Protein Engineering, Design & Selection vol. 26 no. 2 pp. 123–131, 2013
Published online November 14, 2012 doi:10.1093/protein/gzs091

Engineering of α1-antitrypsin variants with improved specificity for the proprotein convertase furin using site-directed random mutagenesis

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Received May 14, 2012; revised October 1, 2012; accepted October 12, 2012

Furin, PACE4, PC5/6 and PC7 are members of the subtilisin-like proprotein convertase (SPC) family. Although these enzymes are known to play critical roles in various physiological and pathological events including cell differentiation, tumor growth, virus replication and the activation of bacterial toxins, their distinct functions are yet to be fully delineated. α1-PDX is an engineered α1-antitrypsin variant carrying the RXXR consensus motif for furin within its reactive site loop. However, α1-PDX inhibits other SPCs in addition to furin. In this work, we prepared various rat α1-antitrypsin variants containing Arg at the P1 site within the reactive site loop, and examined their respective selectivity. The novel α1-antitrypsin variant AVNR (AVPM^{352}/AVNR) was identified as a highly selective inhibitor of furin. This variant formed a sodium dodecyl sulfate- and heat-stable furin/α1-antitrypsin complex and inhibited furin activity *ex vivo* and *in vitro*. Other SPC members including PACE4, PC5/6 and PC7 were not inhibited by the AVNR variant. Furin-mediated maturation of bone morphogenetic protein-4 was completely inhibited by ectopic expression of the AVNR variant. The AVNR variant should prove to be a useful inhibitor in identifying the specific role of furin.

Keywords: α1-antitrypsin/α1-PDX/furin/PACE4/serpin

Introduction

Furin is a Ca^{2+}-dependent serine protease and a member of the subtilisin-like proprotein convertase (SPC) family (Nakayama, 1997; Thomas, 2002). Furin has been shown to play a key role in the proteolytic activation of proproteins through RXKR, RXRR or RXXR sequences (Remacle *et al.*, 2008). To date, seven SPC family members have been identified in mammals, and include furin, PC1/3, PC2, PACE4, PC4, PC5/6 and PC7 (Seidah *et al.*, 1994; Thomas, 2002). Although these enzymes possess similar cleavage specificities, their cellular localization and expression profiles differ. Furin and PC7 are expressed ubiquitously, whereas PACE4 and PC5/6 show cell-specific expression. PC1/3, PC2 and PC4 are located in secretory vesicles of endocrine cells, while furin is mainly localized in the trans-Golgi network as a membrane-bound enzyme. Furin cycles to the cell surface and is then shunted to the trans-Golgi network *via* an endosomal compartment (Thomas, 2002). PACE4 and PC5/6 are secretory enzymes and are localized in the extracellular matrix via interaction with heparan sulfate proteoglycan (Tsuji *et al.*, 2003). Given the expression profile of furin and its cleavage preference, it is likely that this enzyme is a major protease which plays a role in the activation of bacterial toxins (Tsuneoka *et al.*, 1993), viral pathogens (Stieneke-Gröber *et al.*, 1992; Anderson *et al.* 1993), proteins involved in tumor growth and the metastasis of tumor cells such as adhesion molecules (Defilles *et al.*, 2011), growth factors (Wick *et al.*, 2006) and metalloproteases (Sato *et al.*, 1996). However, the involvement of other SPCs has not been fully understood. Although RNA interference has been employed to identify functions of furin (Anders *et al.*, 2006), PC5/6 (Stein *et al.*, 2006) and PACE4 (Yuasa *et al.*, 2009, 2012) in cell cultures, the design of species-specific shRNA target sequences and complete knockdown of the enzyme by RNA interference is generally difficult. However, development of a specific enzyme inhibitor could be utilized as an effective tool in identifying SPC functions, and may be used across different species of organisms.

α1-Antitrypsin is a major serine protease inhibitor (serpin) in plasma whose target enzyme is leukocyte elastase (Huntington, 2006). Following cleavage at Ala-Ile-Pro-Met^{358}↓ within the reactive site loop of human α1-antitrypsin by target protease, cleaved α1-antitrypsin immediately forms a 1 : 1 molar complex (acyl intermediate) with protease that is stable on boiling in 1% sodium dodecyl sulfate (SDS). The cleavage of α1-antitrypsin, complex formation and loss of protease activity are tightly coupled. The protease specificity of α1-antitrypsin can be altered by mutation within the reactive site loop. The α1-PIT (α1-antitrypsin Pittsburgh) variant was identified in a patient with a fatal bleeding disorder (Owen *et al.*, 1983). α1-PIT possesses Ala-Ile-Pro-Arg^{358} within the reactive site loop, which changes its specificity from an inhibitor of elastase to a potent inhibitor of thrombin. High levels of unprocessed proalbumin were found in patient serum (Brennan *et al.*, 1984), suggesting that α1-PIT inhibits furin-catalyzed processing (AGVFRR↓) of proalbumin. α1-Antitrypsin Portland (α1-PDX) is an engineered antitrypsin variant designated as a furin-specific inhibitor (Anderson *et al.*, 1993). This variant possesses a minimum consensus sequence (RXXR) for efficient cleavage by furin within the reactive site loop. α1-PDX is a 3000-fold more effective furin inhibitor than α1-PIT. Intracellular expression of α1-PDX or the exogenous addition of recombinant α1-PDX protein was shown to block tumor growth (Bassi *et al.* 2001), virus replication (Anderson *et al.*, 1993; Jean *et al.*, 2000) and the activation of bacterial toxin (Jean *et al.*, 1998). Successful use of α1-PDX in the inhibition of tumorigenesis has been reported (Nejjari *et al.*, 2004; Scamuffa *et al.*, 2008). α1-PDX was also able to inhibit the invasiveness of squamous cell carcinoma cell lines by blocking the activation of proproteins such as matrix metalloprotease and insulin-like growth factor-1 receptor (López de Cicco *et al.*, 2007). However, PACE4 and PC5/6 are also inhibited by α1-PDX (Tsuji *et al.*, 1999, 2002).

We have continued our efforts to create specific inhibitors of individual SPCs by the engineering of rat α1-antitrypsin, and have shown that the development of specific inhibitors is possible by mutagenesis of the reactive site loop (Tsuji *et al.*, 2002, 2007). Substituting Arg-Arg-Arg-Arg for Ala-Val-Pro-Met^{352} at P4-P1 and Ala for Leu^{354} at P2′ created a potent PACE4- and PC5/6-specific inhibitor (Tsuji *et al.*, 2007). The AVRR α1-antitrypsin variant (Ala-Val-Pro-Met^{352}/Ala-Val-Arg-Arg) exhibited surprisingly unique specific inhibitory activity. This variant inhibited furin and PC5/6, but not PACE4.

