) is a splicing variant. In HEK-293 cells, an 800 bp PCR product was also detected. A larger PCR product (1050 bp) was detected in BeWo, Dami and SHSY5Y cells. We can not rule out that the 1050 and 800 bp species may represent non-specific PCR products. Further analysis is necessary to identify these transcripts.

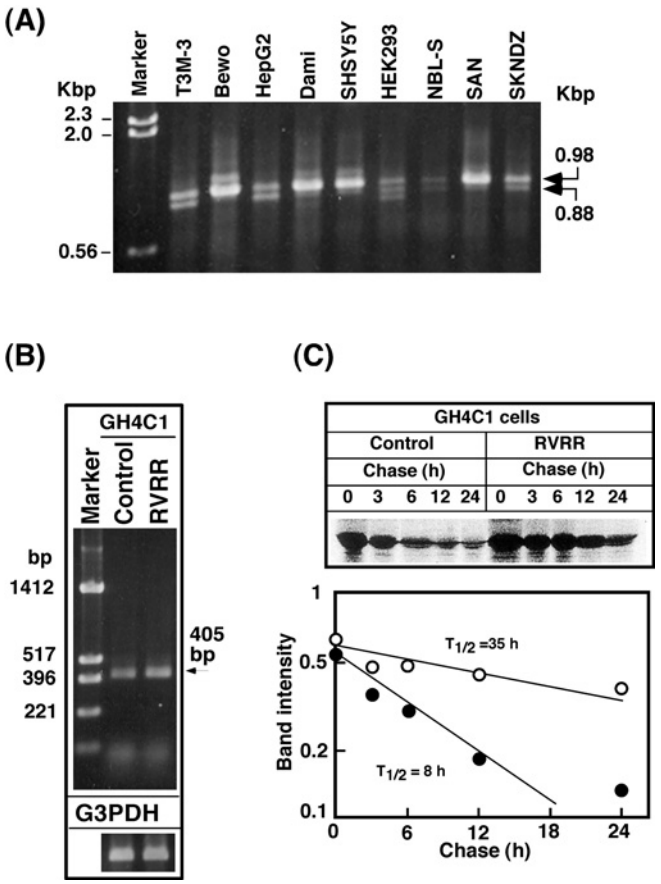

Figure 5 Expression of *RCN-3* transcripts in human culture cells and effect of α1AT RVRR variant on half life of RCN-3 protein in GH4C1 cells

(**A**) PCR was performed on cDNA synthesized from total RNA derived from various culture cells as described in the Experimental section. Sizes are give in kb. The 980 and 880 bp bands are indicated by arrows. (**B**) Total RNA from control GH4C1 cells and cells expressing the α1AT RVRR variant was isolated and analysed by RT–PCR as described in the Experimental section. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) transcript is shown as a positive control. Sizes are given in bp. (**C**) Control GH4C1 cells (●) and cells expressing the α1AT RVRR variant (○) were labelled with [^{35}S]Met/[^{35}S]Cys for 3 h and then chased for 3, 6, 12 and 24 h. Cell extracts were subjected to immunoprecipitation using anti-RCN-3 antibody and were analysed by SDS/PAGE (10% gel). The RCN-3 48 kDa band intensity was quantified using a BAS-1500 image analyser. The experiment was repeated twice with similar results.

Effects of RVRR variant expression on other ER resident proteins

To determine whether expression of other ER proteins is influenced by the ectopic expression of the RVRR variant, protein spots resolved by 2D-PAGE were screened by sequence analysis. As shown in Table 1, three ER-resident proteins were identified as calreticulin (spot a) [27], RCN-1 (spots b and c) [19] and ERP29 (ER protein 29 kDa, spot d) [28]. Calreticulin functions as a lectin-like molecular chaperone for newly synthesized glycoproteins in the lumen of the ER [29]. RCN-1 is a member of the CREC family [18,19]. ERP29 is a member of the protein disulphide-isomerase family [28]. As shown in Figure 1(B), no differences in the expression level of these proteins was observed between control and RVRR cells. Like RCN-3, RCN-1 possesses six EF-hand motif repeats and a C-terminal HDEL sequence. The amino acid sequences are very similar, possessing 58% amino acid identity. However, unlike RCN-3, the expression level of RCN-1 was unaffected by the RVRR variant. It is likely that RCN-3 had accumulated specifically in GH4C1 cells by expression of the RVRR variant.

