J. Biochem. 125, 627-633 (1999)

Subtilisin-Like Proprotein Convertases, PACE4 and PC8, as Well as Furin, are Endogenous Proalbumin Convertases in HepG2 Cells[1]

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Received October 12, 1998; accepted December 15, 1998

Serum albumin is synthesized as a larger precursor form, proalbumin, which undergoes proteolytic processing at a dibasic site by a hepatic proprotein convertase within the secretory pathway to generate the mature form. Although furin, a member of the subtilisin-like proprotein convertase (SPC) family, was thought to be the only candidate hepatic convertase for proalbumin, SPC family members other than furin were recently suggested to also be involved in proalbumin processing. This study was designed to identify the endogenous proprotein convertases involved in proalbumin processing. Since human hepatoma HepG2 cells are highly differentiated and produce major plasma proteins, this cell line was used as a model for hepatocytes. Northern blot analysis revealed that PACE4, furin and PC8 of the SPC family were expressed in HepG2 cells as well as in the liver. Ribonuclease protection assay showed that PACE4A-II mRNA is the major transcript in HepG2 cells among the PACE4 isoforms. The coexpression studies showed that furin, PACE4A-II and PC8 were all able to convert proalbumin to albumin correctly. To elucidate the roles of these endogenous SPC family members in proalbumin processing, the antisense RNA for PACE4, furin and PC8 was stably expressed in HepG2 cells, respectively. The expression of each antisense RNA resulted in approximately 30% inhibition of endogenous proalbumin processing. We therefore concluded that PACE4 and PC8, as well as furin, are involved in the processing of proalbumin in HepG2 cells, and that these SPC family members are functionally redundant in this processing.

Key words: furin, PACE4, PC8, proalbumin processing, subtilisin-like proprotein convertase.

Serum albumin is the most abundant plasma protein. It is multifunctional, and plays roles in maintenance of the colloid osmotic pressure of the plasma and transport of the metabolites. Like many other plasma proteins, albumin is initially synthesized as preproalbumin, a larger precursor form, and then cotranslationally converted to proalbumin, which has an amino terminal hexapeptide extension (Arg-Gly-Val-Phe-Arg-Arg, called the propeptide). Subsequently, proalbumin is converted to serum albumin through cleavage of the propeptide at a dibasic site (*1, 2*). Such proteolytic processing at this dibasic site is the most common mechanism for the modification of a variety of bioactive peptides and proteins including peptide hormones, growth factors, receptors and viral envelope glycoproteins, and is an essential step for producing the fully active products. Therefore, proprotein convertase plays an important role in many biological events, such as the maintenance of homeostasis and hormonal regulation, through activation of the precursor proteins. Moreover, impaired or aberrant processing of the plasma protein precursors, proalbumin, pro-factor IX and fibrinogen, results in disorders, proalbuminemia (*3-5*), hemophilia B (*6, 7*), and mild bleeding tendency (*8*), respectively. Although there are a number of potential candidates, the hepatic convertase involved in the processing of the plasma protein precursors has not yet been fully determined.

Previously, we and other groups identified the Ca^{2+}-dependent endoprotease activity responsible for precursor (*e.g.* proalbumin and viral envelope fusion glycoprotein F0) processing at the dibasic cleavage sites in *trans*-Golgi membrane prepared from rat liver (*9-11*). On the other hand, mutations of proalbumin at the dibasic site, such as −2 Arg→His, and −1 Arg→Gln, cause proalbuminemia (*3-5*), indicating that hepatic proprotein convertase ex-

[1] This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.
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Abbreviations: PMSF, phenylmethylsulfonyl fluoride; SPC, subtilisin-like proprotein convertase.

hibits strict substrate specificity toward the dibasic site in the propeptides. These data suggest that the subtilisin-like proprotein convertase (SPC) family may be involved in this processing, since the enzymatic character and strict sequence requirement of the cleavage site are very similar to those determined biochemically for the putative hepatic proprotein convertase (*9-11*).

