Inactivation of Proprotein Convertase, PACE4, by α1-Antitrypsin Portland (α1-PDX), a Blocker of Proteolytic Activation of Bone Morphogenetic Protein during Embryogenesis: Evidence That PACE4 Is Able to Form an SDS-Stable Acyl Intermediate with α1-PDX[1]

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PACE4 (SPC4), a member of the subtilisin-like proprotein convertase (SPC) family of proteases that cleave at paired basic amino acids, exhibits a dynamic expression pattern during embryogenesis and colocalizes with bone morphogenetic proteins (BMPs). Recently Cui *et al.* reported that the ectopic expression of α1-antitrypsin variant Portland (α1-PDX), an engineered serpin that contains the minimal SPC consensus motif in its reactive loop, blocks the proteolytic activation of BMP4, leading to abnormal embryogenic development [Cui, Y. *et al.* (1998) *EMBO J.* 17, 4735-4743]. TGFβ-related factors such as BMPs are synthesized as inactive precursors and activated by limited proteolysis at multibasic amino acids. Therefore, an α1-PDX-inhibitable protease is thought to participate in BMP activation. However, conflicting properties, including sensitivity to α1-PDX, have been reported for PACE4. In this study, we examined whether α1-PDX is responsible for the inhibition of PACE4 by measuring the protease/inhibitor complex directly. Here we show that α1-PDX has the ability to form an SDS-stable acyl-intermediate (180 kDa) with PACE4 *in vivo* and *in vitro*. Further, we characterized the PACE4 secreted into the culture medium from Cos-1 cells by a specific immunological assay. An α1-PDX-insensitive and decanoyl-RVKR-chloromethylketone-sensitive 60-kDa protease(s) is greatly activated in conditioned medium by PACE4 overexpression, suggesting that the activation of an unknown protease(s) other than PACE4 is the cause of the variation in the properties of PACE4. PACE4 is a Ca^{2+}-dependent protease with an optimal Ca^{2+} requirement of 2 mM, and shows its highest activity at weakly basic pH. PACE4 activity is completely inhibited by EDTA and EGTA, but not by leupeptin. These results show that PACE4 activity can be inhibited by α1-PDX as well as furin (SPC1) and suggest that the inhibition of PACE4-mediated activation of factors such as BMPs by α1-PDX causes abnormal embryogenic development.

Key words: α1-antitrypsin, BMP processing, PACE4, α1-PDX, SPC.

PACE4 (SPC4) is one of the subtilisin-like proprotein convertases (SPCs) involved in the proteolytic processing of proproteins, propeptide hormones, and proneuropeptides at paired and single basic amino acid sites (*1, 2*). This post-translational processing is a common mechanism in the production of biologically active peptides and proteins. In mammals, seven members of this SPC family have been identified to date by cDNA cloning based on PCR strategy. These include furin (also known as SPC1, PACE) (*3*), PC2 (also known as SPC2) (*4*), PC1/3 (also known as SPC3) (*5*), PACE4 (also known as SPC4) (*6*), PC4 (also known as SPC5) (*7*), PC5/6 (also known as SPC6) (*8, 9*), and PC8 (also known as LPC, PC7, SPC7) (*10-13*). All these SPCs are Ca^{2+}-dependent serine proteases and share such structural similarities as a signal peptide, propeptide, subtilisin-like catalytic domain, and homoB domain in their basic domain structure. However, they differ in their tissue distribution profiles, especially PACE4, which exhibits a unique restricted distribution in both endocrine and nonen-

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Abbreviations: SPC, subtilisin-like proprotein convertase; SCD, subtilisin-like catalytic domain; BMP, bone morphogenetic protein; TGF, transforming growth factor; *pyr*, L-pyroglutamyl; Boc, *t*-butyloxycarbonyl; *Suc*, succinyl; MCA, 4-methylcoumaryl 7-amide; DFP, diisopropyl fluorophosphate; PMSF, phenylmethanesulfonyl fluoride; PCMB, *p*-chloromercuribenzoate; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; α1-AT, α1-antitrypsin; α1PIT, α1-antitrypsin Pittsburgh; α1-PDX, α1-antytrypsin Portland; Dec-RVKR-CMK, decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone; PBS, phosphate buffered saline; vWF, von Willebrand factor; FCS, fetal calf serum.

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docrine cells. PACE4 is expressed at high levels in the anterior pituitary (*14*), B cells of pancreatic islets (*15*), heart (*16*), and liver (*6*). Recently we showed that PACE4 is highly expressed in the mitral cells of the rat olfactory bulb, where it is involved in its developmental regulation (*17*). Furin and PC8, which have transmembrane domains in their carboxy terminal regions, exhibit ubiquitous tissue distribution (*2, 10-13*). In contrast, PC1/3 and PC2 are expressed mainly in endocrine cells such as pituitary (*18*), thyroid (*19*), pancreatic islets (*15*), and adrenal gland (*2*). PC4 is restricted to spermatogenic cells (*20*). PC5/6, like PACE4, has been detected in both endocrine and nonendocrine cells, however, their distribution profiles are clearly distinct (*21*). In embryonic development, the expressions of PACE4 and PC5/6 are highly regulated, whereas furin and PC8 (SPC7) are expressed ubiquitously (*13*). In fact, PACE4 displays dynamic expression patterns throughout development and is upregulated in some tissues that exhibit high level expressions of bone morphogenetic proteins (BMPs) (*13*). PACE4 is coexpressed with BMP2, 4, and 7 in the primitive heart, in the apical ectodermal ridge of developing limb buds, and in the interdigital mesenchyme of embryogenic limbs. During neural tube patterning, PACE4 is coexpressed with BMP6 in the floor plate. Like peptide hormones, PACE4 and furin can acivate TGFβ-related differentiation factors such as BMPs and Nodal by proteolytic cleavage at paired basic amino acids (*22*). These observations suggest that PACE4 is a key enzyme regulating the activities of various differentiation factors such as BMPs by proteolytic activation. Recently, the proteolytic activation of BMP4 in *Xenopus* oocytes was shown to be blocked by the ectopic expression of an engineered α1-antitrypsin variant Portland (α1-PDX), leading to abnormal embryonic development (*23*). α1-Antitrypsin (α1-AT) is a major serine protease inhibitor in plasma and its primary function is to control the activity of neutrophil elastase (*24*). The sequence of the reactive site of human α1-antitrypsin is Ala-Ile-Pro-Met, but its enzyme specificity is known to be changed by engineered mutations at the reactive site. α1-Antitrypsin Pittsburgh (α1-PIT) contains, Ala-Ile-Pro-Arg in its active site and changes its specificity from an inhibitor of elastase to a potent inhibitor of thrombin (*25*). α1-Antitrypsin Portland (α1-PDX) contains Arg-Ile-Pro-Arg in its reactive site, which is the minimal consensus motif (RXXR) for efficient cleavage by furin. The interaction of α1-antitrypsin with serine protease occurs through strong interactions presumably caused by proteolytic attack on the inhibitor, the latter acting initially as a substrate for the enzyme. This inhibitor forms a 1:1 molar complex (acyl-intermediate) with serine protease that is stable on boiling in 1% sodium dodecyl sulfate. The cleavage of the inhibitor, the complex formation, and the loss of protease activity are tightly coupled. Therefore the formation of an SDS-stable protease/α1-AT complex is evidence that α1-AT is a potent inhibitor of this protease. α1-PDX is known to be a potent inhibitor of furin (*26*), however the specificity of this variant toward other SPCs such as PACE4 has not been clarified. It is important to evaluate the ability of α1-PDX to inhibit PACE4 activity in order to understand the mechanism of abnormal embryogenic development caused by α1-PDX.

Among SPC family members, furin, PC2, and PC1/3 have been purified to a homogeneous state from overexpressed culture cells (*27-29*) or native endocrine tissue (*30*), and their enzyme activities characterized. Individual SPC family members exhibit distinct but overlapping substrate specificities. As a general rule, these SPCs hydrolyze peptide bonds adjacent and distal to clusters of multiple basic residues. PACE4 has not been purifed to date. Therefore its cleavage preference has been characterized following the coexpression of PACE4 and proprotein as a potential substrate in various cultured cells. By this method, PACE4 has been shown to be able to convert various proproteins, such as pro-von Willebrand factor (cleavage site: RSKR↓) (*31*), pro-complement C3 (RRRR) (*31*), pro-renin mutant (RTKR) (*31*), HIV gp-160 (REKR) (*32*), prosomatostatin (RLELQR) (*33*), somatostatin-28 (RERK) (*33*), pro-nerve growth factor (RSKR), (*34*), and wild type (RKKR) and mutants (RAAR, FTKR, STRR) anthrax toxin protective antigen (*35*) to bioactive mature forms. More recently, coexpression studie have shown precursors of BMP4 (RAKR) and Nodal (RQRR), TGFβ-related growth factors, to be processed to active mature forms by PACE4 or furin (*22*). On the other hand, Inocencio *et al.* reported that PACE4 participates in the activation of E-64-sensitive protease, which has a substrate specificity similar to that of SPC, in CHO-K1 cells (*36*). Hubbart *et al.* showed that PACE4-transfected cells acquired the ability to process prostromelysin-3 to its active form (*37*). The conditioned medium of cultured cells that overexpress SPC is often used as the enzyme source for characterization because SPC, which does not have a membrane-binding domain, is secreted into the medium. In the case of PACE4, care must be taken in the interpretation of these data because the activity of other proteases might be elevated by the artificial overexpression of PACE4. Previously Benjanet *et al.* showed through coexpression experiments that α1-PDX is a general inhibitor of SPC family members (*38*). Mains *et al.* (*39*) and Jean *et al.* (*26*) analyzed the specificity of α1-PDX using conditioned medium from cells expressing SPC as an enzyme source, and reported that α1-PDX is selective for furin and, to a lesser extent, PC6B, but not PACE4. However, they did not prove that the elevated activity level in the conditioned medium is derived from PACE4, although some protease might be activated in cultured cells by PACE4 expression, as mentioned above. Therefore it is difficult to evaluate the interaction of α1-PDX with PACE4 based on the inhibition of activity toward synthetic substrates using medium from PACE4-expressing cells as an enzyme source.

