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Furin has the proalbumin substrate specificity and serpin inhibitory properties of an in situ hepatic convertase

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Abstract

Furin, a KEX2 protease homolog with high RNA expression in the liver is an excellent candidate as a hepatic proprotein convertase. Here we show that purified recombinant furin has the same proalbumin specificity and serpin inhibitory properties as the in situ hepatic convertase. There was rapid cleavage at the -RRD- site of normal human proalbumin but there no significant cleavage of natural unprocessed variants with cleavage site sequences of -RRV-, -HRD-, -RQD-, or -CRD-. Cleavage of the latter was not increased by S-aminoethylation. Furin was specifically inhibited by α_1-antitrypsin Pittsburgh (358 Met→Arg), ($K^1/_2$ = 3 μM) but not by 50 μM normal antitrypsin M or by antithrombin, however, antithrombin/heparin was a good inhibitor ($K^1/_2$ = 9 μM). The pH optimum for proalbumin cleavage was between pH 5.5 and 6.0, indicating that furin is potentially fully active within secretory vesicles, the site of proalbumin cleavage.

Key words: Furin; Proalbumin conversion; Serpin inhibition; pH optimum

1. Introduction

Since the discovery of the structure of proinsulin 25 years ago [1], there has been an intensive search for the endoprotease involved in the cleavage of prohormones and proproteins at di- and multibasic sequences prior to their secretion. The identification of the yeast KEX2 gene product as being responsible for the cleavage of pro-α-factor and prokiller toxin at Lys Arg sequences [2] led to the identification of a series of mammalian cDNA homologs (furin, PC2, PC1/3, PC4, PACE4 and PC6) of this subtilisin-like protease [3–10]. Co-expression of these putative convertases with proprotein substrates established that they were indeed capable of cleaving target substrates [11–13], and more recently, recombinant forms have been expressed in mammalian cells and the encoded proteases isolated from cell supernatants [14–17]. This advance has facilitated the characterization of their pH optima, substrate specificities and inhibitory profiles with class-directed inhibitors. Not too surprisingly, in general terms, these enzymes have been found to be KEX2-like but with differences in the basic sequences that they recognize.

Just as the *kex2* mutants of *S. cerevisiae* led to the identification of the yeast convertase, mutations in humans that prevented the processing of proalbumin at its diarginyl site, permitted the prediction of the specificity and serpin inhibitory profile of the in situ hepatic convertase. These predictions were that: (i) the protease should cleave the propeptide Arg^{-6}-Gly-Val-Phe-Arg-Arg^{-1} from the N-terminal of proalbumin, but not cleave the variants proalbumin Lille (−2 Arg→His) and proalbumin Christchurch (−1 Arg→Gln), which make up 50% of the serum albumin in heterozygous carriers; (ii) it should be a serine protease inhibitable by the reactive center variant α_1-antitrypsin Pittsburgh (358 Met→Arg) but not by normal antitrypsin M or the other major liver-derived Arg serpin, antithrombin [18–20]. A Ca^{2+}-dependent KEX2-like protease with these precise properties was subsequently identified in hepatic Golgi secreting vesicles [21]. The existence of a similar, if not identical, KEX-2-like hepatic protease has been confirmed by others [22,23].

The more recent detection of the circulating proalbumin variants, Blenheim (1 Asp→Val) and Kaikoura (−2 Arg→Cys) further refine the predicted specificity of the in situ convertase. Heterozygous carriers of the Blenheim mutation have 10% proalbumin, and 40% mature albumin Blenheim in circulation [24], but it is not known whether this reflects a total failure of in situ processing with subsequent partial circulating cleavage by an unknown protease, or simply reflects partial cleavage by the hepatic convertase. It has, however, been shown that hepatic secretory vesicle extracts fail to cleave this substrate [24] and that transfected COS-1 cells secrete the unprocessed proalbumin [25]. Proalbumin Kaikoura (−2 Arg→Cys) comprises only 3–5% of the circulatory albu-