SPC family members are Ca^{2+}-dependent serine proteases which contain a highly conserved subtilisin-like catalytic domain, and have been shown to cleave several precursors at dibasic sites to produce bioactive proteins. To date, the known members of this family are furin (also called SPC1), PC2 (SPC2), PC1/3 (SPC3), PACE4 (SPC4), PC4 (SPC5), PC5/6 (SPC6), and PC7/8/LPC (SPC7) [for reviews see (*12-14*)]. The expression of PC2 and PC1/3 is restricted to neuroendocrine and endocrine tissues, such as the central nervous system, pancreatic islets, and thyroid and adrenal glands, suggesting that these enzymes process peptide hormones within a regulated secretory pathway. PC4 is only expressed in the testis. Furin and PC7/8 show ubiquitous expression. On the other hand, PACE4 and PC5/6 exhibit unique restricted expression in both non-endocrine and endocrine tissues. Among the members of this family, furin, PACE4, PC5/6, and PC7/8 are the prime candidates for the hepatic proprotein convertase, since these enzymes are expressed strongly in the liver (*12*) and are active within the constitutive secretory pathway through which the plasma protein precursors are routed. Indeed, furin correctly cleaves proalbumin *in vitro* and in coexpression experiments (*15, 16*). However, a kinetic study involving proalbumin processing revealed that endogenous hepatic convertase and furin have different enzymatic characters (*17*). Moreover, anti-furin antibodies were able to absorb only half of the activity in the hepatic convertase preparation. These data indicated that, in a *trans*-Golgi membrane extract, endoproteases other than furin are also responsible for proalbumin processing. However, the contribution of other members of the SPC family has not yet to be clarified.

PACE4 was first cloned from a cDNA library of human hepatoma HepG2 cells (*18*), and this enzyme has a unique tissue distribution unlike furin. In particular, high level expression of PACE4 mRNA was found in the liver, heart and pituitary (*12*). We previously reported that PACE4 has eight isoforms (A-I, A-II, B, C, CS, D, E-I, and E-II) (*19-21*), that are produced as a consequence of alternative splicing events (*22*), and that they show unique expression patterns (*23-25*). However, the physiological substrate for PACE4 has not yet been fully determined.

In the present study, to evaluate the roles of PACE4 and other SPC family members in the processing of proalbumin, we first examined which of the SPC family members and their isoforms are expressed in HepG2 cells by Northern blot analysis and ribonuclease protection assay, and then examined the processing activities of SPC family members toward proalbumin by means of coexpression experiments. In addition, the effects of the expression of antisense RNAs for SPC family members in this cell line were analyzed. Using these approaches, we show here that PACE4, as well as furin and PC7/8, are involved in the processing of proalbumin *in vivo* and that these enzymes are functionally redundant in this processing.

MATERIALS AND METHODS

Northern Blot Analysis and Ribonuclease Protection Assay—Total RNA from HepG2 cells was isolated using ISOGEN (Nippon Gene, Tokyo) according to the manufacturer's instructions. For Northern blotting, 10 μg of total RNA was resolved by electrophoresis on a 1% agarose gel containing 6.7% formaldehyde and then transferred to a Hybond-N+ nylon membrane (Amersham, England). cDNA probes were labeled with [α-^{32}P]dCTP (Amersham) using a *Bca* BEST™ Labeling Kit (Takara, Kyoto). The sites of the probes were as follows: human furin (nucleotides 1561-2050) (*26*), human PACE4 (nucleotides 1687-2754) (*18*), human PC5/6 (nucleotides 1801-2385) (*27*), and human PC7/8 (nucleotides 1315-1992) (*28*). Hybridization and washing were performed as described previously (*25*). The blots were examined using a BAS-1500 bioimaging analyzer (Fuji Photo Film, Tokyo).