Table 1 Identification of proteins separated by 2D-PAGE

Protein spots were digested with lysyl endopeptidase. Released peptide was purified by reverse-phase HPLC and was sequenced as described in the Experimental section. Homology search showed that all peptide sequences from the 48 kDa protein were highly similar to human RCN-3. Spots a, b, c, and d were shown to be calreticulin, RCN-1, RCN-1 and ERP29 respectively. Numbers in parentheses are the amino acid numbers of the corresponding regions of identified proteins.

		Amino acid sequence	Identified protein
48 kDa protein			
Peptide 1		DGYVQVEEYIADLYS	RCN-3 (217–231)
Peptide 2		GRLDGSEVGYWVLPPS	RCN-3 (259–274)
Peptide 3		EILSNTNMFVGSQA	RCN-3 (301–314)
Spot	a	DPAIYFK	Calreticulin (18–24)
		DDEFTHLYTL	Calreticulin (165–174)
	b	ATYGYYLGNPA	RCN-1 (137–147)
	c	WEEYK	RCN-1 (131–135)
	d	RLAENSASSXXL	ERP29 (75–86)

Prolongation of the half-life of RCN-3 by the inhibition of SPCs

To determine the mechanism responsible for the specific accumulation of RCN-3 protein in cells expressing the RVRR variant, the expression levels of the *RCN-3* transcript and half-life of the RCN-3 protein was compared in control and RVRR GH4C1 cells. The half-life of the RCN-3 protein was determined by employing a pulse–chase experiment. As shown in Figure 5(B), a single *RCN-3* transcript of the predicted size (405 bp) was amplified in both cell types; however, no difference in the expression level was detected. In contrast, the half-life ($t_{1/2}$) of the RCN-3 protein was 4-fold longer in cells expressing the RVRR variant (35 h) than in control cells (8 h) (Figure 5C). These results clearly indicate that accumulation of RCN-3 protein in RVRR GH4C1 cells results from prolongation of the half-life of the RCN-3 protein.

Transient interaction between proPACE4 and RCN-3

Co-expression experiments were employed to examine the effect of expression of SPC members on the turnover of RCN-3. The cell extract and conditioned medium were subjected to immunoprecipitation using anti-RCN-3 antibody and were analysed by SDS/PAGE (10% gel) as shown in Figure 6(A). Most of the RCN-3 was retained in the cells, while a small amount was secreted into the medium. The amount of secreted RCN-3 decreased when RCN-3 and SPC were co-expressed. In particular, expression of furin resulted in substantial reduction in the amount of RCN-3 secreted. As shown in Figure 6(A) (right-hand panel), RCN-3 degradation (processed) products (34 and 17 kDa) were detected in the conditioned medium from cells co-transfected with furin and RCN-3 when the autoradiogram was overexposed. These results suggest that some of the RCN-3 was cleaved by SPC and that the degradation products were secreted. In contrast, we found that the protein band (indicated by the arrow) with the same molecular mass as proSPC that had been transfected into the cells co-immunoprecipitated with anti-RCN-3 antibody in the cell extracts. A 95 kDa band co-immunoprecipitated from the furin co-expressing cells, while 110 and 115 kDa bands co-immunoprecipitated from the PACE4- and PC6A-co-expressing cells respectively, suggesting that RCN-3 was associated with proSPCs during their biosynthesis. To confirm the binding of RCN-3 to proPACE4, the cell extract and conditioned medium were subjected to immunoprecipitation using anti-PACE4 (anti-homoB) or anti-RCN-3 antibodies and were then analysed by SDS/PAGE (10% gel) (Figure 6B). Endogenous RCN-3 was detected in HEK-293 cells, and the amount markedly increased