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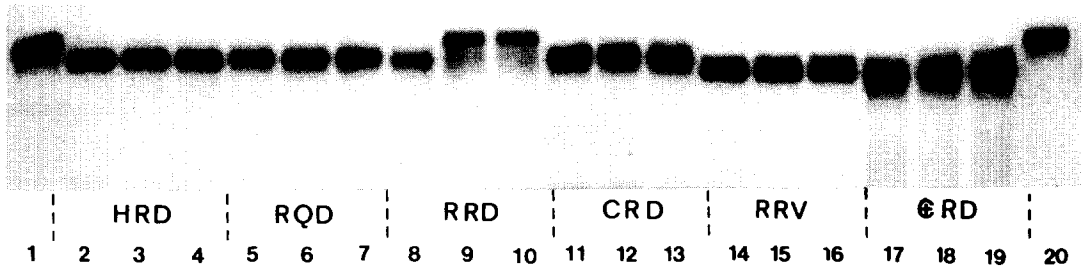

Fig. 1. Cleavage of human proalbumin variants by Δ704 furin. Each reaction contained 10 μg of proalbumin in 3 μl 20 mM MES, 1 mM $CaCl_2$ pH 7.0 containing 1 mg/l ml α globin. To this was added 0.33 μl (4.5 m units) of furin and incubation was carried out at 30°C for 0, 3 and 6 h. The reactions were stopped by freezing and then analyzed by agarose gel electrophoresis at pH 8.6 with Coomassie blue staining, anode at top. Lanes 1 and 20: mature serum albumin marker. Lanes 2–4: proalbumin Lille 0, 3 and 6 h incubation. Lanes 5–7: proalbumin Christchurch 0, 3 and 6 h incubation. Lanes 8–10: normal human proalbumin 0, 3 and 6 h incubation. Lanes 11–13: proalbumin Kaikoura incubated 0, 3 and 6 h incubation. Lanes 14–16: proalbumin Blenheim 0, 3 and 6 h incubation. Lanes 17–19: aminoethylated proalbumin Kaikoura incubated 0, 3 and 6 h. The cleavage site sequence of each variant is indicated.

min in heterozygotes, the remainder being Arg-albumin (30%) and mature albumin (63–64%) [26]. The Arg-albumin results from the new Cys acting as a site for cleavage by the signal peptidase, but of the expected 20% proalbumin that would reach the secretory vesicles, it is not clear if there is partial processing to give the observed plasma level, or no processing with subsequent slow cleavage during the 18 day half-life of albumin. Notwithstanding these limitations, it is clear that, at best, proalbumin Blenheim and Kaikoura are poor substrates of the in situ convertase.

Although furin mRNA is ubiquitously expressed, its high level in the liver makes it a candidate hepatic convertase. Here we investigate the proalbumin specificity and serpin inhibitory profile of a purified truncated (soluble) form of furin in order to determine if it has the predicted properties of the in situ proalbumin convertase.

2. Materials and methods

Human proalbumin and antitrypsin Pittsburgh were isolated from the plasma of the affected child as described earlier [19]. Antithrombin was isolated from normal human plasma by heparin affinity and QAE-Sephadex chromatography [27]. Antitrypsin M was also purified from normal plasma, and variant proalbumins were isolated from the plasma of heterozygous carriers [26]. S-Aminoethylation of the Cys residues at positions −1 and 34 of proalbumin Kaikoura was carried out as previously described [28].

The establishment of the CHO cell line, CHO/Δ704, expressing the Δ704 mutant of mouse furin has been previously described as has the purification of this C-terminally truncated form of the enzyme [16]. The major 81/83 kDa doublet form of the protease was used in this investigation and it had a specific activity of 178 units/mg [16]. The purified furin was stored at a concentration of 13.3 U/ml at −100°C in 10 mM MES, 1 mM $CaCl_2$ containing 5 mg/ml ovalbumin until required.