In this work, we prepared rat α1-antitrypsin variants by the random substitution of a residue within the reactive site loop, and compared the *in vitro* and *ex vivo* sensitivity of furin, PACE4 and PC5/6 against these variants. As a result, the AVNR variant (Ala-Val-Asn-Arg^{352}) was shown to be a highly specific inhibitor of furin among the SPCs.

Materials and methods

Materials

Pyroglutamyl-Arg-Thr-Lys-Arg-4-methylcoumaryl 7-amide, leupeptin, E-64 [trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane], pepstatin and bestatin were purchased from the Peptide Institute (Osaka, Japan). Rabbit anti-rat α1-antitrypsin antiserum was from ZYMED (San Francisco, CA, USA). Easy Tag™ Express Protein Labeling Mix [^{35}S] was from PerkinElmer (MA, USA). SuperSignal West Pico Stable Peroxide Solution and SuperSignal West Pico Luminol/Enhancer Solution were from Thermo Scientific (Rockford, IL, USA). KOD Plus DNA polymerase was from TOYOBO (Osaka, Japan). Recombinant mouse soluble furin was obtained from R&D systems (Minneapolis, MN, USA). Human α1-antitrypsin, TPCK-treated trypsin, bovine plasma kallikrein and anti-human α1-antitrypsin antibody were from Sigma (St Louis, MO, USA). Bovine thrombin was from Wako (Osaka, Japan). Bovine factor Xa was from Novagen (Madison, WI, USA). Other reagents used were of the highest grade available.

Construction of expression plasmids

Human PACE4A (A-I isoform) was subcloned into mammalian expression vector pALTER-MAX (Promega Corp., Madison, WI), as previously described (Nagahama *et al.*, 1998). Expression vectors for truncated mouse furin (△704/pAC) and PC5/6A (PC5/6A/pRcCMV) were provided by Prof K. Nakayama (Kyoto University). Soluble PC7 (human PC7 lacking membrane-binding domain) cDNA was prepared by polymerase chain reaction (PCR) using EcoRI-linked sense primer (5′-AAGAATTCCTGATGCCGAAGGGGAGGCAGAAAG-3′, initiation codon underlined) and XhoI-linked antisense primer (5′-CCGCTCGAGTCAGGTGTAACCATCTTCCTCAGGAATTTT-3′, stop codon underlined). Human PC7 (PC8) cDNA was kindly provided by Prof M.T. Gillespie (University of Melbourne) (Bruzzaniti *et al.*, 1996) and used as a template. Amplified fragment was subcloned into pcDNA3 expression vector (Invitrogen, Carlsbad, CA, USA). The pMT21 expression vector containing Myc-tagged BMP-4 (bone morphogenetic protein-4) was a gift from Prof D.B. Constam (Ecole Polytechnique Fédérale de Lausanne) (Constam and Robertson, 1999). Rat wild-type α1-antitrypsin (AVPM^{352}, P1 site is Met^{352}), α1-PIT (AVPM^{352}/AVPR), α1-PDX (AVPM^{352}/RVPR), AVRR (AVPM^{352}/AVRR) and R4SA (AVPMSL^{354}/RRRRSA) were subcloned into pcDNA3 vector (Invitrogen), as previously described (Tsuji *et al.* 2002, 2007). Novel variants of rat α1-antitrypsin were generated by a two-step PCR process, as previously described (Tsuji *et al.*, 2002). The sense mutagenic primers used for introducing the mutations within the reactive site loop of α1-antitrypsin are shown in Table I. First, the 3′-terminal cDNA fragment containing the mutated reactive site loop was generated by PCR with KOD Plus DNA polymerase using a sense mutagenic primer and EcoRI-linked antisense primer (5′-CGGAATTCGTGATTAACGTGTGGGATCTATC-3′, stop codon underlined). The amplified fragment was used as an antisense primer for a second PCR. The full-length variant cDNA was then generated by a second PCR using BamHI-linked sense primer (5′-ATGGATCC

Table I. Sense primers used in the mutagenesis of the reactive site loop of rat α1-antitrypsin

Sense primer no.						Nucleotide (amino acid) sequence							
						P4	P3	P2	P1	P1′		P2′	
(1)	5′-	ACT	GTG	GTG	GAG	**D**CC	**N**TC	**N**CC	AGG	TCT	CTG	CCC	-3′
		T	V	V	E	X1	X2	X3	R	S	L	P	
(2)	5′-	ACT	GTG	GTG	GAG	G**D**C	G**N**C	C**N**C	AGG	TCT	CTG	CCC	-3′
		T	V	V	E	X4	X5	X6	R	S	L	P	
(3)	5′-	ACT	GTG	GTG	GAG	GCC	GTC	**NN**C	AGG	TCT	CTG	CCC	-3′
		T	V	V	E	A	V	X7	R	S	L	P	
(4)	5′-	ACT	GTG	GTG	GAG	**D**CC	GTC	CCC	**DN**G	TCT	CTG	CCC	-3′
		T	V	V	E	X8	V	P	X9	S	L	P	

Mutated nucleotides are shown in bold. The bait amino acid that interacts with the S1 site of the protease is referred to as the P1 residue (rat α1-antitrypsin P1: residue 352), with the amino acids on the amino terminal side of the scissile reactive center bond labeled P1, P2, P3 and P4, and the amino acids on the carboxyl side labeled P1′, P2′ (Schechter and Berger, 1967).

X1: Ala/Thr/Ser; X2: Ile/Leu/Phe/Val; X3: Ala/Pro/Ser/Thr; X4: Asp/Gly/Val; X5: Ala/Asp/Gly/Val; X6: Arg/His/Leu/Pro; X7: Ala/Asn/Asp/Arg/Cys/Gly/His/Ile/Leu/Phe/Pro/Ser/Thr/Tyr/Val; X8: Ala/Thr/Ser; X9: Ala /Arg/Glu/Gly//Lys/Met/Ser/Stop/Thr/Trp/Val.