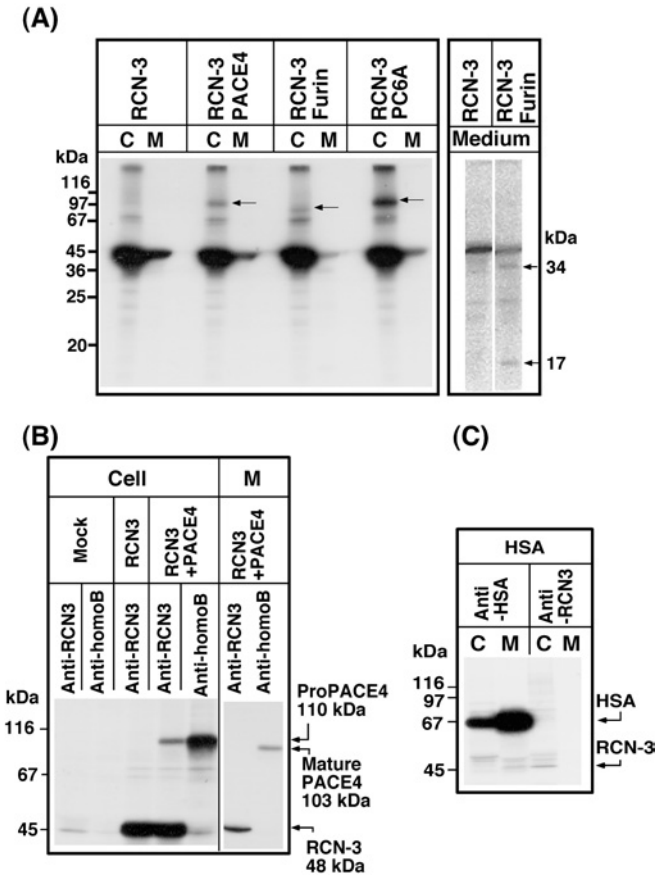

Figure 6 Co-expression of RCN-3 and SPC (furin, PACE4 and PC6A) in HEK-293 cells

(**A**) HEK-293 cells (35-mm-diameter dish) were transiently transfected with the expression vector (1.0 μg) for RCN-3, furin, PACE4 or PC6A. Following radiolabelling for 6 h, the cell extract (C) and conditioned medium (M) were subjected to immunoprecipitation using anti-RCN-3 antibody and were subsequently analysed by SDS/PAGE (10% gel). Arrows indicate a 95 kDa band. The autoradiogram (immunoprecipitate of conditioned medium from cells expressing furin and RCN-3) was overexposed to reveal the weaker degradation products. The 34 and 17 kDa degradation products are indicated by arrows. (**B**) HEK-293 cells were transfected with the expression vector for RCN-3 in the absence or presence of the expression vector for PACE4. Following labelling as described in (**A**), the cell extract and conditioned medium were subjected to immunoprecipitation using anti-RCN-3 and anti-PACE4 (anti-homoB) antibodies. (**C**) HEK-293 cells were transfected with expression vector for HSA. After labelling, the cell extract and conditioned medium were subjected to immunoprecipitation with anti-HSA or anti-RCN-3 antibody. Molecular-mass sizes are given in kDa.