The ribonuclease protection assay was performed using an RPA II™ Kit (Ambion, TX, USA). The cDNA fragment of PACE4 (nucleotides 1687-2754) (*18*) was cloned into pCR™II (Invitrogen, CA, USA) to synthesize an antisense cRNA probe labeled with [α-^{32}P]CTP (Amersham) using T7 RNA polymerase (Gibco BRL, MD, USA). In brief, 7.0×10^5 cpm of probe was hybridized to 20 μg of total RNA at 42°C for 12 h. Unhybridized RNA molecules were then digested with 112.5 units of RNase A and 4,500 units of RNase T1. The protected molecules were resolved by electrophoresis and then visualized by autoradiography.

Coexpression Experiment—The expression plasmids for human PACE4A-II, mouse furin and human PC7/8 were used as described previously (*20, 28*). The expression plasmids for mouse PC5/6A were kindly provided by Drs. K. Nakayama and K. Murakami (University of Tsukuba, Tsukuba). The full-length cDNA for human serum albumin was obtained by RT-PCR using the total RNA from HepG2 cells as a template, and then was cloned into pcDNA3. The plasmids for both enzyme (2.5 μg) and substrate (2.5 μg) were cotransfected into COS-1 cells using DEAE-dextran as described previously (*20*). After transfection for 60 h, the cells were labeled with 200 μCi/ml [^{35}S]methionine and cysteine for 12 h, and then the conditioned medium was mixed with the protease inhibitor mixture (final concentrations, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml pepstatin A) and the immunoprecipitation buffer (final concentrations, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 2 mM EDTA). The sample was pretreated with 20 μl of a 50% suspension of protein A-Sepharose (Pharmacia, Uppsala, Sweden) for 1 h and then centrifuged. Samples were incubated with goat antiserum for human serum albumin (Cappel, NC, USA) on ice. After 1 h, 30 μl of a 50% suspension of protein A-Sepharose was added and the mixture was incubated by rotary mixing at 4°C for 1 h. After centrifugation, the immune complex was washed three times with Buffer A (10 mM Tris-HCl, pH 7.5, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.05% SDS, and 150 mM NaCl), once with Buffer B [10 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40, 150 mM NaCl, and 2 mM EDTA], three times with Buffer B containing 500 mM NaCl instead of 150 mM NaCl, and then twice with Buffer B. The samples were resolved by polyacrylamide gel isoelectric focusing (pH 5-8) (*29*). The

gel was then treated with Amplify (Amersham) and dried. The gel bands exhibiting radioactivity were quantified using a BAS-1500 bioimaging analyzer.

Establishment of a HepG2 Cell Line Expressing Antisense RNA for SPC—The various plasmids used were derived from expression vector pcDNA3 (Invitrogen, CA, USA) as follows. pPACE4fullAS was constructed by inserting the full length human PACE4A-II cDNA in the antisense orientation. pPACE4-5′AS containing the 5′-portion of PACE4 cDNA (nucleotides −27-511, corresponding to the signal peptide and propeptide) (*18*), which shows very low sequence homology with cDNAs for other SPC, was derived from the above vector. pFurinAS and pPC7/8AS contain the 5′-portion of mouse furin (nucleotides −94-370) (*30*) and human PC7/8 (nucleotides −21-488) (*28*) cDNA, respectively, in the antisense orientation. The 5′-portions of the cDNAs, which exhibit no significant homology with each other, was used to avoid cross-hybridization of mRNA within the cells.

These plasmids were transfected into HepG2 cells, respectively, by means of lipofection using *Trans* IT™-LT1 transfection reagent (Mirus, WI, USA) according to the manufacturer's instructions. The transfected cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 0.8 mg/ml G418 for approximately 3 weeks. The colonies were subcloned on 24-well plates in duplicate; one was used for confirmation of the antisense RNA expression by reverse transcriptase-polymerase chain reaction analysis. The established cell lines were maintained in the presence of 0.4 mg/ml G418.

To analyze the processing of proalbumin, each HepG2 cell line (1.0×10^6 cells) in a 35-mm dish was labeled with 150 μCi/ml [^{35}S]methionine and cysteine in the minimum essential medium for 6 h, and then the conditioned medium was subjected to immunoprecipitation as described above.