ATGGCACCCTCCATCTCACG-3′, initiation codon underlined) and the first PCR product as the antisense primer. Wild-type α1-antitrypsin cDNA was used as the template. The α1-antitrypsin variants AVNK and AVNA cDNAs were created using mutation primers 5′-GTCAACAAGTCTCTG CCCCCTCAAGTG-3′ and 5′-GTCAACGCGTCTCTGCC CCCTCAAGTG-3′ (mutations underlined) as described above. AVNR variant cDNA was used as a template. Human α1-antitrypsin cDNA was amplified by PCR using an N-terminal sense primer (5′-TGATCAACCATG CCGTC TTCTGTCTCG-3′, initial codon underlined) and antisense primer (5′-TTATTTTGGGTGGGATTCACCAC-3′, terminal codon underlined). Total human liver cDNA library was used as a template for PCR. Amplified cDNA was subcloned into pGEM-T Easy vector (Promega). Complete insert sequences confirmed by sequencing were isolated by digestion with restriction enzymes (BamHI and EcoRI) and then inserted into pcDNA3.1. Human α1-antitrypsin cDNA was ligated into pcDNA3.1 vector (Invitrogen) (hAT/pcDNA). Expression vector for human α1-antitrypsin AINR variant (AIPM^{382}/AINR) was prepared by PCR using the PrimeSTAR mutagenesis Basal Kit (Takara-Bio, Kyoto, Japan) with hAT/pcDNA as a template. 5′-GCCATAAATAGATCTATCCCC CCCGAGGTC-3′ (mutation underlined) was used as a sense mutation primer. The mutation was confirmed by DNA sequencing. cDNAs coding for α1-antitrypsin variants devoid of a signal peptide were generated and subcloned into pQE30 vector (Qiagen, Hilden, Germany), as previously described (Tsuji *et al.*, 1999).

Formation of enzyme–inhibitor complex ex vivo

HEK293 and Cos-7 cells were grown at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. Expression plasmids for SPC (1 μg) and α1-antitrypsin variants (1 μg) were cotransfected into cultured cells in a 35-mm dish using Lipofectamine 2000 (Invitrogen). After 48 h, cells were labeled with 100 μCi/ml [^{35}S]Met/Cys for 8 h. The α1-antitrypsin variant and SPC/inhibitor complex in the conditioned medium were detected by immunoprecipitation with anti-α1-antitrypsin antibody and SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970), as previously described (Tsuji *et al.* 2002). As a negative control, wild-type α1-antitrypsin was also cotransfected with the expression vector for SPC. Radioactive bands in the gel were analyzed using a BAS-1500 bio-imaging analyzer (Fuji Film, Tokyo, Japan). Experiments were performed at least three times to ensure reproducibility. The ability of the human AINR variant to form an SDS- and heat-stable complex with furin was analyzed by cotransfecting Cos-7 cells with expression vectors for furin and the AINR variant. Expression vectors for soluble furin (0.5 μg) and the AINR variant (0.5 μg) were cotransfected into cultured cells in a 35-mm dish. Following transfection for 24 h, cells were rinsed with serum-free Opti-MEM and incubated in the same medium (1.0 ml/dish). After 24 h, the conditioned medium was collected and concentrated by ultrafiltration (100 μl). The concentrated conditioned medium (20 μl) was used for the western blot analysis of the AINR variant using anti-human α1-antitrypsin antibody.

Enzyme assays

SPC activity in the conditioned culture medium was assayed in a 0.2-ml reaction mixture comprising 0.1 M Tris-HCl buffer (pH 7.5), 2 mM $CaCl_2$, 0.1 mM *pyr*-RTKR-MCA, protease inhibitors (10 μg/ml each of leupeptin, E-64, bestatin and pepstatin, 1 μg/ml of chymostatin, 10 μg/ml ovoinhibitor and 30 ng/ml TIMP-2) and an appropriate amount of enzyme at 37°C. Purified furin activity was assayed with *pyr*-RTKR-MCA in the absence of protease inhibitors. Trypsin, thrombin, factor Xa and plasma kallikrein activities were assayed with t-butyloxycarbonyl Phe-Ser-Arg-MCA (Boc-FSR-MCA), t-butyloxycarbonyl Val-Pro-Arg-MCA (Boc-VPR-MCA), t-butyloxycarbonyl Ile-Glu-Gly-Arg-MCA (Boc-IEGR-MCA) and Pro-Phe-Arg-MCA (PFR-MCA), respectively. The reaction mixture (total volume 0.2 ml) contained 0.1 M Tris-HCl buffer (pH 7.5) and 50 μM MCA-substrate. Following incubation, the reaction was terminated and the MCA liberated was measured fluorometrically as previously described (Tsuji *et al.*, 1999). Protein content was determined by the method of Bradford using Bio-Rad protein assay reagent with bovine serum albumin as the standard (Bradford, 1976).

Purification of recombinant α1-antitrypsin variants and antithrombin III

α1-Antitrypsin variants α1-PDX and AVNR were expressed in *Escherichia coli* strain BL-21 transformed with expression vectors. Protein expression was induced by the addition of 0.1 mM isopropyl β-D-thiogalactoside as previously described (Tsuji *et al.*, 1999). Cells from a 500-ml culture were disrupted by sonication and then centrifuged. The resulting supernatants (20 ml) were applied to a Ni^{2+}-NTA agarose (Qiagen, Germany) column (1.0 cm × 2.0 cm) equilibrated with the homogenization buffer. The column was washed extensively with 20 mM imidazole buffer (pH 7.85) containing 0.5 M NaCl. Bound proteins were eluted with 50 and 100 mM imidazole (pH 7.85) containing 0.5 M NaCl. α1-Antitrypsin variant eluted in the 100 mM imidazole fraction and was further purified by MonoQ chromatography. Following dialysis against 20 mM Tris-HCl buffer (pH 7.5), the fraction was applied to a MonoQ HR5/5 column (GE Healthcare, Uppsala, Sweden) equilibrated with the dialysis buffer, and eluted with a linear gradient of NaCl (0–0.5 M) in the same buffer. Final preparations of these variants yielded a single band (45 kDa) on SDS-PAGE.

Bovine antithrombin III was purified from fetal calf serum as follows. Fetal calf serum (50 ml) was mixed with the same volume of 0.1 M Tris-HCl buffer (pH 7.5) containing 0.225 M NaCl and then applied to a heparin-Sepharose (GE Healthcare, Uppsala, Sweden) column (1.5 cm × 5.0 cm) equilibrated with 0.1 M Tris-HCl buffer (pH 7.5) containing 0.225 M NaCl. After washing with the same buffer followed by 0.1 M Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl, antithrombin was eluted with 0.1 M Tris-HCl buffer (pH 7.5) containing 2.0 M NaCl. Purified antithrombin III was then dialyzed against 50 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl. The final preparation yielded a single band (58 kDa) on SDS-PAGE.

Formation of enzyme–inhibitor complex in vitro

Recombinant α1-PDX and the ANVR variant (3 μg) were incubated with recombinant furin (1 μg) in 20 μl of 20 mM

Tris-HCl buffer (pH 7.5) containing 2 mM $CaCl_2$ at 4°C. Following incubation for 2 h, the reaction mixture was mixed with 20 μl of SDS-PAGE loading buffer and heated at 95°C for 5 min. Aliquots (10 μl) were subjected to 10% SDS-PAGE and bands were visualized by silver staining. The concentrate (furin activity: 0.055 nmol/min) of the conditioned culture medium from HEK293 cells transiently expressing soluble furin was incubated with purified α1-PDX (5 μg) or the AVNR variant (5 μg) at 37°C. After 0.5–4 h, the reaction mixture was subjected to SDS-PAGE followed by western blotting using anti-α1-antitrypsin antibody.