Proalbumin converting activity was assayed by electrophoresis in 1% agarose gels using Tris-barbitol buffer pH 8.6 [19]. Where ^{125}I-proalbumin was used, approximately 100,000 cpm was incubated with the protease and the reaction was terminated by freezing. Prior to electrophoresis, 0.5 μl of heterozygous proalbumin Christchurch plasma was added as a carrier/internal protein marker. In experiments with cold proalbumin, 10 μg of substrate was used and in some cases, 0.2 μCi $^{63}Ni^{2+}$ was added after incubation, ^{63}Ni autoradiography was performed prior to Coomassie blue staining [19].

Automated protein sequence analysis was performed on an Applied Biosystems 471A sequencer using a Problott cartidge.

3. Results

Furin specifically cleaves the propeptide Arg-Gly-Val-Phe-Arg-Arg from the N-terminus of purified human proalbumin to generate the more anodal band of mature serum albumin (Fig. 1, lanes 8–10). Authenticity of cleavage to the mature N-terminal sequence of Asp-Ala-His- was confirmed by $^{63}Ni^{2+}$ autoradiography (not shown) [19] and N-terminal sequence analysis of the product after transfer to a PVDF membrane. There was greater than 90% conversion of normal proalbumin to albumin after 3 h incubation (Fig. 1, lanes 8–10), representing a reaction rate of greater than 0.2 nmol cleaved/min/unit of furin under these conditions. There was, however, no detectable cleavage of the circulating variants proalbumin Lille (−2 Arg→His), Christchurch (−1 Arg→Gln) or Blenheim (1 Asp→Val) even after twice this incubation period (6 h). But there was very minor cleavage of proalbumin Kaikoura (−2 Arg→Cys) and its S-aminoethylated derivative after the 6 h incubation (Fig. 1). This was confirmed with prolonged incubations

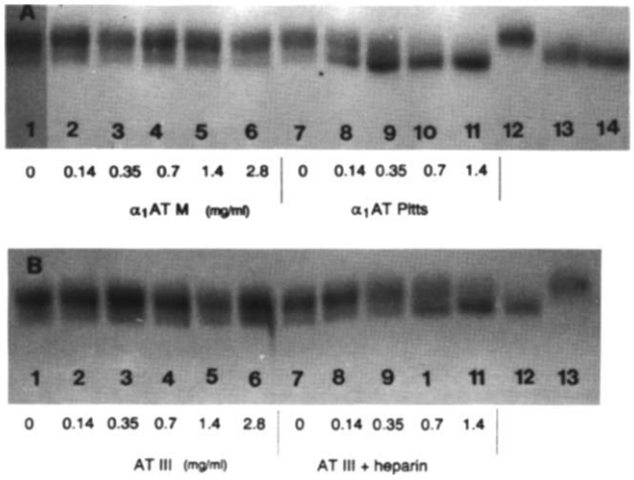

Fig. 2. (A and B) Serpin inhibitory profile of furin catalyzed cleavage of ^{125}I-human proalbumin. Increasing amounts of the specified serpin (0–10 μg) were freeze-dried and redissolved in 2.76 μl 50 mM MES, 1 mM $CaCl_2$ pH 6.5, 1 mg/ml α globin and 0.24 μl of furin. The enzyme and inhibitor were incubated for 15 min prior to the addition of 0.5 μl of substrate; ^{125}I-human proalbumin (10^5 cpm). The reaction was then incubated at 30°C for 3 h and 0.5 μl of plasma added as a carrier/marker prior to electrophoresis and autoradiography. (A) lanes 1–6: 0, 0.14, 0.35, 0.7, 1.4 and 2.8 mg/ml final concentration of antitrypsin M, respectively. Lanes 7–11: 0, 0.14, 0.35, 0.7 and 1.4 mg/ml antitrypsin Pittsburgh. (B) Lanes 1–6: as for (A) but using antithrombin. Lanes 7–11: as for (A) but using antithrombin with 0.2 U heparin. Positive trypsin controls were included; 0.9 μg trypsin in 3 μl 25 mM phosphate buffer pH 7.0 was pre-incubated with the specified serpin and treated as above. Lane A12: no added serpin. Lane A13: 0.7 mg/ml antitrypsin M. Lane A14: 0.7 mg/ml antitrypsin Pittsburgh. Lane B12: 0.7 mg/ml antithrombin. Lane B13: 0.2 U heparin.