In this study, we analyzed directly the formation of the acyl-intermediate of α1-PDX with PACE4 by coexpression and *in vitro* studies. Furthermore the enzymatic properties of PACE4, including the inhibitor profile and substrate specificity, were also determined using immunopurified PACE4. Our results demonstrate that α1-PDX has the ability to form an acyl-intermediate with PACE4 as well as furin, suggesting the involvement of PACE4 in BMP4 activation during embryonic development.

MATERIALS AND METHODS

Materials—Peptidyl-MCAs, leupeptin, E-64, pepstatin, and chymostatin were purchased from Peptide Institute (Osaka). TLCK and TPCK were from Boehringer-Mannheim (Mannheim, Germany). Polystyrene beads (3.2-mm

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diameter) were from Immunochemical (Okayama). Protease-free BSA and human α1-antitrypsin were from Sigma (St Louis, MO, USA). the chemiluminescence detection kit (Super Signal Substrate Western Blotting) was obtained from Pierce (Rockford, IL, USA). Rabbit anti-human α1-antitrypsin antiserum was from ZYMED (San Francisco, CA, USA). Purified kexstatin and soluble mouse furin were a gift from Drs. K. Oda and Y. Shibano (Kyoto Institute of Technology, Kyoto), and Dr. K. Nakayama (Tsukuba University, Tsukuba). Easy Tag™ Express Protein Labeling Mix [^{35}S] was from NEN Life Science, (Boston, MA. USA). The other reagents used were of the highest grade available.

Preparation of Antibodies—The polyclonal anti-PACE4 catalytic domain (Cys^{263}-Leu^{448}) antiserum (anti-SCD) was prepared as described previously (*31*), and the antibody was purified by the standard method including sodium sulfate fractionation, DEAE-cellulose chromatography, and gel filtration on Ultrogel AcA-34. Monoclonal antibody (anti-CT) against the carboxyl region (Thr^{920}-Asp^{939}) of PACE4A-I was purified by sodium sulfate fractionation and protein A-Sepharose chromatography (*40*). The polyclonal anti-PACE4 HomoB domain (Ala^{456}-Asn^{688}) antiserum (anti-HomoB) was prepared as described previously (*40*).

Construction of Expression Plasmids and Coexpression Experiments—The wild type and active site-mutant (S/A420, active serine^{420} converted to alanine) of human PACE4 (A-I isoform) cDNAs were subcloned into the mammalian expression vector, pALTER-MAX (Promega, Madison, WI, USA) as described previously (*40*). The rat α1-antitrypsin and its variant (Pittsburgh, M^{352}/R) cDNAs, mouse truncated soluble furin cDNA (Δ704), and pro-von Willebrand factor cDNA were kindly provided by Dr. Y. Ikehara (Fukuoka University, Fukuoka), Dr. K. Nakayama (Tsukuba University, Tsukuba), and Dr. J.A. van Mourik (Central Laboratory of the Nertherlands Red Cross, Nertherlands), respectively. The rat α1-antitrypsin Portland (α1-PDX, AVPM^{352}/RVPR) expression vector was constructed by two steps of polymerase chain reaction (PCR) as follows. The 3′-terminal cDNA fragment containing the mutated reactive site was generated by PCR (30 cycles, 95°C 1 min, 55°C 1 min, 72°C 2 min) using *Pst*I-linked sense mutagenic primer [5′-GCTGCAGGAGCCACTG-TGGTGGAG**CG**CGTCCCC**AGG**-3′ coding for Arg (bold) at positions 349 and 352] and *Eco*RI-linked antisense primer (5′-TTGAATTCGTGATTAACGTGTGGGATCTATC, stop codon underlined). Wild type α1-antitrypsin cDNA was used as the template. The amplified fragment (155 bp) was purified by agarose gel electrophoresis and used as an antisense primer for a second PCR. The full length α1-PDX cDNA was generated by the second PCR using *Bam*HI-linked sense primer (5′-GGATCCTGAAAATGGCACCCT-CCATCTCACGG-3′, initial codon underlined) and the first PCR product as the antisense primer. The amplified fragment (1.2 kbp) was purified by agarose gel electrophoresis and subcloned into the *Bam*HI and *Eco*RI sites of pcDNA3 vector (Invitrogen, Netherland). The full sequence of the insert was confirmed with an automated ALF DNA sequencer (Pharmacia, Sweden).

Transfection and immunoprecipitation were performed as described previously (*31*). Monkey kidney Cos-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics and 10% fetal calf serum under 5% CO_2. The expression plasmids for both PACE4A (2.5 μg) and α1-antitrypsin variant (2.5 μg) were cotransfected into Cos-1 cells in a 35 mm dish using DEAE-dextran. After transfection for 60 h, the cells were labeled with 100 μCi/ml [^{35}S]methionine and [^{35}S]cysteine for 8 h. Then the conditioned medium and cell lysate were immunoprecipitated with anti-PACE4 SCD, anti-PACE4 HomoB, or anti-α1AT antibodies.

Preparation of ^{35}S-Radiolabeled PACE4, Furin, and α1-Antitrypsin Variants—For binding experiments of PACE4 with α1-antitrypsin variants, ^{35}S-labeled human PACE4, soluble furin, rat native α1-antitrypsin, α1-PIT, and α1-PDX were prepared using Cos-1 cells transiently transfected with these cDNAs as described previously (*31, 40*). These constructs were transfected into Cos-1 cells using DEAE-dextran, and the cells were grown in 100-mm dishes and labeled with [^{35}S]methionine and cysteine (100 μCi/ml) after transfection for 60 h. After 8 h, the conditioned medium was collected, centrifuged to remove insoluble materials, and used for binding experiments. Conditioned media (0.5 ml) containing PACE4A and the α1-antitrypsin variant were coincubated at 37°C for 2 h, and then the complex of PACE4 with inhibitor was immunoprecipitated with anti-HomoB or anti-human α1AT antibodies as described previously (*40*).

Western Blots—Media and cell extracts from Cos-1 cells were subjected to 7.5% SDS-PAGE (*41*) and the electrophoresed proteins were transferred onto a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk in 20 mM Tris-HCl buffer, pH 7.4, containing 0.5 M NaCl, and then incubated with a anti-HomoB or anti-CT antibody (5 μg/ml), followed by a peroxidase-linked goat anti-rabbit IgG second antibody (1:5,000 final dilution). PACE4 was detected using a chemiluminescence kit and Konica X-ray film.

Expression and Purification of Recombinant α1-Antitrypsin Variants—The cDNAs coding for rat α1-antitrypsin variants devoid of signal peptides were generated by PCR using *Bam*HI-linked sense primer (5′-CTAGAGGAT-CCGAGGATGCCCAGGAAACCGATACC-3′) and *Hin*dIII-linked antisense primer (5′-TATAAGCTTTTAA-CGTGTGGGATCTATCAC-3′). The pcDNA3 expression vectors mentioned above were used as a template. The amplified cDNA fragments were subcloned into the *Bam*HI and *Hin*dIII sites of pQE30 vector (Quiagen, Germany). The full sequence of the insert was confirmed. The purification of α1-antitrypsin variant from *Escherichia coli* was carried out by the method of Jean *et al.* (*26*) with slight modification as follows. These variants were expressed in *E. coli* strain BL-21 transformed with expression vectors. Protein expression was induced by the addition of 1 mM isopropyl β-D-thiogalactoside, and the cultures were grown at 30°C for 6 h. The cells from 1 liter of culture were collected by centrifugation, and homogenized with 60 ml of 20 mM Tris-HCl, pH 7.85, containing 0.5 M NaCl and 5 mM imidazole. The cells were disrupted with a French pressure cell press (1,300 psi, SLM Instruments, USA) and diluted 3-fold with the same buffer. After centrifugation (27,000 $\times g$ for 15 min), the supernatants were applied to a Ni^{2+}-NTA agarose column (1.5 $\times$ 3.0 cm, Quiagen, Germany) equilibrated with the homogenization buffer. The column was washed extensively with the same buffer and eluted with 20, 50, and 100 mM imidazole in the same

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buffer. α1-Antitrypsin variants were eluted in the 50 mM imidazole fraction. The fractions with high specific activity were kept and concentrated (2 mg/ml) by ultrafiltration. The final preparation of these variants yielded a single band (45 kDa) on SDS-PAGE and this band cross-reacted with anti-human α1-antitrypsin antibody on Western blots. In addition, we confirmed that rat α1-antitrypsin Pittsburgh (α1-PIT) and Portland (α1-PDX) inhibits thrombin and furin, respectively, like human α1-antitrypsin variants.

Preparation of Conditioned Medium for the Immunopurification of PACE4—The expression plasmids for the wild type or active site mutant (S/A420) were transfected into Cos-1 cells (30 μg/100-mm dish) using DEAE-dextran as described previously (*31*). The transfected cells were incubated in DMEM supplemented with 10% fetal calf serum. After transfection for 48 h, the cells in 100-mm dishes were rinsed twice with PBS and incubated in 4 ml of serum-free opti-MEM (Gibco-BRL, Rockville, MD, USA). After 17 h, the conditioned medium (80 ml) was collected and centrifuged for 10 min at 3,000 rpm to remove floating cells. The supernatant was concentrated (2.0 ml) by ultrafiltratiom using an Amicon YM-10 filter.

Enzyme Assays—The protease activity was assayed with *pyr*-RTKR-MCA unless otherwise stated. The reaction mixture (total volume 0.2 ml) contained 0.1 M Tris-HCl buffer, pH 7.5, 2 mM $CaCl_2$, and 50 μM *pyr*-RTKR-MCA. The reaction was initiated by adding enzyme solution or 5–10 immunobeads. After incubation at 37°C for 6–18 h, the reaction was terminated by the addition of 1 ml of 0.2 N acetic acid, and the MCA liberated was determined fluometrically with a Hitachi F-2000 spectrofluorometer (380 nm excitation, 460 nm emission). The protein concentration was determined by the method of Bradford using a Bio-Rad protein assay reagent with BSA as a standard (*42*).