Western Blot Analysis—Each HepG2 cell line (1.0×10^6 cells) in a 35-mm dish was cultured in 0.6 ml of OPTI-MEM I (Gibco BRL) for 24 h. The conditioned medium was mixed with trichloroacetic acid at the final concentration of 10%, incubated for 10 min on ice and then centrifuged. The precipitated protein was subjected to standard Western blotting with an anti-PACE4A monoclonal antibody, A121G09 (*31*). The PACE4A band was visualized using SuperSignal Substrate, Western Blotting (PIERCE, IL, USA), and Konica X-ray film. The PACE4A band was quantified using MacBAS software (Fuji Photo Film).

RESULTS

Expression of mRNAs for SPC Family Members in HepG2 Cells—The processing of proalbumin was analyzed in HepG2 cells. To determine the endoprotease responsible for proalbumin processing in HepG2 cells, Northern blot analysis of the SPC family members was performed. As shown in Fig. 1, furin (4.4 kb; lane 2) mRNA was strongly expressed in HepG2 cells. PACE4 (4.4 kb; lane 1) and PC7/8 (4.4 kb; lane 3) mRNAs were also expressed moderately, whereas PC5/6 mRNA expression was undetectable (lane 4), indicating that PACE4, furin and PC7/8 are candidates for the hepatic proprotein convertase in HepG2 cells. These three convertases are actually expressed in the liver, however, PC5/6 is also expressed (*12*).

Previously, we reported that PACE4 has eight isoforms (A-I, A-II, B, C, CS, D, E-I, and E-II), all of which are products of alternative splicing of the primary transcript of a single gene (*19-22*). A ribonuclease protection assay was performed to determine the PACE4 isoform expressed in HepG2 cells. PACE4 isoforms have different mRNA 3′-regions. Type-I PACE4A and PACE4E mRNAs contain exon 18 (39 bp), while type-II mRNAs do not. In this experiment, although only one cRNA probe was used, the alternatively spliced isoforms gave respective specific gel bands. PACE4A-II mRNA (677 bp) was detected most abundantly, as shown in Fig. 2. PACE4A-I mRNA (1,068 bp) was also detected at a moderate level, and PACE4E-II mRNA (624 bp), and PACE4C, CS and D mRNA (175 bp) weakly. On the other hand, no PACE4E-I mRNA (1,015 bp) was detected. These data indicated that PACE4A-II mRNA is the major transcript among these isoforms in HepG2 cells.

Processing Activities of SPC Family Members toward Proalbumin—Although furin was able to process proalbumin to albumin (*15, 16*), the activities of PACE4, PC7/8, and PC5/6 toward proalbumin remain unknown. The proalbumin processing activities of PACE4A-II, furin, PC7/8, and PC5/6 in COS-1 cells were examined by means of a coexpression experiment. As shown in Fig. 3, when proalbumin alone was expressed in these cells, it was poorly processed to the mature form (lane 1). However, proalbumin was further processed to albumin on coexpression with PACE4A-II (39%, lane 2). Furin and PC5/6 were also able to process it more efficiently (59 and 57%, lanes 3 and 5, respectively). PC7/8 also processed proalbumin (41%, lane 4). These data indicated that PACE4A-II, PC7/8, and PC5/6, as well as furin, possess processing activity toward proalbumin.

Effect of Expression of Antisense RNAs for SPC on the Processing of Proalbumin—The physiological function of SPC family members was analyzed by an antisense RNA approach. The 5′-portion of each cDNA, which exhibits no significant homology with the cDNAs for other SPC family members, was used for expression to avoid cross-hybridization with other endogenous mRNAs. The expression of antisense RNA was confirmed by reverse transcriptase-polymerase chain reaction analysis using specific primers (data not shown). As a result, 3 clones of PACE4fullAS, 10 of PACE4AS, 12 of FurinAS, and 11 of PC7/8AS were obtained. These clones show no differences (*e.g.* protein synthesis and secretion, and growth rate) compared with wild type and control HepG2 cells (data not shown). As