by transfection with the RCN-3 expression vector. In cells co-transfected with RCN-3 and PACE4, the 48 kDa RCN-3 major band protein and a 110 kDa minor band protein were immunoprecipitated with the anti-RCN-3 antibody. Conversely, the 110 kDa band corresponding to proPACE4 and a faint 48 kDa RCN-3 band immunoprecipitated with the anti-PACE4 (anti-homoB) antibody. The 48 kDa band was not co-immunoprecipitated with anti-PACE4 antibody in the control cell extract (mock). In contrast, secreted mature PACE4 did not co-immunoprecipitate with the anti-RCN-3 antibody (Figure 6B). The amount of proPACE4 bound to RCN-3 did not increase, even when RCN-3 was overexpressed in cells. The most likely explanation is that the amount of endogenously expressed RCN-3 was sufficient to associate with proPACE4. To determine the binding specificity of RCN-3, HSA was expressed transiently and the binding activity of RCN-3 to HSA was examined. HEK-293 cells efficiently synthesized HSA and secreted the

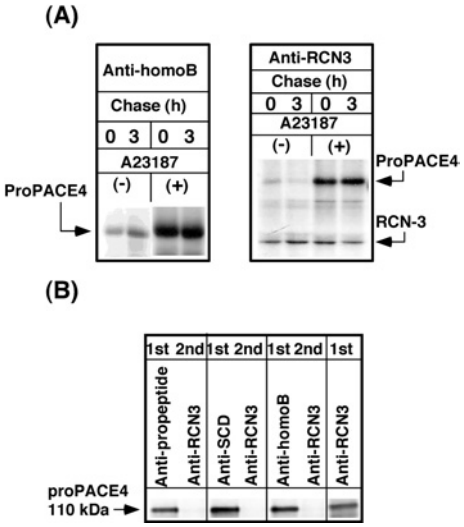

Figure 7 Effects of Ca^{2+} ionophore, A23187, on formation of the RCN-3–proPACE4 complex in HEK-293 cells

(**A**) Cells were transiently transfected with PACE4 and were cultured in the presence of 0.4 μM A23187 and [^{35}S]Met/[^{35}S]Cys (100 μCi/ml). Following an incubation for 3 h, the medium was changed to radioisotope-free medium and cells were cultured for an additional 3 h in the presence or absence of A23187. The cell extract was subjected to immunoprecipitation with anti-PACE4 (anti-homoB) or anti-RCN-3 antibody and were subsequently analysed by SDS/PAGE (10 % gel). (**B**) Cells were transiently transfected with PACE4 and labelled in the presence of A23187 as described in (**A**). The cell extract was initially immunoprecipitated with anti-PACE4 antibody (anti-propeptide, anti-SCD or anti-homoB antibody) (1st) and the resulting supernatants were subjected to immunoprecipitation with anti-RCN-3 antibody (2nd), or immunoprecipitated directly with anti-RCN-3 without pre-absorption with anti-PACE4 antibody. The position of the 110 kDa band is indicated.

protein into the culture medium. A 48 kDa band was detected in immunoprecipitate of cell extract with anti-RCN-3 antibody. However, this band was not detected in immunoprecipitate of cell extract with the anti-HSA antibody (Figure 6C). Furthermore, HSA in cell extract was not co-immunoprecipitated with anti-RCN-3 antibody. A faint 48 kDa band was detected in immunoprecipitate of culture medium with anti-HSA antibody; however, this band was not immunoprecipitated with anti-RCN-3 antibody, suggesting that the faint band is a non-specific band.

Addition of the Ca^{2+}-specific ionophore A23187 into the culture medium can inhibit autoactivation of SPCs in the cells [16]. If RCN-3 binds to proPACE4 transiently during its biosynthesis, the maturation of proPACE4 to PACE4 can be blocked by this reagent. Consequently, the proPACE4–RCN-3 complex is expected to accumulate in cells when cells are cultured in the presence of a Ca^{2+} ionophore. We evaluated whether the presence or absence of Ca^{2+} ionophore had any effect on the association of RCN-3 with proPACE4 in transfected HEK-293 cells. The cells were transiently transfected with PACE4 expression vector, radiolabelled for 3 h, and then chased for 3 h in the presence or absence of A23187. The amount of both proPACE4 (110 kDa band) immunoprecipitated with anti-PACE4 or anti-RCN-3 antibody increased markedly by treatment with A23187 (Figure 7A). To confirm whether the 110 kDa protein co-immunoprecipitated with anti-RCN-3 antibody was proPACE4, cell extracts from A23187-treated cells were pre-absorbed with anti-PACE4 antibodies (anti-propeptide, anti-SCD and anti-homoB antibodies) and then immunoprecipitated with anti-RCN-3 antibody. The anti-SCD and anti-homoB antibodies recognize both precursor and mature PACE4, whereas the anti-propeptide antibody only recognizes proPACE4. As shown in Figure 7(B), the amount of 110 kDa protein immunoprecipitated with anti-RCN-3 antibody decreased markedly when the cell extract was pre-treated with three anti-PACE4 antibodies, indicating that the 110 kDa protein that co-immunoprecipitated with anti-RCN-3 antibody was proPACE4.