Inhibition of processing of pro-bone morphogenetic protein-4 by α1-antitrypsin variant ex vivo

HEK293 cells in 35-mm dishes were transfected with 0.5 μg of expression vector containing Myc-tagged pro-bone morphogenetic protein-4 (proBMP-4), soluble furin or the α1-antitrypsin variant as mentioned above. Following 24 h of transfection, the conditioned medium was immunoprecipitated using mouse anti-Myc monoclonal antibody (Nacalai Tesque, Inc., Kyoto, Japan) and protein G-Sepharose at 4°C for 3 h (GE Healthcare). Immunoprecipitated protein was then analyzed by western blotting using rabbit anti-Myc polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

Results

Screening of furin-specific α1-antitrypsin variant

In the present study, site-directed random mutagenesis was applied using degenerate primers targeting the reactive site loop of α1-antitrypsin to introduce variability at the P1-P4 site of the bait region. As shown in Table I, variants carrying XXXR, AVXR or XVPX sequences at the P4-P1 site were created. Following site-directed random mutagenesis, cDNAs coding α1-antitrypsin variants were ligated into a mammalian expression vector (pcDNA3). Following transformation, expression vectors were isolated from 37 ampicillin-resistant *E.coli* clones and then transfected into HEK293 cells. The conditioned medium was collected 48 h after transfection and the expression level of the α1-antitrypsin variant was examined by western blotting using anti-α1-antitrypsin antibody. Eight clones showed relatively high expression of the α1-antitrypsin variant. These variants possessed the amino acid sequences AVFR, SFSR, SLSR, DALR, AVNR, AAVR, GGHR and AVPE at the P4-P1 site of the reactive site loop.

These variants were tested for their ability to form SDS-stable complexes with furin *ex vivo*. Truncated soluble furin was used since the complex in conditioned medium could be detected with higher sensitivity than the complex in cells. Furin and the α1-antitrypsin variant were co-expressed in HEK293 cells in the presence of radiolabeled amino acids, and the complex in the conditioned medium was then immunoprecipitated with anti-α1-antitrypsin antibody as shown in Fig. 1. The α1-PDX variant was secreted in a 55-kDa form when furin was not co-expressed. On the other hand, in addition to the band representing cleaved α1-PDX (51 kDa), a band corresponding to the protease/inhibitor complex was observed when furin was co-expressed with α1-PDX. This band was also detected in the immunoprecipitate with anti-furin antibody (Tsuji *et al.*, 2007). A 105-kDa band was seen only when furin and α1-PDX were co-expressed. This band did not react with anti-furin antibody. As in the case with co-expression of α1-PDX and furin, SDS- and heat-stable furin/inhibitor complex was formed by co-expression of AFVR, SFSR, SLSR, DALR, AVNR or AAVR with furin. However, a complex comprising GGHR or AVPE with furin was not detected. Although expression levels of the 55-kDa native α1-antitrypsin variants were almost the same, the AVNR variant formed a complex with furin the most readily. On the other hand, α1-PDX (55 kDa, native form) was completely converted to its cleaved form (51 kDa) by furin, whereas these novel variants were secreted in their native form (55 kDa). Previously, we showed that the sequence of the reactive site loop of the α1-antitrypsin variant does not necessarily reflect the substrate preference of furin for complex formation activity (Tsuji *et al.*, 2007). Although these variants were more resistant to cleavage by furin compared with α1-PDX, cleaved variants could form an acyl complex with furin more effectively than with α1-PDX. Since the efficiency of stable complex formation of the AVNR variant with furin was the highest among these variants, its selectivity against SPCs was examined.

Complex formation of PACE4, PC5/6 and PC7 with AVNR variant ex vivo

The AVNR variant and SPC (PACE4, PC5/6 or PC7) were co-expressed in HEK293 cells and the variant in the

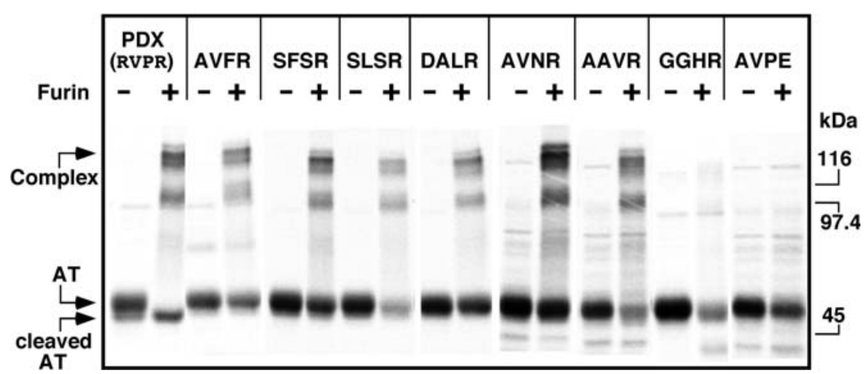

Fig. 1. Formation of SDS- and heat-stable serpin-enzyme complex. The conditioned medium from HEK293 cells transiently expressing furin and the α1-antitrypsin variant was immunoprecipitated with anti-α1-antitrypsin antibody as described in Materials and methods. The immunoprecipitate was analyzed by SDS-PAGE and a BAS 1500 bio-imaging analyzer. Serpin/furin complex, native α1-antitrypsin (AT) and cleaved α1-antitrypsin variants are indicated by arrows.