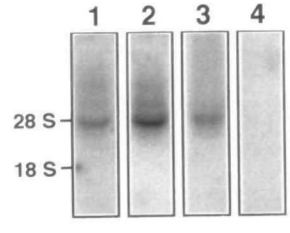

Fig. 1. **Northern blot analysis of the SPC expression in HepG2 cells.** Ten micrograms of total RNA from HepG2 cells was analyzed by Northern blotting as described under "MATERIALS AND METHODS." The blots were hybridized with approximate cDNA probes, respectively, as follows: PACE4 (lane 1), furin (lane 2), PC7/8 (lane 3), and PC5/6 (lane 4).

shown in Fig. 4, the effect of expression of antisense RNA on SPC synthesis was examined by Western blot analysis. When the antisense RNA for PACE4 was stably expressed, the secretion of PACE4A (95 kDa) was reduced by approximately 50% (lanes 2, 3, and 4) compared with in control cells (lane 1). However, PACE4A secretion was not affected by expression of the antisense RNAs for furin and PC7/8 (lanes 5 and 6). These data suggested that the expression of an antisense RNA specifically inhibits the synthesis of the corresponding SPC. The effect of expression of antisense RNAs for other SPC family members was confirmed by Northern blot analysis. When the antisense RNAs for PACE4, furin and PC7/8 were stably expressed, respectively, the amounts of the corresponding mRNAs specifically decreased (PACE4AS-43, 69.2%; PACE4AS-46, 59.0%; FurinAS-3, 66.5%; PC7/8AS-17, 56.7%), whereas the amounts of mRNAs for other SPC family members were not affected by antisense RNA expression in these cell lines (Fig. 5). These data suggested that the syntheses of furin and PC7/8, as well as PACE4, were also specifically suppressed by expression of the corresponding antisense RNAs.

The effect of expression of antisense RNAs for SPC on the processing of endogenous proalbumin was analyzed (Fig. 6). Both the wild type (lane 1) and control (transfected with the empty vector; lane 3) HepG2 cells secreted fully processed albumin (93%) with a low amount of proalbumin (7%), and this processing was blocked completely in the presence of 0.25 μM A23187, a calcium-specific ionophore (lane 2), indicating the involvement of a Ca^{2+}-dependent protease as reported previously by Oda (*32*). However, expression of the antisense RNAs for PACE4A-II, furin and PC7/8 caused a reduction of proalbumin processing, as

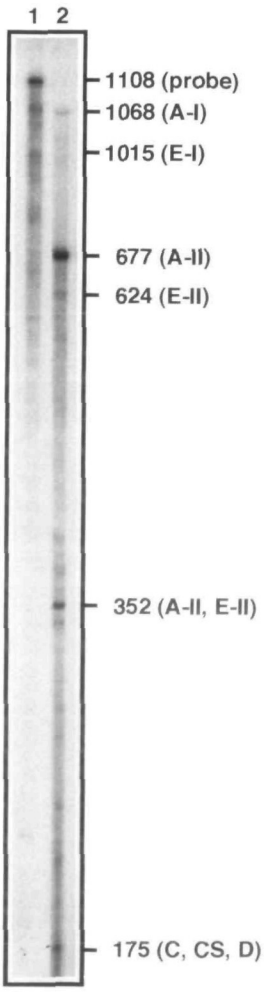

Fig. 2. **Ribonuclease protection assay of the PACE4 isoform expression in HepG2 cells.** Twenty micrograms of total RNA from HepG2 cells was analyzed by ribonuclease protection assay as described under "MATERIALS AND METHODS." The cRNA probe (lane 1) and products (lane 2) were resolved on a 7 M urea, 4% polyacrylamide denaturing gel, followed by autoradiography. The sizes (in base) of the gel bands are indicated on the right.