Preparation of Immunobeads and Immunopurification of PACE4—Purified antibodies were immobilized with polystyrene beads by incubation with 0.1 mg/ml antibody in 0.1 M sodium phosphate buffer, pH 7.5, 0.1% NaN_3 at 4°C. After 16 h, the beads were washed 5 times with ice-cold 0.1 M sodium phosphate buffer, pH 7.0, containing 0.1% protease-free BSA, 0.1 M NaCl, 1 mM $MgCl_2$, and 0.1% NaN_3, and stored in the same solution at 4°C. Approximately 0.05-0.1 μg of IgG was bound to one polystylene bead. The concentrated conditioned medium (2 ml) from Cos-1 cells expressing PACE4 was incubated with 100 immunobeads in an ice-bath for one day. The beads were rinsed 3 times with ice-cold Ca^{2+}-free PBS and used immediately for PACE4 assay. The specificity of the immunobeads was confirmed by immunoprecipitation of radiolabeled PACE4 as follows. HEK293 cells stably expressing PACE4A-I (*39*) were grown in 100-mm dishes and incubated in 10 ml of methionine- and cysteine-free DMEM supplemented with 10% dialyzed FCS. After 1 h, the cells were washed with PBS and then radiolabeled with 400 μCi of Protein labeling mix (a mixture of [^{35}S]methionine and [^{35}S]cysteine) in 4 ml of methionine and cysteine-free DMEM supplemented with 5% FCS for 18 h. The medium was frozen at −80°C until used. The medium was thawed and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant (0.5 ml) was incubated with 5 immunobeads overnight at 4°C. The immunobeads were then washed 3 times with PBS, boiled in loading buffer, and subjected to 7.5% SDS-PAGE. The gel was treated with Amplify (Amersham, Buckinghamshire, England) and dried. The bands of radioactivity were analyzed using a BAS-1500 bioimaging analyzer (Fuji Film, Tokyo).

Determination of the Molecular Mass of PACE4 Secreted into the Culture Medium—The molecular mass of PACE4 secreted into the culture medium was estimated by gel filtration and sucrose density gradient centrifugation. The conditioned medium (80×concentrate, 50 μl) was applied to Superose 12PC 3.2/30 (Pharmacia, Sweden) equilibrated with 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl, and eluted with the same buffer at flow rate of 40 μl/min. Fractions of 30 μl were collected. ^{35}S-Labeled PACE4 prepared as described above was concentrated by ultrafiltration (Amicon centricon-10) and applied to a gradient of 5 to 20% sucrose (weight per volume) in 50 mM Tris-HCl, pH 7.5, and centrifuged for 17 h at 4°C in an SW41 rotor at 35,000 rpm. Fraction of 450 μl were collected and subjected to immunoprecipitation with anti-SCD antibody. Catalase (250 kDa), aldolase (160 kDa), and bovine serum albumin (67 kDa) were used as internal size standards.

Purification of PACE4A by Immunoaffinity Column Chromatography—Purified anti-CT antibody was immobilized with CH-Sepharose 4B (Pharmacia Biotech AB, Uppsala, Sweden). About 2.7 mg of protein was bound to 1 ml of resin. The conditioned medium (200 ml) from HEK293 cells stably expressing PACE4A was applied to an immunoaffinity column (bed volume 1.0 ml) at a flow rate of 20 ml/h. The column was washed with 50 ml of 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl. PACE4A was eluted with 50 mM glycine-HCl, pH 2.7. The eluate was immediately neutralized with 1 M Tris-HCl buffer, pH 7.5, and used for α1-PDX inhibition studies.

RESULTS

Formation of the SDS-Stable Complex of PACE4 and α1-PDX—Rat α1-AT (*43*) has the same inhibitory activity as human α1-AT and its α1-PIT variant (Met^{352}/Arg); like α1-PIT, it inhibits the intracellular processing of proalbumin in transfected cells (*44*). Recombinant rat α1-PIT and α1-PDX were purified to homogeneity from transformed *E. coli* as shown in Fig. 1A, and the action of rat α1-PDX on PACE4 was examined. When ^{35}S-labeled PACE4A secreted into the culture medium from Cos-1 cells and α1-PDX purified from *E. coli* were coincubated, a single high molecular weight band (180 kDa) corresponding to the kinetically trapped, SDS-stable α1-PDX/PACE4A complex, in addition to mature PACE4 (103 kDa), was immunoprecipitated with anti-PACE4 HomoB antibody as shown in Fig. 1B. In contrast, the α1-antitrypsin/PACE4A complex was not generated when wild or Pittsburgh type α1-antitrypsin was incubated with PACE4A. The complex formation was completely abolished by pretreating of PACE4A with Dec-RVKR-CMK, a general inhibitor of SPC family proteases (Fig. 1C).

The same results were obtained when both PACE4A and α1-PDX secreted into the culture medium from Cos-1 cells were coincubated (Fig. 1D). A single band (180 kDa) corresponding to the complex was detected although its binding efficiency was low. This band was not detected when PACE4A was incubated with α1-PIT. In addition, EDTA inhibited the formation of the complex. These

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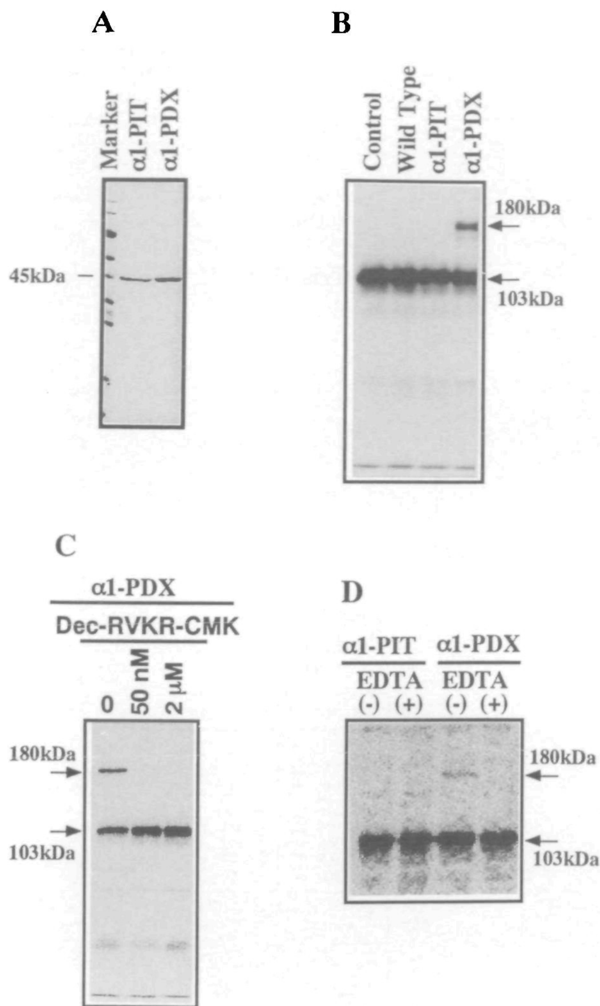

Fig. 1. **Acyl-intermediate complex formation of α1-PDX with PACE4A.** (A) SDS-PAGE of purified α1-PIT and α1-PDX from transfected *E. coli*. One microgram of α1-antitrypsin variants were applied to 10% SDS-PAGE and stained with Coomasie Brilliant Blue. (B) Complex formation between PACE4A secreted into the culture medium and α1-PDX. Purified α1-AT, α1-PIT and α1-PDX, (10 μg) were coincubated in 0.5 ml of ^{35}S-labeled conditioned medium from Cos-1 cells expressing PACE4A at 0°C for 16 h, and immunoprecipitated with anti-PACE4 HomoB antibody. Immunoprecipitates were boiled in 2% SDS in the presence of 10% β-mercaptoethanol, resolved by 10% SDS-PAGE, and analyzed by fluorography. (C) Effect of Dec-RVKR-CMK on complex formation. ^{35}S-labeled conditioned medium (0.4 ml) was incubated in the presence or absence of Dec-RVKR-CMK at room temperature for 30 min before coincubation with purified α1-PDX (10 μg). (D) Complex formation by incubation of PACE4A and α1-PDX secreted into the culture medium. ^{35}S-labeled conditioned media (0.2 ml each) from Cos-1 cells expressing α1-PIT, α1-PDX, or PACE4A were mixed, incubated in the presence or absence of 10 mM EDTA at 37°C for 2 h, and immunoprecipitated with anti-PACE4 HomoB antibody.

results suggest that the complex formation is mediated by the proteolytic action of PACE4A. The ability of α1-PDX to form the SDS-stable complex with PACE4A was further confirmed by coexpression experiments. PACE4A and α1-PDX were coexpressed in Cos-1 cells and then PACE4A in the cell extract and conditioned medium was immunoprecipitated. As shown in Fig. 2A (left), a single band (180 kDa) corresponding to the protease/inhibitor complex, in addition to mature PACE4 (103 kDa), was observed in the medium, whereas the complex was not detected in the cell extract. About 60% of the PACE4 secreted into the culture medium existed in complex. Neither the maturation nor secretion of PACE4 was inhibited by α1-PDX. Figure 2B shows the immunoprecipitation of the complex in the culture medium with either anti-PACE4 HomoB antibody or anti-α1AT antibody. A single band (180 kDa) that was cross-reactive with both antibodies was detected. When anti-α1AT antibody was used, a faint band corresponding to cleaved α1-PDX (51 kDa) in addition to native α1-PDX (55 kDa) was detected. The truncated α1-PDX was throught to be the N-terminal fragment cleaved at arginine[352].

In contrast, the ability of α1-PDX to form complexes with soluble furin was also examined under the same conditions to compare the efficiency of complex formation. Similarly, the α1-PDX/furin complex was immunoprecipitated with anti-α1AT antibody and truncated α1-PDX (51 kDa) was detected (Fig. 2A, right). These results clearly show that α1-PDX can form a kinetically trapped, SDS-stable complex with PACE4 as well as with furin.