Effect of RCN-3 on the autoactivation and secretion of PACE4

Although PACE4 is a putative secretory enzyme, it matures and is secreted much more slowly than general secretory proteins [14]. To test whether RCN-3 can facilitate the autoactivation and secretion of PACE4, RCN-3 was co-expressed with PACE4 in Cos-7 cells. To examine the role of the RXXR SPC-recognition sequence and ER-retrieval signal in RCN-3, three mutants of RCN-3, M1 (RMDR^{89} was replaced by KMDR), M2 (RHIR^{115} was replaced by KHIR) and delC (C-terminal HDEL was deleted) were constructed (Figure 8A). When PACE4 was transfected in Cos-7 cells without RCN-3, 32 ± 11.4 % of PACE4 was secreted as the mature enzyme into the medium (Figure 8B). However, the efficiency of PACE4 secretion increased 2-fold (68 ± 7.8 %) by co-expression with wild-type RCN-3. In contrast, when M1 or M2 mutant was co-expressed with PACE4, the efficiency of PACE4 secretion was 49 ± 6.2 % and 26 ± 9.8 % respectively. In particular, mutation of the second RXXR site (RHIR^{115} to KHIR) resulted in complete loss of facilitation effect on PACE4 secretion. In contrast, secretion efficiency of PACE4 markedly reduced (14 ± 5.9 %) by co-expression with the delC mutant. Further effects of RCN-1 co-expression relating to other members of the CREC family, was also examined. The efficiency of PACE4 secretion was reduced (21 ± 1.9 %) by co-expression with RCN-1 (Figure 8C). These results strongly suggested the presence of a specific interaction between RCN-3 and proPACE4 that plays an important role in the processing and secretion of proPACE4.

DISCUSSION

This is the first report characterizing RCN-3 at the protein level by applying a proteomic approach using an engineered α1AT RVRR variant. Inhibition of PACE4, PC6A and furin by the α1AT RVRR variant resulted in specific accumulation of RCN-3 in GH4C1 cells. RCN-3 possesses Ca^{2+}-binding activity and, like other CREC family proteins, is found mainly in the ER. The *RCN-3* transcript is expressed ubiquitously in various culture cells as shown by RT–PCR analysis. It was also found that inhibition of PACE4, PC6A and furin prolonged the half-life of RCN-3, resulting in the accumulation of RCN-3 in cells. The expression level of the *RCN-3* transcript was not affected by the α1AT variant. These results strongly suggest that RCN-3 accumulation results primarily from the inhibition of RCN-3 degradation, and not from the induced elevated transcription of RCN-3. This inhibitor did not affect the expression levels of RCN-1 or other ER-resident proteins such as calreticulin and ERP29. Compared with other members of the CREC family, the most interesting structural feature of RCN-3 relates to the presence of five RXXR sequences that represent SPC-recognition sequences, suggesting a specific interaction between RCN-3 and SPC. Furthermore, we showed that co-expression of RCN-3 and SPC, and especially furin, decreased the amount of secreted RCN-3. When furin was co-expressed with RCN-3, 34 kDa and 17 kDa products were detected in the medium. If RCN-3 is cleaved at the second RXXR site (RHIR^{115}) by furin, the molecular mass of the C-terminal fragment of RCN-3 is calculated to be 34 kDa. This is similar to the size of RCN-3 fragment detected in the culture medium. Although further *in vitro* analysis is needed, we conclude that RCN-3 is likely to be cleaved by SPC.