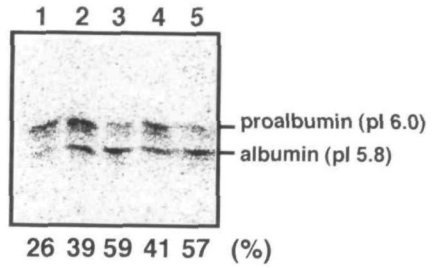

Fig. 3. **Analysis of the processing activity of SPC toward proalbumin.** COS-1 cells were transfected with the expression plasmids for proalbumin in either the absence (lane 1) or presence of the expression plasmids for PACE4A-II (lane 2), furin (lane 3), PC7/8 (lane 4), and PC5/6 (lane 5). After transfection for 60 h, the cells were radio-labeled for 12 h, and then the resulting culture medium was immunoprecipitated as described under "MATERIALS AND METHODS." The immunoprecipitants were resolved by isoelectric focusing. The processing efficiency is indicated below each panel. The positions of the precursor (pro-) and mature forms of the substrate are indicated on the right. Two independent experiments yielded similar results.

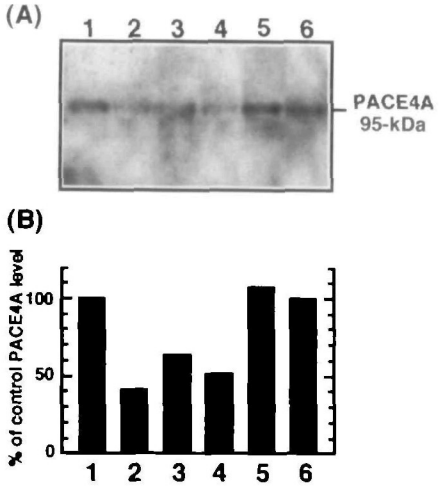

Fig. 4. **Inhibition of PACE4A synthesis by antisense RNA.** (A) PACE4A protein secreted into the culture medium by each cell line was analyzed by Western blotting as described under "MATERIALS AND METHODS." (B) The density of the PACE4A band (95 kDa) was quantified. The data are expressed as percentages of the PACE4A protein level in the control (lane 1 in panel A). Lane 1, control (transfected with the empty vector); lane 2, PACE4fullAS-30; lane 3, PACE4AS-43; lane 4, PACE4AS-46; lane 5, FurinAS-3; lane 6, PC7/8AS-17.

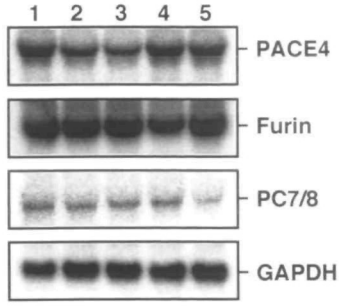

Fig. 5. **Decrease in SPC mRNA levels caused by antisense RNA in transfected HepG2 cells.** Ten micrograms of total RNA from transfected HepG2 cells was analyzed by Northern blotting as described under "MATERIALS AND METHODS." The bands exhibiting radioactivity were quantified using a BAS-1500 bioimaging analyzer. The cDNA probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control. Lane 1, control; lane 2, PACE4AS-43; lane 3, PACE4AS-46; lane 4, FurinAS-3; lane 5, PC7/8AS-17.

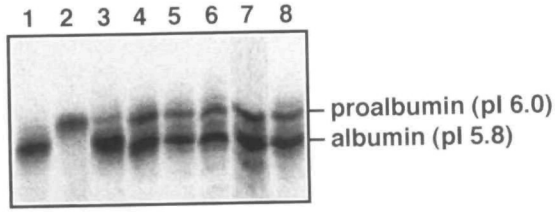

Fig. 6. **Effect of antisense SPC RNA expression on the processing of proalbumin in transfected HepG2 cells.** Each cell line was radio-labeled for 6 h, then the resulting culture medium was immunoprecipitated as described under "MATERIALS AND METHODS." The immunoprecipitants were resolved by isoelectric focusing. The gel was exposed to an imaging plate for 12 h. Lane 1, wild type; lane 2, wild type in the presence of 0.25 μM A23187; lane 3, control (transfected with the empty vector); lane 4, PACE4fullAS-30; lane 5, PACE4AS-43; lane 6, PACE4A-46; lane 7, FurinAS-3; lane 8, PC7/8AS-17. The percentages of the precursor secreted by each cell line are summarized in Table I.