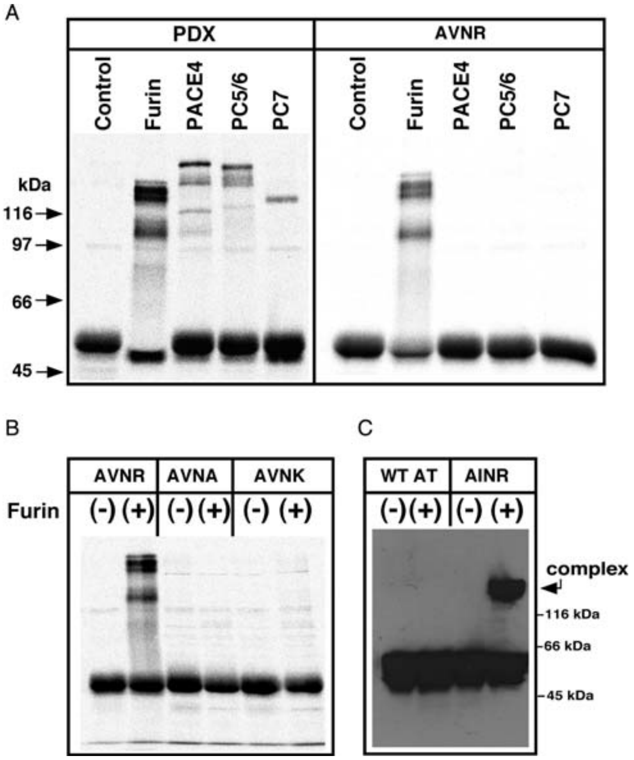

Fig. 2. Specificity of α1-antitrypsin variant AVNR towards furin, PACE4, PC5/6 and PC7. (**A**) Radiolabeled conditioned medium from HEK293 cells transiently expressing furin, PACE4, PC5/6 or PC7 and α1-PDX or the AVNR variant was immunoprecipitated with α1-antitrypsin antibody as described in Materials and methods. As a control, the α1-PDX or AVNR variant was expressed in the absence of SPC. The immunoprecipitate was analyzed by SDS-PAGE and a BAS-1500 bio-imaging analyzer. (**B**) Effect of mutation of the P1 residue of the AVNR variant on SDS-stable complex formation with furin. HEK293 cells were transfected with expression plasmid for furin in the presence of expression plasmid for the AVNR, AVNA or AVNK variant. Serpin/furin complex formation was analyzed as described above. (**C**) SDS-stable complex formation of furin with human α1-antitrypsin AINR variant, (AIPM^{358}/AINR). Cos-7 cells were transfected with expression plasmid for furin in the presence of human AINR variant. Complex formation was analyzed by western blotting using anti-human α1-antitypsin antibody as described in Materials and methods.

conditioned medium was immunoprecipitated with anti-α1-antitrypsin antibody. The AVNR variant failed to form a complex with PACE4, PC5/6 or PC7, and was secreted in a 55-kDa uncleaved form, unlike the case with α1-PDX (Fig. 2A). To confirm cleavage at the P1 site of the AVNR variant by furin, the arginine residue at the P1 site was substituted by Ala (AVNA variant) or Lys (AVNK variant) and complex formation activity was compared. Neither the AVNA nor the AVNK variant were able to form a complex with furin (Fig. 2B). We also examined complex formation activity of the human α1-antitrypsin variant (AIPM^{358}/AINR). As shown in Fig. 2C, the human AINR variant also formed an SDS-stable complex with furin, as was the case with the rat AVNR variant.

Effect of AVNR variant on SPC activity ex vivo

To examine any differences in selectivity of the AVNR variant toward furin, PACE4 and PC5/6, the effect of these variants on furin activity was examined *ex vivo*. The expression vector containing wild-type, α1-PIT, α1-PDX, R4SA, AVRR or AVNR variants of α1-antitrypsin and SPC were co-transfected into HEK293 cells, and secreted SPC activity was examined using *pyr*-RTKR-MCA. This fluorogenic compound is a common substrate for furin, PACE4 and PC5/6. SPC activity was assayed in the presence of various protease inhibitors (leupeptin, bestatin, pepstatin, ovoinhibitor and TIMP-2) to exclude interference by other proteases present in the conditioned culture medium. Furthermore, the activity was assayed in the absence or presence of 20 mM EDTA. SPCs are Ca^{2+}-dependent; therefore, EDTA-sensitive activity was used as a measure of the SPC activity. As shown in Fig. 3, α1-PDX inhibited all SPCs examined. PACE4 and PC5/6 activity was inhibited by the R4SA and AVRR variant, respectively. On the other hand, furin activity was markedly inhibited by expression of the AVNR variant, as was the case with α1-PDX, although it had no effect on PACE4 or PC5/6 activity, unlike the case with α1-PDX. A rat α1-PIT variant containing the AVPR sequence at the P4-P1 site has been shown to have very weak inhibitory activity against furin (Anderson *et al.*, 1993), and did not form an SDS-stable complex with furin (Jean *et al.*, 1998). The R4SA variant (AVPMSL^{354}/RRRRSA) was shown to form a stable inhibitor/protease complex with PACE4 or PC5/6 (Tsuji *et al*., 2007). The AVRR variant (AVPM^{352}/AVRR), which formed an SDS-stable complex with furin and PC5/6, inhibited furin and PC5/6, but not PACE4 (Tsuji *et al.*, 2002). Thus, the inhibitory activity of the AVNR variant toward individual SPCs *ex vivo* is well correlated with its complex formation activity.

Inhibition of furin by AVNR variant and formation of SDS-stable inhibitor/furin complex in vitro

To confirm the inhibitory activity of the AVNR variant toward furin, we first examined the inhibitory activity of purified recombinant AVNR variant against furin in conditioned culture medium from HEK293 cells transiently expressing soluble furin (Fig. 4A and B). Purified AVNR variant was incubated with the conditioned medium and furin activity was then assayed. The AVNR variant (3.6 μg/ml, 80 nM) inhibited 60% of furin activity (0.6 nmol/min), while α1-PDX (0.94 μg/ml, 21 nM) inhibited 80% of furin activity (0.8 nmol/min). The inhibitory activity of the AVNR variant against furin was weaker compared with that of α1-PDX. In an effort to delineate the inhibitory mechanism, the time- and concentration-dependent inactivation of purified furin by the AVNR variant was examined. Progress curves for the hydrolysis of *pyr*-RTKR-MCA by furin in the absence or presence of the AVNR or α1-PDX variant are shown in Fig. 4C. As the concentration of the AVNR variant increased, the initial burst phase of the progress curve shortened and steady-state equilibrium was achieved more rapidly, as was the case with α1-PDX, which indicates slow-binding inhibition. Fig. 4C also shows that the AVNR variant is a markedly less effective inhibitor of furin than α1-PDX *in vitro*.