The inhibitory effect of α1-PDX on the processing of pro von Willebrand factor (pro-vWF) mediated by PACE4A in Cos-1 cells was also examined (Fig. 3). Pro-vWF contains an RSKR sequence at the processing site. When pro-vWF alone was expressed in Cos-1 cells, pro-vWF (320 kDa) was processed to mature vWF (220 kDa) inefficiently (16%) by an endogenous furin-like activity. However pro-vWF was processed to mature vWF efficiently (60%) by coexpression with PACE4A. This PACE4A-mediated processing of pro-vWF was inhibited by α1-PDX (30% processing). In contrast, α1-PIT used as control showed no inhibitory effect.

Characterization of the Protease Activity in Conditioned Culture Medium from Cells Expressing PACE4—We characterized the protease activity in conditioned medium from Cos-1 cells transiently expressing PACE4 in more detail. Wild type and active site mutant (S/A, Ser[420] converted to Ala) PACE4A were expressed transiently in Cos-1 cells, and the cell extracts and media were analyzed by Western blotting. As shown in Fig. 4, the wild type PACE4A was efficiently secreted into the culture medium in a mature form (103 kDa). In contrast, a small amount of both proform (110 kDa) and mature form (103 kDa) PACE4A were detected in the cell extract. It is important to use conditioned medium collected 48 to 65 h after transfection from earlier passages of Cos-1 cells. Prolonged incubation (72-96 h after transfection) appeared to result in a loss of secreted mature PACE4A. The secreted PACE4A seemed to be susceptible to proteolytic modification. Degradation products (46 and 43 kDa) were observed in the culture medium as shown in Fig. 4. In contrast to wild type PACE4A, the active site mutant was hardly secreted into the culture medium and only the proform was detected in the cell extract. The mature form (103 kDa) of the mutant was not detected in the cells or medium. These results indicate that most of the wild type proPACE4A synthesized in Cos-1 cells is processed rapidly to the mature form and then secreted into the culture medium, however, the active site mutant is degraded without maturation.

Similarly, in HEK293 cells expressing PACE4, pro-

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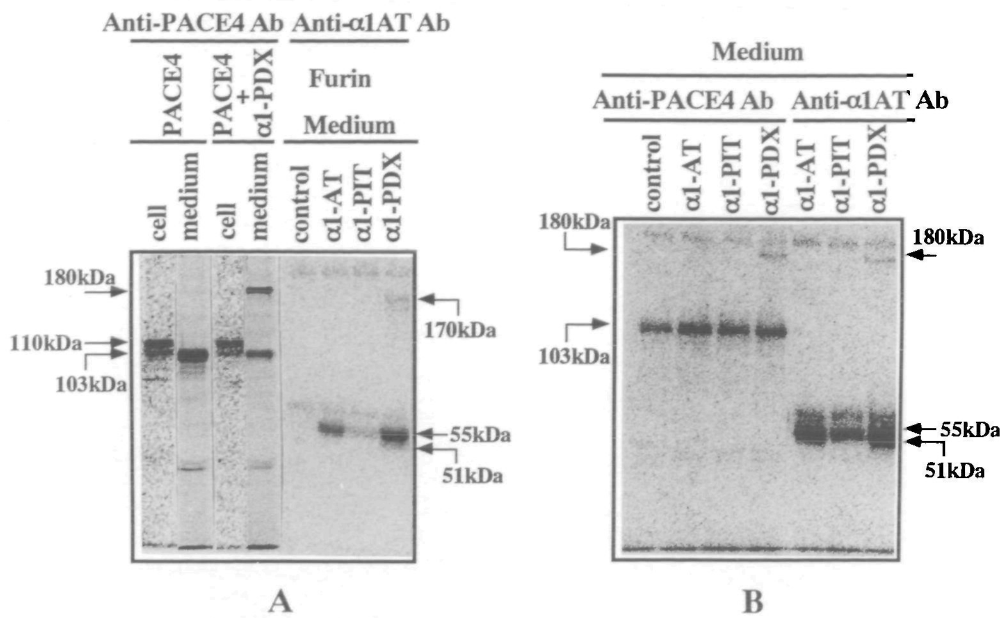

Fig. 2. **Coexpression of PACE4A and α1-antitrypsin variants.** Cos-1 cells were transfected with expression plasmids for PACE4A or furin in either the absence or presence of the expression plasmids for α1-AT, α1-PIT, or α1-PDX. After transfection for 60 h, the cells were radio-labeled for 8 h as described in "MATERIALS AND METHODS." The cell lysates and conditioned media were immunoprecipitated with anti-PACE4 HomoB or anti-α1AT antibodies. The immunoprecipitates were analyzed by SDS-PAGE (7.5%) and fluorography.

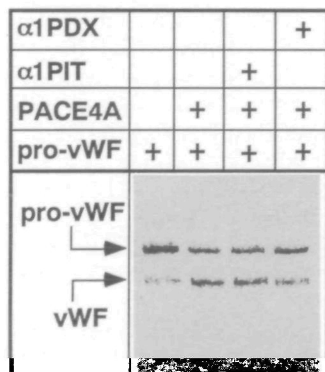

Fig. 3. **Inhibitory effect of α1-PDX on the processing of pro-von Willebrand factor mediated by PACE4.** Cos-1 cells were transfected with expression plasmids for pro-vWF (2 μg) or PACE4A (2 μg) in the presence or absence of the expression plasmids for α1-antitrypsin variants (2 μg, α1-PIT, or α1-PDX). The resulting culture media were immunoprecipitated with anti-vWF antiserum as described in "MATERIALS AND METHODS." The immunoprecipitates were analyzed by SDS-PAGE (5%) and fluorography.

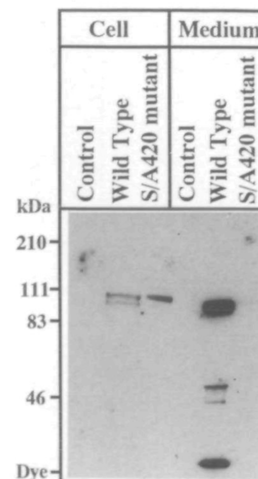

Fig. 4. **Western blot analysis of PACE4A in Cos-1 cells and culture media.** Cos-1 cells were transfected with pSVL vector (control), or the wild type or active site mutant (S/A) of the PACE4A expression plasmid. After transfection for 48 h, the cells were incubated in serum-free media for 17 h. Cells were extracted with 50 mM Tris-HCl buffer, pH 7.5, containing 0.3% CHAPSO and 0.1 M NaCl. Conditioned culture media were concentrated 40-fold by ultrafiltration. Equal amounts of cell extracts and media were subjected to 7.5% SDS-PAGE under reducing conditions, and PACE4A was immunodetected using anti-CT antibody as described in "MATERIALS AND METHODS."

PACE4A of the expected size was synthesized and the mature form was secreted into the medium following removal of the propeptide region by autoactivation as reported previously (*40*). However a smaller amount of mature PACE4A was secreted compared with Cos-1 cells, because the maturation of PACE4A was much slower in HEK 293 cells. Therefore we used the conditioned medium from Cos-1 cells transiently expressing PACE4A as the enzyme source. The medium from Cos-1 cells expressing the mutant enzyme was used as a negative control. We examined the effects of inhibitors of protease activities at pH 7.5 in the presence of 2 mM $CaCl_2$ on *pyr*-RTKR-MCA in conditioned culture media from Cos-1 cells expressing the wild type PACE4A (Table I). The activity of this medium was several-fold higher than that from cells expressing the mutant enzyme. The activity was completely inhibited by Dec-RVKR-CMK, EDTA, and EGTA, whereas PMSF, TLCK, TPCK, E-64, leupeptin, and pepstatin had no effect (data not shown). These results suggest that the protease(s) is a metal-dependent serine protease.

Figure 5 (left) shows gel filtration profiles of the *pyr*-RTKR-MCA cleaving activity in conditioned medium from cells expressing wild type PACE4A and from control cells. The protease activity (fractions 12–16) was 8-fold that of the control. The molecular mass of the enzyme was estimated to be 60 kDa. This value is smaller than the expected

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mass of PACE4 (about 100 kDa). The same activity elution profile was observed when *Boc*-RVRR-MCA was used as a substrate. In contrast, the PACE4 protein (103 kDa) was detected in fractions 8 to 11 by Western blotting as shown in Fig. 5B. The 60-kDa protease activity was not immunoprecipitated with anti-SCD, anti-HomoB, or anti-CT antibodies. Moreover, the 60-kDa protease activity was not inhibited by α1PDX at all. To confirm that the 60 kDa protease is not a truncated PACE4A, the conditioned medium was analyzed by density gradient centrifugation as shown in Fig. 5 (right). Individual gradient fractions were immunoprecipitated with anti-SCD antibody. PACE4A was sedimented as a single 100 kDa peak. No truncated PACE4A was detected. These findings clearly indicate that most of the elevated metal-dependent serine protease activity identified in the conditioned medium from Cos-1 cells expressing PACE4A is not due to PACE4A or truncated PACE4A, although the activity is inhibited by Dec-RVKR-CMK. We could not distinguish PACE4 activity from the 60-kDa protease activity using any of the fluorogenic substrates tested. Thus the specific assay of PACE4 in conditioned medium is hampered by the significant activity of an unknown metal-dependent serine protease that is activated by artificial PACE4 expression. Therefore we attempted to exclude this enzyme activity by an immunological method to identify PACE4 activity.

TABLE I. **Characterization of the protease activity in the conditioned culture medium from Cos-1 cells transiently expressing wild type or active site mutant (S/A⁴²⁰) PACE4A.** The medium was concentrated 40-fold by ultrafiltration and activity was determined in the absence and presence of various inhibitors as described in "MATERIALS AND METHODS."