We previously compared the molecular structure of proPACE4, the latent form which remains associated with the cleaved

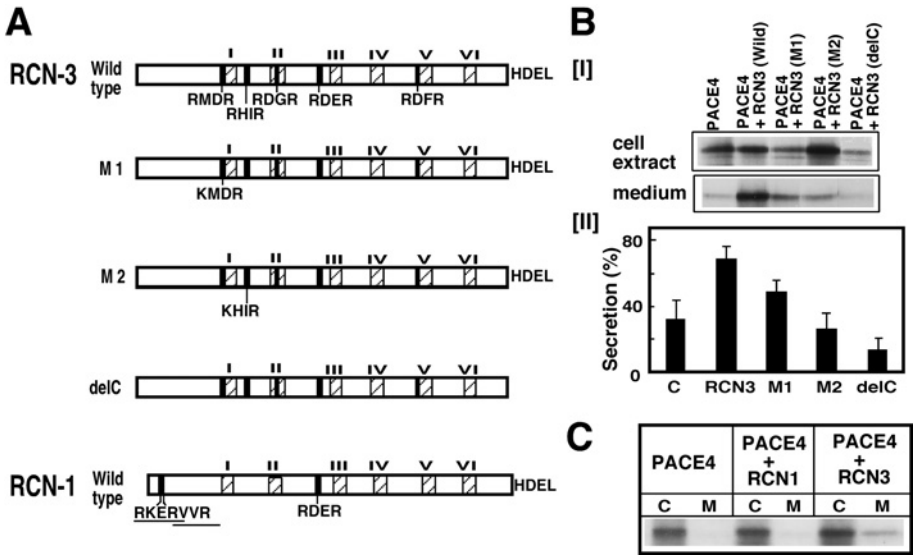

Figure 8 Effects of expression of RCN-3, its mutants and RCN-1 on the secretion of PACE4

(**A**) Schematic representation of the structure of wild-type, and M1, M2 and delC mutants of RCN-3, and wild-type RCN-1. The RXXR site and EF-hand motif (I–VI) are shown. (**B**) I: Cos-7 cells (35-mm-diameter dish) were transfected with expression vector for PACE4 (0.5 μg) in the absence or presence of wild-type or mutant (M1, M2 or delC) RCN-3 expression vector (0.5 μg). Control cells were transfected with PACE4 expression vector (0.5 μg) and pcDNA3 (0.5 μg). Following transfection, cells were labelled for 6 h as described in the Experimental section. The cell extract and conditioned medium were then immunoprecipitated using anti-PACE4 (anti-homoB) antibody. The immunoprecipitate was analysed by SDS/PAGE (10 % gel) and fluorography. The density of each band on the X-ray film was quantified using NIH (National Institutes of Health) Image software. II: the results of quantitative analysis obtained from three independent experiments are shown. Each value is expressed as the mean $\pm$ S.D. C, PACE4; RCN3, PACE4 + wild-type RCN-3; M1, PACE4 + RCN-3 M1 mutant; M2, PACE4 + RCN-3 M2 mutant; delC, PACE4 + RCN-3 delC mutant. (**C**) Effect of RCN-1 on PACE4 secretion efficiency. Transfection of PACE4 and wild-type RCN1, labelling of cells and immunoprecipitation were performed as described in (**B**). The experiments were repeated three times to ensure reproducibility. C, cell extract; M, conditioned medium.

propeptide and mature form, by sedimentation velocity analysis [14]. ProPACE4 (110 kDa) in the cell extract sedimented as a 150–160 kDa molecule, and both the latent form (103 kDa) detected using the propeptide antibody in the cell extract and mature PACE4 secreted into the medium migrated at a size corresponding to a monomer. These results suggest that proPACE4 is retained in the ER by interacting with a specific binding protein, which is released after the first cleavage of the propeptide.