(not shown) which showed that both of these substrates were cleaved at approximately 1% of the rate of normal proalbumin.

The recent finding of circulating proalbumin in association with a second case of the Pittsburg mutation [29] testifies to the validity of the earlier proposal that the in situ proalbumin convertase should be inhibited by antitrypsin Pittsburgh but not normal antitrypsin M or other liver-derived Arg-serpins. In order to test whether purified furin could meet these criteria, ^{125}I-proalbumin was incubated with furin under conditions that allowed 95% conversion (Fig. 2A, lanes 1 and 7). Increasing concentrations of normal antitrypsin M from 0.14 mg/ml to 2.8 mg/ml had no effect on conversion (Fig. 2A, lanes 2–6). In the case of antitrypsin Pittsburgh, however, 50% inhibition was achieved at an inhibitor concentration of 0.14 mg/ml (Fig. 2A, lanes 8–11). By way of positive controls, lane 12 shows the trypsin catalyzed cleavage of proalbumin which, as expected, was completely inhibited by the presence of 0.7 mg/ml antitrypsin M (lane 13) and antitrypsin Pittsburg (lane 14). This concentration of inhibitor represents a 1.4 molar excess over trypsin. The furin catalyzed cleavage of proalbumin was resistant to inhibition by antithrombin at concentrations from 0.14 mg/ml to 2.8 mg/ml (Fig. 2B, lanes 2–6). While 0.2 units of heparin itself had no effect on furin (lane 7), heparin activated antithrombin produced a 50% inhibition of proalbumin conversion at a concentration of about 0.5 mg/ml (lanes 8–11). Again, as a positive control, the trypsin catalyzed cleavage of proalbumin was inhibited fully by 0.7 mg/ml antithrombin even in the absence of heparin (lane 12).

Serpins usually act as suicide inhibitors with the P_1 residue acting as a bait for the target serine protease [30]. The result of this reaction is usually the formation of a tetrahedral complex between the active site serine of the protease and the carbonyl carbon of the P_1 residue. This reaction can then proceed to an acyl protease complex and becomes irreversible. We examined the reversibility of formation of the antitrypsin-furin complex by attempting to regenerate proalbumin cleavage ability in the presence of excess thrombin. Antitrypsin Pittsburgh is an excellent inhibitor of thrombin. Thrombin itself, however, does not cleave proalbumin (Fig. 3, lane 5) nor does it impede furin's ability to do so (lane 4). The ability of antitrypsin Pittsburgh to inhibit furin (lane 2) was reversed if excess thrombin was added to the Pittsburgh–furin complex prior to the addition of substrate (lane 3). Thrombin is, in effect, able to titrate the antitrypsin Pittsburgh off furin, indicating that there is unlikely to be a covalent bond between them.

Two separate reports place the pH optimum of furin-catalyzed hydrolysis of Boc-Arg-Val-Arg-Arg-MCA at just over 7, with 50% activity extending between pH 6