Conditioned mediun	Inhibitor	*pyr*-RTKR-MCA cleaving activity [mean±SD (*n*) pmol/min/ml]	Activity (%)
Medium from cells expressing PACE4A	No addition	42.3±15.4 (*n*=6)	100
	1 μM Dec-RVKR-CMK[a]	5.38	13
	0.1 μM Dec-RVKR-CMK[a]	7.99	19
	2 mM EDTA	4.03	9
	2 mM EGTA	4.14	10
Medium from cells expressing Active site mutant (S/A[420])	No addition	17.6±9.63 (*n*=6)	42
Control (vector) 1	No addition	25.0	59
Control (vector) 2	No addition	19.2	45

[a]Decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone.

Development of a Specific Assay for PACE4 Using Anti-PACE4A C-Terminus Monoclonal Antibody—The immunocomplex of PACE4A and anti-CT antibody was presumed to have enzyme activity, because the carboxyl terminal region of PACE4A is not essential for the enzyme activity (*40*). But the immunocomplex of PACE4A and anti-SCD antibody was expected to be inactive because this antibody reacts with the catalytic domain. Previously Lindberg *et al.* demonstrated that an immunocomplex of PC2 and an antibody against the carboxyl-terminal region of PC2 retained proteolytic activities toward MCA substrate and also natural substrates such as proenkephalin (*45*). The enzymatic properties of this complex were shown to be very similar to those of purified PC2 (*28*). Two kinds of antibodies, anti-SCD and anti-C-terminus antibodies, were immobilized on polystyrene beads used for enzyme-immuno assay as a low background solid phase. First, the binding-specificity of these immunobeads was examined by immunoprecipitation of secreted ^{35}S-labeled PACE4A. After incubation with radiolabeled conditioned medium from cells overexpressing PACE4A, the immunobeads were washed 3 times with Ca^{2+}-free PBS and the proteins bound to the beads were analyzed by SDS-PAGE as shown in Fig. 6. A 96 kDa band was seen in all lanes, indicating that it is non-specific. PACE4A (103 kDa) was specifically immunoprecipitated with both immunobeads. No PACE4A

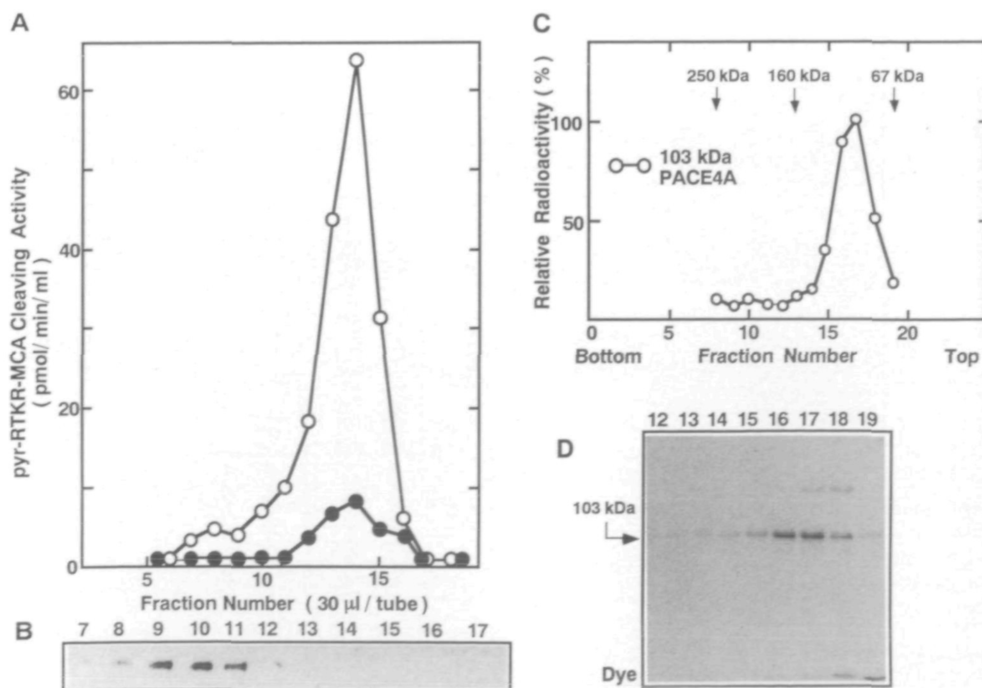

Fig. 5. **Gel filtration and sucrose density gradient centrifugation of conditioned media from control and PACE4-expressing Cos-1 cells.** (A) The medium concentrate (80×, 50 μl) was applied to Superose 12PC 3.2/30 (Pharmacia, Sweden) equilibrated with 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl and eluted with the same buffer at a flow rate of 40 μl/min. Fractions of 30 μl were collected. (B) Western blot analysis of the eluate with anti-PACE4 HomoB antibody. (C) ^{35}S-labeled conditioned medium from Cos-1 cells transiently expressing PACE4A was fractionated by sedimentation on a 5-20% sucrose gradient for 17 h. Individual gradient fractions were immunoprecipitated with anti-SCD antiserum, and then analyzed by SDS-PAGE and fluorography. (D) Radioactivity was analyzed using a BAS-1500.

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protein band was detected in the control lane. These results show that the binding of PACE4A to immunobeads is specific.

Next the immunobeads were incubated with conditioned medium from Cos-1 cells expressing PACE4A, and the protease activity was examined using *pyr*-RTKR-MCA and *Boc*-RVRR-MCA as substrates. As expected, the immunocomplex with the anti-CT antibody was shown to be active toward both MCA substrates as shown in Fig. 7. In contrast, the immunocomplex with the anti-SCD antibody had no enzyme activity.

To confirm the specificity of this assay system, immunobeads bound to anti-CT antibody were incubated with conditioned medium from cells expressing an inactive PACE4A(S^{420}/A) mutant, and the activity was determined. As shown in Fig. 7, the immunocomplex showed no enzyme activity. Figure 8 shows a linear relationship between the dose response and time course of immunopurified PACE4A activity. These results indicate that a quantitative specific PACE4A assay can be achieved with this immunobead system. The enzymatic properties of PACE4A were characterized by this method.

Substrate Specificity toward Fluorogenic Substrates—The ability of PACE4A to cleave a series of MCA substrates is shown in Fig. 9. Of the substrates tested, PACE4A like other SPCs, was found to cleave *pyr*-RTKR-MCA and *Boc*-RVRR-MCA best. The apparent K_m of PACE4A was estimated to be 33 μM. Furin can cleave RXK/RR efficiently and RXXR less efficiently (*27*). An arginine residue at the P1 and P4 or P6 sites of the substrate is essential for cleavage by furin and a basic residue at the P2 position, although not essential, is an important determinant. Furin

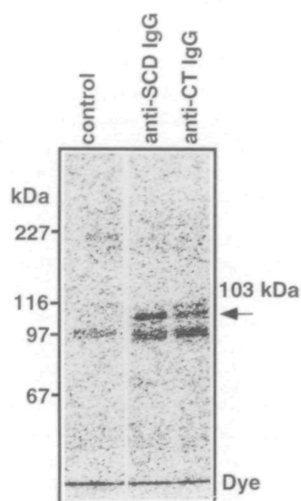

Fig. 6. **Specificity of immunobeads bound to anti-SCD and anti-CT antibody.** Control beads and immunobeads were incubated with ^{35}S-radiolabeled conditioned medium from hEK293 cells stably transfected with the PACE4A expression plasmid. The beads were washed 3 times with PBS, and the protein bound to the beads was analyzed by SDS-PAGE and fluorography as described in "MATERIALS AND METHODS."

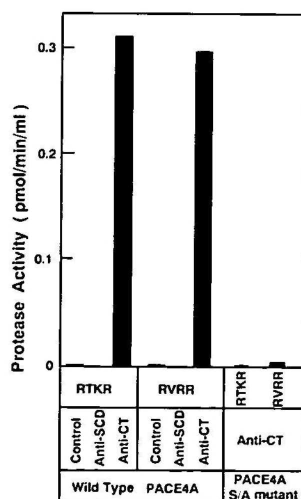

Fig. 7. **Protease activity of immunopurified PACE4A.** Control beads and immunobeads immobilized with either anti-PACE4 C-terminus or anti-PACE4 SCD antibodies were incubated with conditioned culture media from Cos-1 cells transfected with expression plasmids for PACE4A or its mutant (active site mutant) for 18 h at 4°C. The activities toward *pyr*-RTKR-MCA and *Boc*-RVRR-MCA bound to beads were determined as described in "MATERIALS AND METHODS."

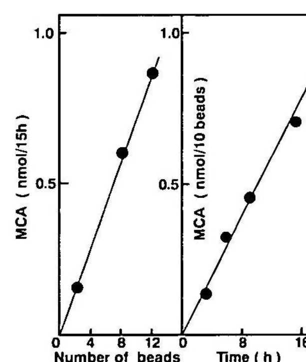

Fig. 8. **Does-response and time-course of immunopurified PACE4A activity.** Protease activity was assayed using *pyr*-RTKR-MCA.

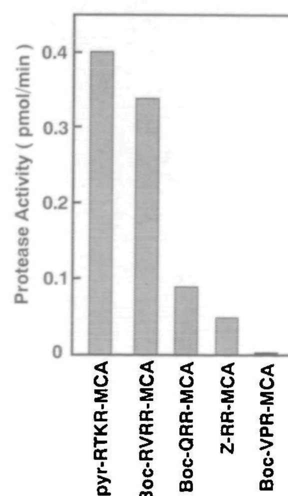

Fig. 9. **Substrate specificity of PACE4A.** The enzyme activity was determined at pH 7.5 with various substrates at a concentration of 50 μM.