In the present study, the results of three different experimental approaches showed that RCN-3 interacts with proPACE4. First, it was found that proPACE4 co-immunoprecipitated with the anti-RCN-3 antibody, whereas mature PACE4 did not. Secondly, inhibition of autocatalytic maturation of PACE4 by the Ca^{2+} ionophore, A23187, markedly increased the amount of proPACE4 co-immunoprecipitated with anti-RCN-3. Thirdly, RCN-3 increased PACE4 secretion efficiency. This effect was decreased by mutation of the RXXR SPC-recognition site or deletion of the HDEL sequence, ER-retrieval signal of RCN-3. The effect of RCN-3 on the secretion of PACE4 appears to represent a specific interaction, as RCN-1 did not display any effect. In the biosynthesis of PACE4, initial cleavage of the propeptide $RVKR^{149}$ that occurs in the ER represents the rate-limiting step in the secretion of activated PACE4 into the medium [13,14]. Like PACE4, the precursors of other SPCs are retained in the ER [30]. In the case of other proteases, such as coagulation factors and cathepsins, the propeptide does not affect their intracellular transport and the proform (latent form) is secreted in addition to the mature enzyme. Thus a unique feature of SPC biosynthesis is that cleavage of the N-terminal propeptide is essential for both activation and intracellular transport. The molecular mechanism that controls retention of the SPC precursor in the ER remains poorly understood. This study clearly demonstrates a specific association of RCN-3 with proPACE4 during its biosynthesis and suggests that RCN-3 plays a role in the retention of proPACE4 in the ER.

The neuroendocrine polypeptide 7B2 possesses an SPC-recognition sequence and is required for the proper transport of the neuroendocrine-specific SPC, the PC2 precursor, from the ER to the *trans*-Golgi network-immature secretory granules and for its maturation [31]. Like 7B2, RCN-3 is an acidic protein that possesses SPC-cleavage sites and is located in the ER. By analogy with the 7B2 protein, we propose that RCN-3 may function as a molecular chaperone for PACE4 within the constitutive secretory pathway. RCN-3 could recognize structural elements exposed by newly synthesized and partially folded proPACE4. As shown in Figure 8(B), RCN-3 M2 mutant ($RXXR^{115}$ was replaced by KXXR) had no effect on PACE4 secretion. KXXR sequence is not recognized by SPC [1,2]. Our results suggest that the RXXR site could bind to the negatively charged substrate-binding site of proPACE4. Although proPACE4 is catalytically inactive with respect to substrate cleavage, its propeptide remains non-covalently bound to the active site of SPC until it reaches its final cellular destination. The propeptide is presumed to act both as an intramolecular chaperone and as an inhibitor of its parent enzyme. The C-terminal multibasic amino acid sequence is critical for the inhibitory function of the propeptide. Therefore it is likely that RCN-3 is released from proPACE4 by competition with the C-terminal $RVKR^{149}$ sequence of the propeptide following completion of proPACE4 folding. Alternatively, RCN-3 may play a role in the retrieval of proPACE4 to the ER. Luminal ER proteins that exit the ER are captured and retrieved to the ER via COPI (coatamer protein I)-coated vesicles that contain KDEL receptors [32]. The proPACE4–RCN-3 complex might be shunted back to the ER from the Golgi compartment through a retrieval process mediated by the RCN-3 HDEL signal. As a consequence of this retrieval system, proPACE4 may not be secreted.

In conclusion, we revealed for the first time that RCN-3 protein selectively accumulated in GH4C1 cells expressing engineered α1AT designed as a SPC inhibitor. Our results show that

RCN-3 interacts transiently with proPACE4 and can stimulate its activation and secretion. Further studies are necessary to clarify the binding mode of RCN-3 to precursors of PACE4 and other SPCs.

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