To compare complex formation activity of the AVNR and α1-PDX variants with furin, recombinant AVNR or the α1-PDX variant was incubated with purified furin and the formation of an SDS-stable complex was analyzed by SDS-PAGE (Fig. 4D). The AVNR variant formed an SDS-stable complex, as was the case with the co-expression experiments in Figs 1 and 2. Although α1-PDX was converted to an SDS-stable complex and cleaved form (43 kDa)

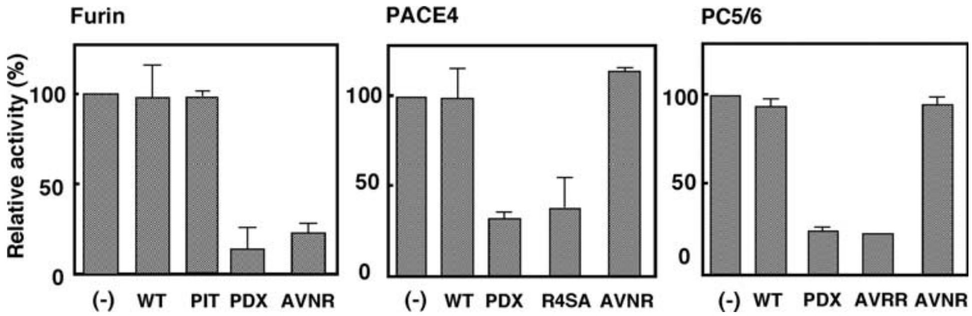

Fig. 3. Effect of α1-antitrypsin variant on furin, PACE4 or PC5/6 activity *ex vivo*. HEK293 cells were transfected with expression vectors for wild-type or antitrypsin variants in the presence of expression vector for furin, PACE4 or PC5/6. After 48 h, *pyr*-RTKR-MCA cleaving activity in the conditioned medium was assayed in the absence or presence of 20 mM EDTA as described in Materials and methods. As a control, HEK293 cells were transfected with pcDNA3 vector and expression vector for furin. EDTA-sensitive *pyr*-RTKR-MCA cleaving activity was used as a measure of the SPC activity. Furin, PACE4 and PC5/6 activity in control conditioned culture medium was 391, 23.6 and 11.7 nmol/h/ml, respectively. Results are shown as means ± SE of three experiments.

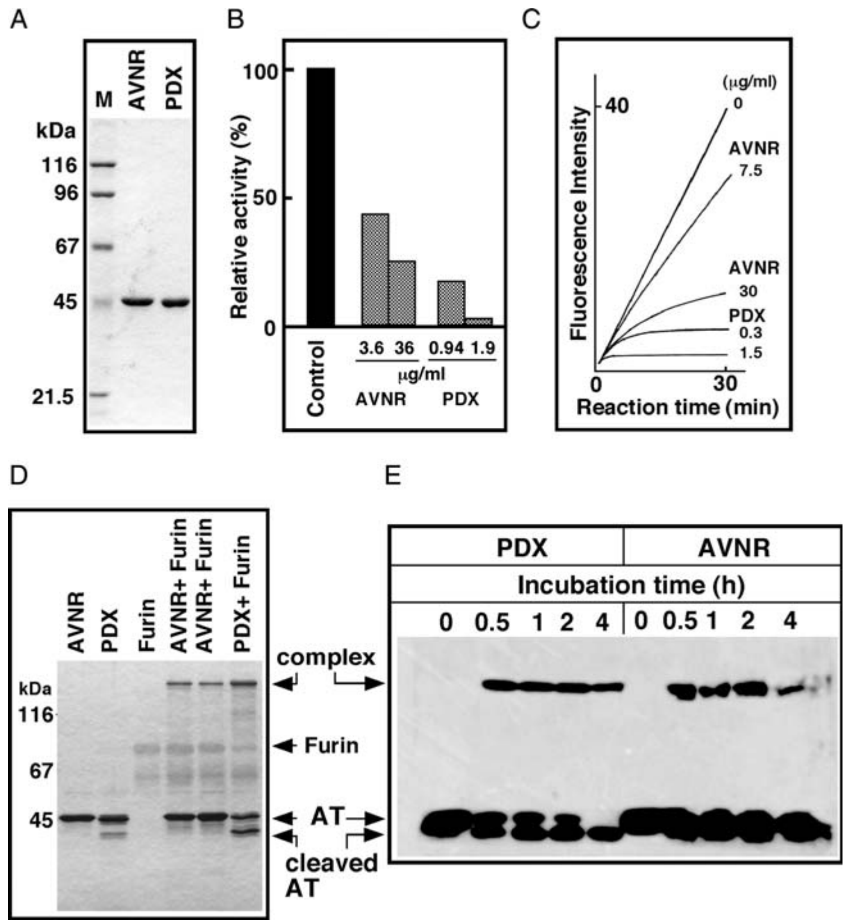

Fig. 4. Inhibition of furin activity by AVNR variant and serpin/furin complex formation *in vitro*. (**A**) SDS-PAGE of purified AVNR and α1-PDX (PDX) variants expressed in *E.coli*. One microgram of each purified variant was subjected to SDS-PAGE (10% gel) and subsequent staining with Coomassie Brilliant Blue. (**B**) Inhibition of furin secreted into conditioned culture medium by the AVNR and α1-PDX variants. The concentrate (furin activity: 1 nmol/min) of conditioned culture medium from Cos-7 cells transiently expressing furin was incubated with purified recombinant AVNR and PDX variants at 4°C for 1 h and furin activity was then assayed. (**C**) Progress curve for the hydrolysis of pyr-RTKR-MCA by furin in the presence of the indicated concentration of recombinant AVNR and PDX variant. Purified furin (0.1 μg, 1.3 nM) was mixed with the AVNR [7.5 μg/ml (167 nM), 30 μg/ml (670 nM)] or PDX [0.3 μg/ml (6.7 nM), 1.5 μg/ml (33 nM)] variant at 30°C in 1.0 ml of 0.1 M Tris-HCl (pH 7.5), 2 μM *pyr*-RTKR-MCA and 2 mM $CaCl_2$. The reaction was monitored continuously by recording the change in fluorescence at 460 nm upon excitation at 380 nm. (**D**) Purifued α1-PDX and the ANVR variant (3 μg) were incubated with purified furin (1 μg) for 2 h, and the formation of furin/serpin complex was analyzed by SDS-PAGE as described in Materials and methods. (**E**) Stability of the furin/AVNR complex *in vitro*. Recombinant α1-PDX or AVNR variant (5 μg) was incubated with conditioned culture medium from HEK293 cells transiently expressing furin at 37°C. After 0, 0.5, 1.0, 2.0 and 4.0 h, the reaction mixture was subjected to SDS-PAGE followed by western blotting using anti-α1-antitrypsin antibody as described in Materials and methods.

by interaction with furin, a cleaved form of the AVNR variant was not detected. In an effort to compare the stability of the complex with furin, the time-course of formation of the SDS-stable complex was analyzed over 4 h by western blotting using the conditioned medium from the HEK293 cells transiently expressing furin and the recombinant AVNR variant. As shown in Fig. 4E, both variants were able to form SDS- and heat-stable complexes at similar levels, with complexes being stable during the 4-h period examined.