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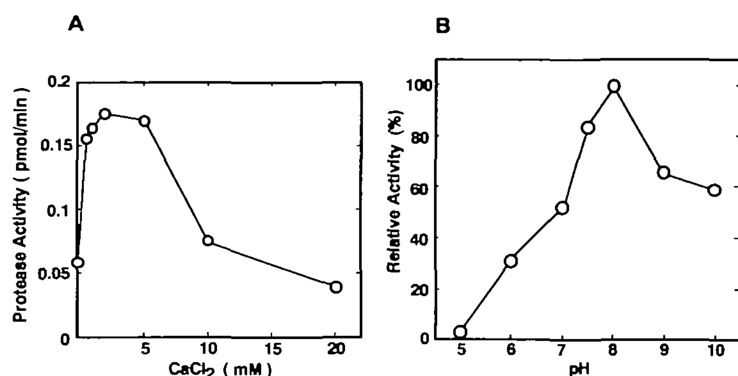

Fig. 10. **Calcium and pH dependence of PACE4A activity.** (A) Immunopurified PACE4A was incubated with *pyr*-RTKR-MCA at pH 7.5 for 18 h in the presence of varying concentrations of $CaCl_2$. (B) The activity of immunopurified PACE4A was assayed in the presence of 2 mM $CaCl_2$ using *pyr*-RTKR-MCA in buffers of various pH. These buffers included acetate buffer (pH 5.0 and 6.0) and Tris-HCl buffer (pH 7.0, 7.5, 8.0, 9.0, and 10.0).

TABLE II. **Inhibitor sensitivity of immunopurified PACE4A.** The enzyme activity was determined with *pyr*-RTKR-MCA in the presence of inhibitor as described in "MATERIALS AND METHODS."

Inhibitor	Concentration	Relative activity (%)
No inhibitor		100
DFP	1 mM	101
PMSF	1 mM	81
Benzamidine	1 mM	78
TLCK	1 mM	89
TPCK	1 mM	18
Leupeptin	25 μg/ml	85
	250 μg/ml	100
Antipain	25 μg/ml	81
Chymostatin	40 μg/ml	91
Iodoacatamide	1 mM	116
PCMB	1 mM	11
E-64	25 μg/ml	89
Pepstatin	25 μg/ml	101
Bestatin	25 μg/ml	87
EDTA	2 mM	3.3
EGTA	2 mM	1.9
$CuSO_4$	2 mM	25
$ZnSO_4$	2 mM	65
$MgCl_2$	2 mM	51

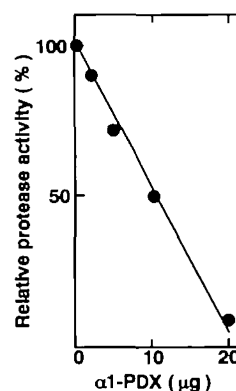

Fig. 11. **Effect of α1-PDX on PACE4 activity.** PACE4A was purified by immunoaffinity column chromatography from the conditioned medium of HEK293 cells stably expressing PACE4A. Increasing amounts of α1-PDX were added to the reaction mixture containing the purified enzyme (31 pmol/h), and the mixtures were incubated at 37°C for 24 h.

and PC7 have no activity toward tripeptidyl- or dipeptidyl-MCA substrates such as *Boc*-QRR-MCA (*27*, *46*). However PACE4A showed significant activity toward *Boc*-QRR-MCA and *Z*-RR-MCA which have no arginine at the P4 position.

Calcium Requirement of PACE4A—Similar to furin (*27*) and PC7 (*46*), PACE4A activity was stimulated by Ca^{2+} with a peak at 2 mM and inhibited by Ca^{2+} at concentrations greater than 5 mM (Fig. 10A). PACE4A showed 20% of the maximum activity when no Ca^{2+} was added to the reaction mixture. Immunobeads were stored in a solution containing BSA after antibody immobilization. Because BSA has Ca^{2+} affinity sites (*47*), immunobeads coated with BSA bound Ca^{2+} during incubation with conditioned medium containing 2 mM $CaCl_2$. This trace amount of Ca^{2+} associated with the immunobeads might stimulate PACE4A activity. The optimum pH for PACE4A was between 7.5 and 8.5, however, 30% of maximum activity was retained at pH 6.0. (Fig. 10B).

Effects of Inhibitors on PACE4A—The effects of various inhibitors on the activity for *pyr*-RTKR-MCA at pH 7.5 were examined (Table II). Similar to furin, PC2, PC1/3, and PC7, EDTA and EGTA inhibited the activity almost completely, whereas E-64 (cysteine protease inhibitor), leupeptin, and antipain (inhibitor of cysteine proteases and trypsin-like serine proteases), chymostatin (inhibitor of cysteine proteases and chymotrypsin-like serine proteases), pepstatin (aspartic protease inhibitor), and bestatin (aminopeptidase inhibitor) had no significant effects. The activity was inhibited by PCMB, although E-64 and iodoacetamido had no effects. The inhibitory effect of PCMB may be due to the presence of a heavy metal ion (Hg^{2+}) in this reagent. Cu^{2+}, Zn^{2+}, and Mg^{2+} also have inhibitory effects, but not as strong as those of furin (*27*) and PC7 (*46*). The most remarkable feature of the inhibitor sensitivity of PACE4A is the effect of TPCK, which is an inhibitor of chymotrypsin-like serine proteases and cysteine proteases. Our enzyme preparation did not cleave *Suc*-LLVY-MCA, which is a good substrate for chymotrypsin-like proteases (data not shown). Furin (*27*), PC2 (*28*), and PC7 (*46*) were not inhibited by TPCK. On the other hand, Mains *et al.* reported the sensitivity of rat PACE4 to inhibition by leupeptin and the relative insensitivity to inhibition by EDTA, as compared with furin (*39*). However, immunopurified PACE4A was not inhibited by leupeptin at all and its sensitivity to inhibition by EDTA was similar to that of other SPCs. The effect of α1-PDX on PACE4 activity toward *Boc*-RVRR-MCA was examined using enzyme purified by immunoaffinity column chromatography. As shown in Fig. 11, α1-PDX inhibited the activity in a dose-dependent manner.

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DISCUSSION

For a long time, PACE4 and furin were thought to have similar physiological functions since both enzymes show a wide tissue distributions. However, recent detailed analyses of the tissue and cell distribution of these enzymes by *in situ* hybridization and immunohistochemistry clearly demonstrated differences in the distribution profiles of PACE4 and furin, indicating distinct functions. Furin is ubiquitously expressed in various tissues and cultured cells, whereas PACE4 is highly expressed in the anterior pituitary, neural tissues, pancreas, heart, and liver (*2, 14-17, 21*). PACE4 displays dynamic expression patterns throughout development and is upregulated in some tissues that exhibit high expression levels of bone morphogenetic proteins (BMPs), suggesting the importance of PACE4 in the regulation of BMP activity. Constam *et al.* showed that BMP2, 4, and 7 are coexpressed with PACE4 (SPC4) in the primitive heart, in the apical ectodermal ridge of developing limb buds, and in the interdigital mesenchyme of embryogenic limbs (*13*).

α1-Antitrypsin Portland (α1-PDX) is a good tool with which to elucidate the physiological function of SPCs *in vivo*. α1-PDX blocks BMP-4 processing by inhibiting the SPCs in *Xenopus* oocytes and disturbs embryogenic development (*23*). A common feature of all TGF-β-related growth factors is that their active forms are dimers of a C-terminal fragment cleaved from a larger precursor (*22*). Generally, the sequence of the processing site is Arg-X-Arg/Lys-Arg, which is a potential cleavage motif for SPC family proteases. Thus these results suggest the significance of SPCs in the regulation of signaling pathways during embryogenesis.

The inhibitory effects of α1-PDX on SPCs have been reported by several groups. Jean *et al.* showed by *in vitro* binding experiments that α1-PDX is able to form an SDS-stable complex with furin (*26*). There was agreement in the results concerning the effects of α1-PDX on furin, PC6, and PC7. Furin and PC6 are inhibited by α1-PDX which PC7 is not. However, the results for PACE4 differ. In coexpression experiments, Benjanet *et al.* compared the inhibitory effects of α1-PDX on the intracellular processing of two model precursors (pro-7B2 and POMC) mediated by six members of the SPC family, furin, PC1, PC2, PACE4, PC5/6, and PC7 (*38*). They concluded that α1-PDX is not a selective but rather a general inhibitor of SPCs other than PC7 in the constitutive pathway. Decroly *et al.* showed that α1-PDX inhibits equally the abilities of furin, PACE4, PC1, and PC5/6 to cleave gp-160 *in vitro* (*32*). Decroly *et al.* prepared these SPCs from the conditioned medium of GH4C1 cells infected with a Vaccinia Virus recombinant. On the other hand, Mains *et al.* (*39*) and Jean *et al.*(*26*) reported that α1-PDX has no effect on PACE4 activity. Mains *et al.* used conditioned medium of an HEK293 stable transfectant expressing and secreting rat PACE4 to evaluate PACE4 activity. Jean *et al.* used the conditioned medium from Lovo cells infected with the Vaccinia Virus recombinant. Both groups used recombinant α1-PDX produced in *E. coli.*

Previously, two groups reported that the activity of a protease other than PACE4 is elevated in cultured cells by the exogenous expression of PACE4. Inocencio *et al.* reported that an E-64-sensitive protease is activated by PACE4 expression in CHO-K1 cells but not in 7.P15 cells (*36*). Hubbart *et al.* showed that SCC (squamous cell carcinoma) cultures expressing PACE4 endogenously or ectopically are able to process stromelysin-3 extracellularly to its active mature form (*37*).