shown in Table I. In clone PACE4fullAS-30, significant inhibition of proalbumin processing was observed (31.6$\pm$1.1%, lane 4), compared with in wild type and control cells (lanes 1 and 3). Similarly, this processing was also inhibited by the expression of the antisense RNA for the 5′-portion of PACE4 cDNA in clones PACE4AS-43 and PACE4AS-46 (28.2$\pm$1.6 and 27.0$\pm$2.9%, lanes 5 and 6, respectively). These data and the results of the coexpression experiments indicated that proalbumin is a physiological substrate of PACE4. The expression of antisense RNA for furin resulted in 31.5$\pm$1.7% inhibition, as shown by clone FurinAS-3 (lane 7). When the antisense RNA for PC7/8 (clone PC7/8AS-17) was expressed, 30.5$\pm$3.2% of the proalbumin processing was inhibited (lane 8). The radioactivity of total albumin (pro+mature) secreted was nearly equal in these clones (Table I). Thus, the proalbumin processing was inhibited by antisense RNA expression for the three SPC family members to similar extents. These results suggest that not only furin but also PACE4 and PC7/8 are responsible for the proalbumin processing in HepG2 cells.

TABLE I. **Effect of expression of antisense RNAs for subtilisin-like proprotein convertases on the processing of proalbumin.** Each value indicates the mean$\pm$SD obtained for three independent experiments.

Cell line	Proalbumin (PSL)	Albumin (PSL)	% of proalbumin
Wild type (−)[a]	219$\pm$33	2,747$\pm$45	7.4
Wild type (+)	2,768$\pm$37	N.D.	100
Control[b]	281$\pm$43	3,671$\pm$58	7.1
PACE4fullAS-30	1,279$\pm$45	2,769$\pm$61	31.6
PACE4AS-43	1,003$\pm$57	2,555$\pm$78	28.2
PACE4AS-46	974$\pm$104	2,633$\pm$142	27.0
FurinAS-3	1,193$\pm$64	2,593$\pm$83	31.5
PC7/8AS-17	1,127$\pm$124	2,568$\pm$163	30.5

[a]In the absence (−) and presence (+) of 0.25 μM A23187. [b]The control cell line was transfected with the empty expression vector. N.D., not determined because it was below the detection limit; PSL, photo-stimulated luminescence.

DISCUSSION

Although we and other groups have tried to purify the hepatic proprotein convertase responsible for proalbumin processing, no homogeneous protease has been isolated yet. However, these studies showed that hepatic proalbumin convertase is a Ca^{2+}-dependent serine protease which resembles SPC family members. Coexpression experiments of protease with substrate proteins have been frequently performed to analyze the function of SPC family members. However, overexpression of these convertases does not conclusively demonstrate their true physiological function, since the ratio of convertase to substrate protein varies considerably in nature and, in particular, PACE4 seems to be activated inefficiently in COS-1 cells compared with furin. On the other hand, the antisense approach was effective in demonstrating the crucial roles of the SPC family in proprotein processing *in vivo*. This approach was first used to elucidate the role of PC1/3 in pro-opiomelanocortin (POMC) processing in AtT-20 cells (*33*). In this case, the expression of the antisense RNA for PC1/3 led to a dramatic and specific decrease in the PC1/3 mRNA level that was correlated with significant inhibition of the processing of endogenous POMC. Subsequently, this expression has been used to demonstrate the role of PC2 in the processing of pro-enkephalin (*34*), POMC (*35*), proglucagon (*36*), and pro-neurotensin (*37*), and the role of PC1/3 in the processing of pro-chromogranin A (*38*) and pro-cholecystokinin (*39*). Although the coexpression study showed that furin was able to convert proalbumin to albumin correctly, there is no direct evidence that this enzyme is the endogenous hepatic proalbumin convertase. Therefore, we used this approach to directly identify the endogenous SPC family member involved in the proalbumin processing in HepG2 cells. The expression of the antisense RNAs for furin, PACE4 and PC7/8 resulted in approximately 30% inhibition of the endogenous proalbumin processing, respectively. Moreover, these enzymes possess processing activity toward proalbumin, as shown by the coexpression experiments. These data indicated strongly and directly that PACE4 and PC7/8, as well as furin, are the endogenous proalbumin convertases in HepG2 cells.