Inhibition of furin-mediated processing of proBMP-4 by AVNR variant

To confirm the inhibitory activity of the AVNR variant toward furin in the cell, HEK293 cells were cotransfected with constructs expressing proBMP-4, the AVNR variant and furin. ProBMP-4 has been shown to be preferentially processed by furin into its mature form (Cui *et al.*, 1998). Fig. 5 shows the effects of various α1-antitrypsin variants on proBMP-4 processing in HEK293 cells. The 25–30-kDa band present in all lanes represents the IgG light chain of the antibody used for the immunoprecipitation of BMP-4. In the absence of furin, most of proBMP remained in the cells and was barely secreted. With the exogenous expression of furin, processed BMP-4 was secreted into the culture medium. Although co-expression with wild-type α1-antitrypsin or the α1-PIT variant had no effect on the processing or secretion of mature BMP-4, processing was completely inhibited by the AVNR variant, as was the case with α1-PDX.

Effect of AVNR variant on other serine protease activity in vitro

α1-Antitrypsin Pittsburgh (α1-PIT, Met^{358} is replaced by Arg) efficiently inhibits thrombin, but lacks the neutrophil elastase-inactivating capacity of normal α1-antitrypsin. In addition to thrombin, α1-PIT inhibits other serine proteases, including factor XIa, plasma kallikrein, plasmin, activated protein C and factor XII fragment involved in the contact system of plasma proteolysis (Scott *et al.*, 1986; Travis *et al.*, 1986; Patston *et al.*, 1990). Similarly, the α1-antitrypsin double-mutant having Ala^{357}-Arg^{358} residues at P2 and P1 also inhibits plasma kallikrein, factor XIIa and plasmin (Patston *et al.*, 1990). The inhibitory activity of the AVNR variant against trypsin, thrombin, factor Xa and plasma kallikrein was examined. Purified human α1-antitrypsin or the recombinant AVNR variant was incubated with serine protease in the absence of substrate, and enzyme activity was assayed using synthetic substrates (Fig. 6). The AVNR variant showed stronger inhibitory activity against trypsin, factor Xa and plasma kallikrein compared with wild-type α1-antitrypsin. The inhibitory activity of the AVNR variant and wild-type α1-antitrypsin against thrombin was markedly weaker compared with that against antithrombin III. The variant exhibited particularly strong inhibitory activity against plasma kallikrein, while wild-type α1-antitrypsin did not inhibit kallikrein. The α1-PDX variant showed no

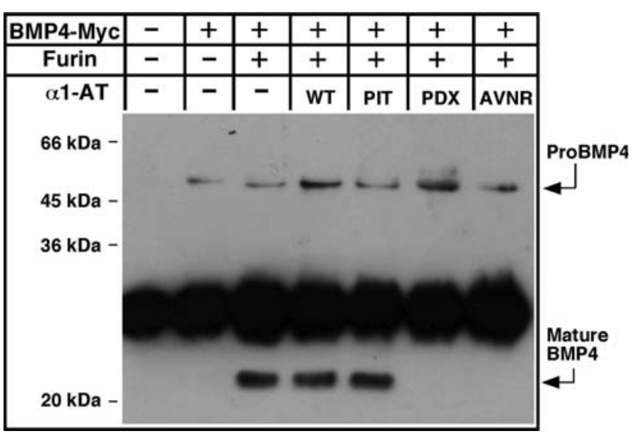

Fig. 5. Inhibition of proBMP-4 maturation by AVNR variant. HEK293 cells were transfected with expression plasmids for proBMP4 (BMP4-Myc), furin and α1-antitrypsin (wild type or variant). After transfection for 24 h, the conditioned medium was immunoprecipitated and then analyzed by SDS-PAGE as described in Materials and methods.

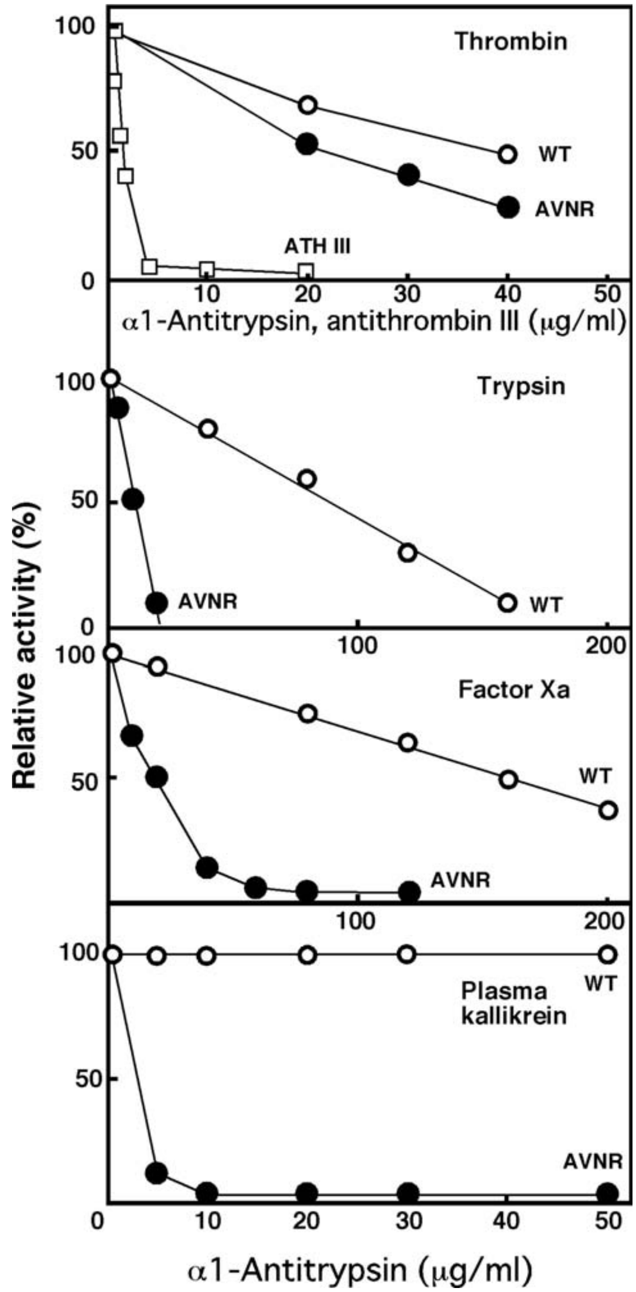

Fig. 6. Inhibition of thrombin, trypsin, factor Xa and plasma kallikrein by AVNR variant. Thrombin (3.35 nmol/min), trypsin (4.35 nmol/min), factor Xa (1.0 nmol/min) and kallikrein (0.634 nmol/min) were incubated with wild-type human α1-antitrypsin (WT, open circles) or the AVNR variant (AVNR, closed circles) in phosphate-buffered saline (0.1 ml) at 25°C. After 1 h, residual thrombin, trypsin, factor Xa and plasma kallikrein activities were assayed using Boc-VPR-MCA, Boc-FSR-MCA, Boc-IFGR-MCA and PFR-MCA, respectively. Antithrombin III was incubated with thrombin in the presence of 20 μg/ml heparin and thrombin activity was assayed (ATHIII, open squares). The measurements were repeated in triplicate. Deviation was <5% of the mean value.

inhibitory activity against thrombin, trypsin, factor Xa or plasma kallikrein.