Thus the kinds of proteases whose expressions are elevated vary among different cells. These findings indicate that it is problematic to evaluate the inhibitory effect (inhibition of enzyme activity) of α1-PDX on PACE4 activity using the medium from PACE4-expressing cells as an enzyme source. Therefore we analyzed directly the action of α1-PDX on the PACE4 molecule by measuring SDS-stable (acyl-intermediate) α1-PDX/PACE4 complex formation. If PACE4 is inhibited by α1-PDX specifically, an acyl-intermediate of PACE4 with α1-PDX has to be generated. A single 180 kDa band corresponding to the SDS-stable complex of PACE4 and α1-PDX was detected by immunoprecipitation when PACE4 secreted into the culture medium was incubated *in vitro* with either purified α1-PDX from transformed *E. coli* or α1-PDX secreted from Cos-1 cells. This complex formation is specific for α1-PDX since it was not detected when α1-AT or α1-PIT was used. Coexpression studies confirmed the ability of α1-PDX to form an SDS-stable complex with PACE4 and the inhibitory effect of α1-PDX on PACE4 activity. Thus these results clearly show that α1-PDX is a potent inhibitor of PACE4 as well as of furin and PC6. PACE4 is highly homologous to PC6 and furin. Alignment of the catalytic domain of PACE4 with those of other SPCs reveals 68, 76, and 53% identity with furin, PC6, and PC8, respectively. Moreover substrates containing an α1-PDX-like RXXR sequence are cleaved efficiently by furin and PACE4 (*35*), but poorly by PC7 (*46*). These results support our conclusions. The molecular mass of α1-PDX/PACE4A (180 kDa) is larger than the sum of the molecular masses of α1-PDX (recombinant form, 45 kDa) and PACE4A (103 kDa). In kinetic studies, Jean *et al.* showed that α1-PDX forms a 1:1 molar complex with furin (87 kDa), although the molecular mass of the α1-PDX/furin complex (160 kDa) as determined by SDS-PAGE is larger than the sum of both molecular masses, as in the α1-PDX/PACE4A complex (*26*). These findings suggest that PACE4 forms a 1:1 molar complex with α1-PDX as well as furin. It is most likely that the α1-PDX/SPC complex migrates more slowly through the gel than a protein with the same molecular mass.

To know why the sensitivity of PACE4 to α1-PDX differs among laboratories, we characterized the human PACE4 secreted into the culture medium as a mature enzyme in more detail. In Cos-1 cells transfected with PACE4A expression vector, mature PACE4A (103 kDa) was efficiently secreted into the culture medium. However, most of the increased *pyr*-RTKR-MCA cleaving activity in the medium is not due to PACE4 activity, even through it was completely inhibited by Dec-RTKR-CMK and EDTA. As shown by gel filtration and sedimentation analyses, the 60-kDa protease activity that was elevated by PACE4 expression did not immunoprecipitate with anti-HomoB and anti-SCD antibodies, indicating that the 60-kDa protease is not a truncated form of PACE4A. Immunopurified PACE4A was inhibited by PCMB and TPCK, however, the 60-kDa protease activity was not. This result also indicates that the 60 kDa protease activity is not due to PACE4

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activity. This activity hampers the specific assay of PACE4. It is highly likely that PACE4 participates in the activation of other metal-dependent serine-proteases. The protease(s) activated by PACE4 expression remains to be identified.

We attempted to develop a specific assay for PACE4 using an immunological method. This method proved to be specific and quantitative as shown in Figs. 6-8. Using this method, the enzymatic properties of PACE4A were examined. The data presented in Fig. 10A and Table II demonstrate that PACE4A is a Ca^{2+}-dependent protease with an optimal Ca^{2+} requirement of 2 mM. PACE4 shows the highest activity under weakly basic conditions (pH 7.5-8.5), however it retains 30% of maximum activity at pH 6.0. Therefore, we can not exclude the possibility that PACE4 may be functional within the Golgi or *trans*-Golgi network area. Very recently, we showed that PACE4 is a hepatic proalbumin processing protease (*48*). The proteolytic processing of proalbumin occurs in either the *trans*-Golgi compartment or secretory vesicles in the liver. These results indicate that PACE4 is not only functional in the extracellular space but also in the Golgi compartment or secretory vesicles. The coexpression of PACE4 with α1-PDX also supports this idea. The fact that the efficiency of complex formation on coexpression was higher than in *in vitro* binding experiments suggests that the PACE4/α1-PDX complex is formed in secretory vesicles or the *trans*-Golgi network where secretory proteins are concentrated.

The cleavage specificity of PACE4 was first examined using a series of fluorogenic peptidyl substrates. PACE4 showed a similar cleavage pattern to that of furin. Tetrapeptides with a basic amino acid at the P1, P2, and P4 positions were found to be the best substrates. However PACE4 cleaved tripeptide and dipeptide substrates containing a pair of basic residues, unlike other SPC members. Similarly, Gordon *et al.* showed that PACE4 recognizes RAAR and, to a much lesser extent, KR and RR sequences in Anthrax toxin protective antigen, while furin does not recognize KR or RR sequence in coexpression studies (*35*). The inhibitor profile of immunopurified PACE4 is generally similar to that of furin (*27*), PC2 (*28, 45*), PC3 (*29*), and PC7 (*46*). PACE4 activity was completely inhibited by EDTA, EGTA, or PCMB, whereas leupeptin, E-64, chymostatin, and pepstatin had no significant effects. The inhibitory effect of α1-PDX on the hydrolysis of fluorogenic substrates was examined using soluble PACE4 purified by immunoaffinity chromatography. As shown in Fig. 11, PACE4 activity was completely inhibited by α1-PDX. Thus, the inhibitory effect of α1-PDX on PACE4 activity was confirmed by SDS-stable complex formation *in vitro* and *in vivo*, and the effects on PACE4-mediated pro-vWF processing and the hydrolysis of fluorogenic substrates.

Recent genetic studies have revealed unique functions for individual SPCs. Embryos lacking furin die at around embryonic day 10.5, exhibiting severe ventral closure defects (*49*). Embryos lacking PACE4 display varying degrees of holoprosencephaly (cyclopia) (*22*). Thus, furin can not compensate for the loss of PACE4. A comparison of the 5′-flanking regions of SPC genes also supports the idea of a unique function for PACE4. The most remarkable feature of its sequence is the presence of many binding sites (E-box) for basic helix-loop-helix (bHLH) transcription factors (*50*). bHLH factors play an essential role in cell differentiation such as in neurogenesis (*51*). As shown by the ectopic expression of α1-PDX in *Xenopus* oocytes, abnormal embryogenic development occurs by inhibition of the proteolytic maturation of BMP4 (*23*). The activities of various TGF β-related factors such as BMPs, activin, and Nodal are very likely to be regulated by proteolytic activation mediated by furin and PACE4 (*22*). Taken together, these findings indicate that part of the effect of α1-PDX on embryonic development is caused by the inhibition the PACE4-mediated activation of TGF β-related factors such as BMPs. The differences in function between PACE4 and furin are yet to be determined.

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REFERENCES

1. Halban, P.A. and Irminger, J.-C. (1994) Sorting and processing of secretory proteins. *Biochem. J.* **299**, 1-18
2. Seidah, N.G., Chrétien, M., and Day, R. (1994) The family of subtilisin/kexin like pro-protein and pro-hormone convertases: Divergent or shared functions. *Biochimie* **76**, 197-209
3. Fuller, R.S., Brake, A.J., and Thorner, J. (1989) Intracellular targeting and structural conservation of a prohormone-processing endoprotease. *Science* **246**, 482-486
4. Smeekens, S.P., Avruch, A.S., LaMendola, J., Chan, S.J., and Steiner, D.F. (1991) Identification of a cDNA encoding a second putative prohormone convertase related to PC2 in AtT20 cells and islets of Langerhans. *Proc. Natl. Acad. Sci. USA* **88**, 340-344
5. Smeekens, S.P. and Steiner, D.F. (1990) Identification of a human insulinoma cDNA encoding a novel mammalian protein structurally related to the yeast dibasic processing protease *Kex2. J. Biol. Chem.* **265**, 2997-3000
6. Kiefer, M.C., Tucker, J.E., Joh, R., Landsberg, K.E., Saltman, D., and Barr, P.J. (1991) Identification of a second human subtilisin-like protease gene in the fes/fps region of chromosome 15. *DNA Cell Biol.* **10**, 757-769
7. Nakayama, K., Kim, W.-S., Torii, S., Hosaka, M., Nakagawa, T., Ikemizu, J., Baba, T., and Murakami, K. (1992) Identification of the fourth member of the mammalian endoprotease family homologous to the yeast Kex2 protease: Its testis-specific expression. *J. Biol. Chem.* **267**, 5897-5900
8. Nakagawa, T., Hosaka, M., Torii, S., Watanabe, T., Murakami, K., and Nakayama, K. (1993) Identification and functional expression of a new member of the mammalian Kex2-like processing endoprotease family: Its striking structural similarity to PACE4. *J. Biochem.* **113**, 132-135
9. Lusson, J., Vieau, D., Hamelin, J., Day, R., Chrétien, M., and Seidah, N.G. (1993) cDNA structure of the mouse and rat subtilisin/kexin-like PC5: A candidate proprotein convertase expressed in endocrine and nonendocrine cells. *Proc. Natl. Acad. Sci. USA* **90**, 6691-6695
10. Bruzzaniti, A., Goodge, K., Jay, P., Taviaux, S.A., Lam, M.H.C., Berta, P., Martin, T.J., Moseley, J.M., and Gillespie, M.T. (1996) C8, a new member of the convertase family. *Biochem. J.* **314**, 727-731
11. Meerabux, J., Yaspo, M.L., Roebroek, A.J., Van de Ven, W.J., Lister, T.A., and Young, B.D. (1996) A new member of the proprotein convertase gene family (LPC) is located at a chromosome translocation breakpoint in lymphomas. *Cancer Res.* **56**, 448-451
12. Seidah, N.G., Hamelin, J., Mamarbachi, M., Dong, W., Tadros,