So far only furin has been thought to be responsible for

proalbumin processing. However, Ledgerwood *et al.* showed recently that normal proalbumin is a relatively poor substrate for this enzyme compared with a mutant proalbumin, and that natural −4 Val is replaced by Arg (*17*). Moreover, in the kinetic study, whereas hepatic convertase and furin exhibited similar K_m values with normal proalbumin, a significant difference of the K_m values with mutant proalbumin between them was found. Moreover, anti-furin antibodies could not completely absorb the proalbumin processing activity in the hepatic convertase preparations. These data suggest that endoprotease activity other than that of furin is also involved in the proalbumin processing in the liver, which is in accordance with our finding that there is a redundant mechanism in proalbumin processing in which three endogenous enzymes (PACE4A-II, furin, and PC7/8) are involved. In our study, the expression of the antisense RNA for PACE4 caused significant inhibition (approximately 30%) of the proalbumin processing and this enzyme was able to process proalbumin in the coexpression experiment, indicating strongly that PACE4 is also involved in this processing.

The proteolytic processing of proalbumin occurs in either the *trans*-Golgi compartment or secretory vesicles (*40*), where the hepatic convertase activity was biochemically identified, but not in the endoplasmic reticulum fraction (*9–11*). Overexpression experiments using mammalian cells have shown that furin and PC7/8 are localized in the *trans*-Golgi compartment (*16, 41*), suggesting that these enzymes probably process proalbumin in this compartment. The intracellular localization of PACE4 has not been determined. However, endogenous PACE4A (95-kDa) was detected in the *trans*-Golgi membrane fraction of rat liver on Western blot analysis using a monoclonal antibody (data not shown). It is therefore considered that proalbumin processing by PACE4 may occur in the *trans*-Golgi network and/or secretory vesicles.

The propeptide sequence of proalbumin is Arg-Gly-Val-Phe-Arg-Arg, and this sequence matches the recognition sequence of PC7/8 (Arg-X-X-X-Lys/Arg-Arg) completely (*41*). Although the recognition sequence of furin (Arg-X-Lys/Arg-Arg) does not match (*42*), this convertase is able to correctly convert proalbumin to albumin. The recognition sequence of PACE4 is Arg-X-Lys/Arg-Arg (*43, 44*). However, PACE4A-II is able to process proalbumin at the dibasic site, whereas the P4 position of proalbumin does not comprise basic amino acid residues. These data are good agreement with the fact that immunopurified recombinant PACE4 exhibits specificity for the dibasic site in several synthetic peptides (Tsuji, A., unpublished results).

The many plasma proteins which require proteolytic activation at the paired or multiple basic amino acid residues include components of the complement system (*e.g.* C3, C4, and C5) and blood clotting system (*e.g.* thrombin and factor IX). Since impaired processing of these precursors causes serious disorders, such as hemophilia B (*6, 7*) occasionally, the processing of plasma protein precursors may be compensated by a redundant mechanism which involves more than one SPC, as shown in this study.

In conclusion, we demonstrated directly that among the members of the SPC family, not only furin but also PACE4 and PC7/8 are endogenous convertases responsible for proalbumin processing in HepG2 cells. The fact that at least three enzymes are involved might reflect some redundancy to ensure the production of a large amount of the plasma protein. In the liver, PC5/6, in addition to these three enzymes, is also expressed, therefore, this enzyme is still a candidate endogenous convertase involved in proalbumin processing.

We are grateful to Drs. K. Murakami and K. Nakayama for the gift of the mouse furin cDNA. We also wish to thank Mrs. Yuka Sasaki for her secretarial assistance, and Miss Yuki Kiyosue and Miss Yukari Kujime for their technical assistance.

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