Discussion

In this study, XXXR variants were constructed by site-directed random mutagenesis and furin-specific α1-antitrypsin variants were screened by examining SDS-stable complex formation activity in HEK293 cells. Six novel α-antitrypsin variants (AVFR, SFSR, DALR, AVNR and AAVR) were identified which could form SDS-stable complexes with furin. All variants lacked Arg at the P4 site. Among these variants, the AVNR variant seems to form a stable complex with furin the most readily. In contrast, GGHR variants containing a single arginine residue at the P1 site (AVPM^{352}/GGHR) did not form stable complexes with furin, indicating the significance of residues at the P2, P3 and P4 sites. Previously, we showed that novel α1-antitrypsin AVRR (AVPM^{352}/AVRR) and ARRR (AVPM^{352}/ARRR) variants could also form SDS-stable complexes with furin and PC5/6 (Tsuji *et al.*, 2002, 2007). On the other hand, variant RRRRSA (AVPMSL^{354}/RRRRSA) was efficiently cleaved by furin without formation of a stable complex (Tsuji *et al.*, 2007). Thus, the sequence of the reactive site loop of the α1-antitrypsin variant which can form a stable complex with furin does not always coincide with the cleavage preference of furin (Dufour *et al.*, 2001; Tsuji *et al.*, 2002, 2007).

α1-PIT, a naturally occurring α1-antitrypsin variant, is a potent inhibitor of thrombin and differs from α1-PDX by a single residue at the P4 site in the reactive site loop. The AVNR variant differs from rat α1-PIT by a single residue at the P2 site. Pro at the P2 site of α1-PIT is substituted by Asn in the AVNR variant, and this mutation results in a marked change of SPC selectivity of the α1-antitrypsin variant. α1-PIT was shown to exhibit very weak inhibitory activity against furin (Brennan *et al.*, 1984; Anderson *et al.*, 1993; Jean *et al.*, 1998), and did not form an SDS-stable protease/inhibitor complex with furin. In contrast, the AVNR variant formed a serpin/furin complex. Thus, the proline residue at the P2 site plays an important role in serpin/furin complex formation.

Co-expression experiments showed that the AVNR variant is specific for furin. PACE4, PC5/6 and PC7 do not form stable complexes with this variant. Unlike α1-PDX, cleaved AVNR variant was not detected in the co-expression and *in vitro* experiments. Following formation of the α1-PDX/furin acyl complex intermediate, half of the complex proceeds toward the substrate pathway and cleaved α1-PDX is released, while the rest of the complex proceeds toward the inhibitor pathway and forms an SDS-stable protease/inhibitor complex (Jean *et al.*, 1998; Huntington, 2006). The relative flux (SI) of the α1-antitrypsin variant through these pathways reflects its efficiency as an inhibitor for a given endoprotease. In the case of α1-PDX and furin, the SI value was 2.0 (Jean *et al.*, 1998). When purified furin was incubated with the α1-PDX or AVNR variant *in vitro*, an SDS-stable inhibitor/furin complex was formed. However, a 43-kDa-cleaved antitrypsin (Glu^{25}-Arg^{352}) fragment of the AVNR variant was not produced by interaction with furin. It is likely that most of the acyl intermediate in the case of the AVNR variant forms a stable complex with furin without cleavage at the P1 site of the variant. The stability of the AVNR variant/furin and α1-PDX/furin complexes with furin did not differ significantly. Furthermore, the AVNR variant completely inhibited the furin-mediated processing of BMP-4 in HEK293 cells. In the cell transfection experiments, since the high concentration of the AVNR variant reacts with furin within the Golgi apparatus and trans-*Golgi* network, furin activity might be efficiently inactivated by complex formation, although its inhibitory activity is lower compared with that of the α1-PDX variant *in vitro*. Our results clearly indicated that the AVNR variant is a highly selective inhibitor of furin among the SPC family. To confirm the specificity of the α-antitrypsin variant possessing Asn and Arg at the P2 and P1 positions, respectively, we created a human α1-antitrypsin variant (AINR variant, AIPM^{358}/AINR) which contained Asn^{357} at the P2 and Arg^{358} at the P1 positions. The amino acid sequence of human α1-antitrypsin is highly homologous with the sequence of rat α1-antitrypsin (amino acid identity: 65%), a notable difference being a six-amino acid residue (PQGDAA) insertion between amino acid positions 26 and 27 of rat α1-antitrypsin. Co-expression experiments showed that the human AINR variant formed an SDS-stable complex with furin, as was the case with the rat AVNR variant.

Furin and other SPC family members (PACE4, PC5/6 and PC7) which function in the processing of proproteins synthesized within the constitutive pathway (Thomas, 2002) are functionally redundant in some cases, such as proalbumin processing in liver (Mori *et al.*, 1999). However, these members possess unique individual roles (Scamuffa *et al.*, 2006; Seidah and Prat, 2012). To date, the unique role of PACE4 has been demonstrated in various cell differentiation systems (Yuasa *et al.*, 2007, 2009, 2012). On the other hand, the opposing functions of furin and PACE4 on breast cancer cell phenotypes have been reported (Lapierre *et al.*, 2007). Based on these studies, PACE4 and PC5/6 in particular seem to be required to maintain the balance between growth factors and receptors and associated antagonistic proteins in order to control cell differentiation and growth. Thus, the precise shared functional roles of furin, PACE4 and PC5/6 remain to be completely elucidated. Although various furin inhibitors including synthetic compounds and engineered proteins have been developed (Seidah and Prat, 2012; Sielaff *et al.*, 2012), a furin-specific inhibitor is yet to be reported. Compared with the α1-PDX variant, the AVNR variant is more selective against furin, although its reactivity is lower. Furin-mediated processing of proprotein in cultured cells is specifically inhibited by exogenous expression of the AVNR variant. Use of the AVNR variant should prove to be a powerful tool in clarifying the specific role of furin in cell cultures.

Acknowledgement

The authors wish to thank Dr K. Nakayama for the gift of the truncated furin cDNA and PC5/6 cDNA, Dr M.T. Gillespie for the gift of PC7 cDNA and Dr Constam for the gift of Myc-tagged BMP4 cDNA.

Funding

Financial support from The University of Tokushima is gratefully acknowledged.

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