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H., Mbikay, M., Chrétien, M., and Day, R. (1996) cDNA structure, tissue distribution, and chromosomal localization of rat PC7, a novel mammalian proprotein convertase closest to yeast kexin-like proteinases. *Proc. Natl. Acad. Sci. USA* **93**, 3388-3393
13. Constam, D.B., Calfon, M., and Robertson, E.J. (1996) SPC4, SPC6, and the novel protease SPC7 are coexpressed with bone morphogenetic proteins at distinct sites during embryogenesis. *J. Cell Biol.* **134**, 181-191
14. Johnson, R.C., Darlington, D.N., Hand, T.A., Bloomquist, B.T., and Mains, R.E. (1994) PACE4: A subtilisin-like endoprotease prevalent in the anterior pituitary and regulated by thyroid status. *Endocrinology* **135**, 1178-1185
15. Nagamune, H., Muramatsu, K., Akamatsu, T., Tamai, Y., Izumi, K., Tsuji, A., and Matsuda, Y. (1995) Distribution of the kexin family proteases in pancreatic islets: PACE4C is specifically expressed in B cells of pancreatic islets. *Endocrinology* **136**, 357-360
16. Beaubien, G., Schafer, M.K.H., Weihe, E., Dong, W., Chétien, M., Seidah, N.G., and Day, R. (1995) The distinct gene expression of the pro-hormone convertases in the rat heart suggests potential substrates. *Cell Tissue Res.* **279**, 539-549
17. Akamastu, T., Daikoku, S., Nagamune, H., Yoshida, S., Mori, K., Tsuji, A., and Matsuda, Y. (1997) Developmental expression of a novel Kexin-family protease, PACE4E, in the rat olfactory system. *Histochem. Cell. Biol.* **108**, 95-103
18. Marcinkiewicz, M., Ramla, D., Seidah, N.G., and Chrétien, M. (1994) Developmental expression of the prohormone convertase PC1 and PC2 in mouse pancreatic islets. *Endocrinology* **135**, 1651-1660
19. Nagamune, H., Muramatsu, K., Higashine, M., Akamatsu, T., Tamai, Y., Yonetomi, Y., Tsuji, A., Izumi, K., Sakaguchi, T., Yoshida, T., and Matsuda, Y. (1997) Cyclic AMP-inducible procalcitonin processing in thyroidal parafollicular cells is regulated by the kexin-family protease, PC1 in *Medical Aspects of Proteases and Protease Inhibitors* (Katunuma, N., Kido, H., and Travis, J. eds.) pp. 22-33, IOS Press, Amsterdam
20. Torii, S., Yamaguchi, T., Murakami, K., and Nakayama, K. (1993) Location of Kex2-like processing endoproteases, furin and PC4, within mouse testis by *in situ* hybridization. *FEBS Lett.* **316**, 12-16
21. Dong, W., Marcinkiewicz, M., Vieau, D., Chrétien, M., Seidah, N.G., and Day, R. (1995) Distinct mRNA expression of the highly homologous convertases PC5 and PACE4 in the rat brain and pituitary. *J. Neurosci.* **15**, 1778-1796
22. Constam, D.B. and Robertson, E.J. (1999) Regulation of bone morphogenetic protein activity by prodomains and proprotein convertases. *J. Cell. Biol.* **144**, 139-149
23. Cui, Y., Jean, F., Thomas, G., and Christian, J.L. (1998) BMP-4 is proteolytically activated by furin and/or PC6 during vertebrate embryogenic development. *EMBO. J.* **17**, 4735-4743
24. Travis, J. and Salvesen, G.S. (1983) Plasma proteinase inhibitors. *Annu. Rev. Biochem.* **52**, 655-709
25. Owen, M.C., Brennan, S.O., Lewis, J.H., and Carrel, R.W. (1983) Mutation of antitrypsin to antithrombin. α1-antitrypsin Pittsburg (358Met-Arg), A fatal bleeding disorder. *New. Eng. J. Med.* **309**, 694-698
26. Jean, F., Stella, K., Thomas, L., Liu, G., Xiang, Y., Reason, A.J., and Thomas, G. (1998) α1-Antitrypsin Portland, a bioengineered serpin high selective for furin: Application as an antipathogenic agent. *Proc. Natl. Acad. Sci. USA*, **95**, 7293-7298
27. Hatsuzawa, K., Nagahama, M., Takahashi, S., Takada, K., Murakami, K., and Nakayama, K. (1992) Purification and characterization of furin, a kex2-like processing endoprotease, produced in Chinese hamster ovary cells. *J. Biol. Chem.* **267**, 16094-16099
28. Lamango, N.S., Zhu, X., and Lindberg, I. (1995) Purification and enzymatic characterization of recombinant prohormone convertase 2: Stabilization of activity by 21 kDa 7B2. *Arch. Biochem. Biophys.* **330**, 238-250
29. Zhou, Y. and Lindberg, I. (1993) Purification and characterization of the prohormone convertase PC 1 (PC 3). *J. Biol. Chem.* **268**, 5615-5623
30. Azayan, A.V., Krieger, T.J., and Hook, V.Y. (1995) Purification and characterization of the candidate prohormone processing protease PC2 and PC1/3 from bovine adrenal medulla chromaffin granules. *J. Biol. Chem.* **270**, 8201-8208
31. Mori, K., Kii, S., Tsuji, A., Nagahama, M., Imamaki, A., Hayashi, K., Akamatsu, T., Nagamune, H., and Matsuda, Y. (1997) A novel human PACE4 isoform, PACE4E is an active processing protease containing a hydrophobic cluster at the carboxy terminus. *J. Biochem.* **121**, 941-948
32. Decroly, E., Wouters, S., Di Bello, C., Lazure, C., Ruysschaert, J.-M., and Seidah, N.G. (1996) Identification of the paired basic convertase implicated in HIV gp-160 processing based on *in vitro* assays and expression in CD4$^+$ cell lines. *J. Biol. Chem.* **271**, 30442-30450
33. Brakch, N., Galanopoulou, A.S., Patel, Y.C., Boileau, G., and Seidah, N.G. (1995) Comparative proteolytic processing of rat prosomatostatin by the convertases PC1, PC2, furin, PACE4 and PC5 in constitutive and regulated secretory pathways. *FEBS Lett.* **362**, 143-146
34. Seidah, N.G., Benjannet, S., Pareek, S., Savaria, D., Hamelin, J., Goulet, B., Laliberte, J., Lazure, C., Chrétien, M., and Murphy, R.A. (1996) Cellular processing of the nerve growth factor precursor by the mammalian pro-protein convertases. *Biochem. J.* **314**, 951-960
35. Gordon, V.W., Rehemtulla, A., and Leppla, S.H. (1997) A role for PACE4 in the proteolytic activation of anthrax toxin protective antigen. *Infect. Immun.* **65**, 3370-3375
36. Inocencio, N.M., Sucic, J.F., Moehring, J.M., Spence, M.J., and Moehring, T.J. (1997) Endoprotease activities other than furin and PACE4 with a role in processing of HIV gp-160 glycoprotein in CHO-K1 cells. *J. Biol. Chem.* **272**, 1344-1348
37. Hubbard, F.C., Goodrow, T.L., Liu, S-C., Brilliant, M.H., Basset, P., Mains, R.E., and Klein-Szanto, A-J-P. (1997) Expression of PACE4 in chemically induced carcinogenesis is associated with spindle cell tumor conversion and increased invasive ability. *Cancer Res.* **57**, 5226-5231
38. Benjanet, S., Savaria, D., Laslop, A., Munzer, J.S., Chrétien, M., Marcinkiewicz, M., and Seidah, N.G. (1997) α1-Antitrypsin Portland inhibits processing of precursors mediated by pro-protein convertases primarily within the constitutive secretory pathway. *J. Biol. Chem.* **272**, 26210-26218
39. Mains, R.E., Berard, C.A., Denault, J.-B., Zho, A., Johnson, R.C., and Leduc, R. (1997) PACE4: a subtilisin-like endoprotease with unique properties. *Biochem. J.* **321**, 587-593
40. Nagahama, M., Taniguchi, T., Hashimato, E., Imamaki, A., Mori, K., Tsuji, A., and Matsuda, Y. (1998) Biosynthetic processing and quoternary interactions of proprotein convertase SPC4 (PACE4). *FEBS Lett.* **434**, 155-159
41. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
42. Bradford, M.M. (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72**, 248-254
43. Misumi, Y., Sohda, M., Ohkubo, K., Takami, N., Oda, K., and Ikehara, Y. (1990) Molecular cloning and sequencing of the cDNA of rat α1-protease inhibitor and its expression in Cos-1 cells. *J. Biochem.* **108**, 230-234
44. Misumi, Y., Ohkubo, K., Sohda, M., Takami, N., Oda, K., and Ikehara, Y. (1990) Intracellular processing of complement pro-C3 and proalbumin is inhibited by rat α1-protease inhibitor variant (Met352-Arg) in transfected cells. *Biochem. Biophys. Res. Commun.* **31**, 236-242
45. Lindberg, I., Van den Hurk, W.H., Bui, C., and Batie, C.J. (1995) Enzymatic characterization of immunopurified prohormone convertase 2: Potent inhibition by a 7B2 peptide fragment. *Biochemistry* **34**, 5486-5493
46. Munzer, J.S., Basak, A., Zhong, M., Mamarbachi, A., Hamelin, J., Savaria, D., Lazure, C., Benjannet, S., Chrétien, M., and Seidah, N.G. (1997) *In vitro* characterization of the novel proprotein convertase PC7. *J. Biol. Chem.* **272**, 19672-19681
47. Kragh-Hansen, V. and Vorum, H. (1993) Quantitative analysis

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of the interaction between calcium ions and human serum albumin. *Clin. Chem.* **39**, 202-208
48. Mori, K., Imamaki, A., Nagata, K., Yonetomi, Y., Kiyokage-Yoshimoto, R., Martin, T.J., Gillespie, M.T., Nagahama, M., Tsuji, A., and Matsuda, Y. (1999) Subtilisin-like proprotein convertase, PACE4 and PC8, as well as furin, are endogenous proalbumin convertases in HepG2 cells. *J. Biochem.* **125**, 627-633
49. Roebroek, A.J.M., Umans, L., Pauli, I.G.L., Robertson, E.J., van Leuven, F., Van de Ven, W.J.M., and Constam, D.B. (1998) Failure of ventral closure and axial rotation in embryos lacking the proprotein convertase furin. *Development* **125**, 4863-4876
50. Tsuji, A., Hine, C., Tamai, Y., Yonemoti, K., Mori, K., Yoshida, S., Bando, M., Sakai, E., Mori, K., Akamatsu, T., and Matsuda, Y. (1997) Genomic organization and alternative splicing of human PACE4 (SPC4), kexin-like processing endoprotease. *J. Biochem.* **122**, 438-452
51. Littlewood, T. and Evan, G.I. (1998) The functions of bHLH proteins in *Helix-Loop-Helix Transcription Factors* (Third edition), pp. 6-26, Oxford University Press, Oxford

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