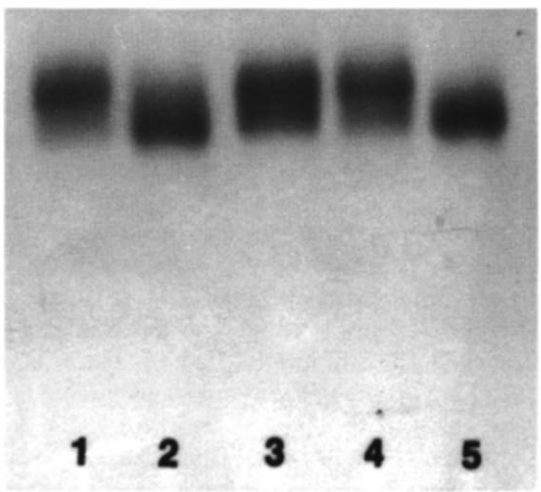

Fig. 3 Autoradiograph showing the reversibility of antitrypsin Pittsburgh inhibition of furin catalyzed cleavage of proalbumin. Lane 1: 0.2 μl furin + proalbumin. Lane 2: 0.2 μl furin + 1.5 μg antitrypsin Pittsburgh + proalbumin. Lane 3: 0.2 μl furin + 1.5 μg antitrypsin Pittsburgh + 2.5 μg thrombin and proalbumin. Lane 4: 0.2 μl furin + 2.5 μg thrombin + proalbumin. Lane 5: 2.5 μg thrombin + proalbumin. Reactions were carried out in a total volume of 3.75 μl 50 mM MES pH 6.5, 1 mM $CaCl_2$ 1 mg/ml α globin. Antitrypsin Pittsburgh and furin were preincubated for 20 min, thrombin was then added and the reaction incubated for a further 20 min to permit possible dissociation of complexes. Proalbumin was then added to all tubes which were incubated for 2.5 h at 30°C prior to electrophoresis and autoradiography.

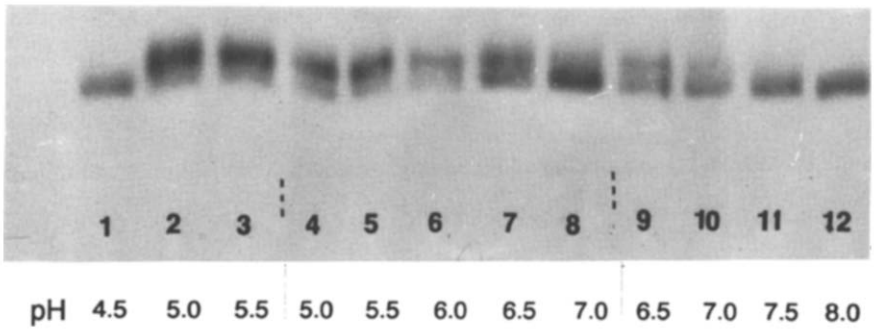

Fig. 4 pH Dependence of furin cleavage of ^{125}I-human proalbumin. All reactions contained 3 μl of the specified buffer with contained 1 mg/l α globin. To this was added 0.3 μl (6×10^4 cpm) ^{125}I-proalbumin and 0.2 μl of furin, incubation was for 2 h at 30°C. Lanes 1–3: 50 mM acetate, 1 mM $CaCl_2$ pH 4.5, 5.0 and 5.5, respectively. Lanes 4–8: 50 mM MES, 1 mM $CaCl_2$ pH 5.0, 5.5, 6.0, 6.5 and 7.0, respectively. Lanes 9–12: 50 mM Hepes/Tris, 1 mM $CaCl_2$ pH 6.5, 7.0, 7.5 and 8.0, respectively.

and 8.5 [16,17]. When assayed by proalbumin conversion, however, the pH optimum was between 5.5 and 6.0 in 50 mM MES buffer; 90% of this activity was retained at pH 5.0 but less than 10% activity was retained at pH 7.0 (Fig. 4). Confirmatory results were obtained in 50 mM acetate which showed 90% activity at pH 5.0 and zero activity at pH 4.5. In 50 mM Hepes/Tris, activity was barely detectable at pH 7.0 and none was detected at pH 7.5 or 8.0.

4. Discussion

The availability of naturally occurring unprocessed variants of human proalbumin allowed a direct comparison of furin to the in situ convertase. Furin has the same proalbumin specificity as the in situ enzyme giving rapid cleavage of normal proalbumin (cleavage site sequence -Arg-Arg-Asp-), but no significant cleavage of the physiologically unprocessed variants Lille, Christchurch or Blenheim with respective processing site sequences of -His-Arg-Asp- , -Arg-Gln-Asp- , or -Arg-Arg-Val- (Fig. 1). The very minor cleavage at the Cys Arg Asp site of proalbumin Kaikoura is unlikely to be of physiological significance, but it is of interest that aminoethylation of this Cys to a Lys analogue did not significantly alter the slow cleavage rate, supporting the proposal [31] that hepatic processing at adjacent dibasic sites requires an Arg-Arg rather than a Lys-Arg sequence. In contrast, the yeast KEX2 protease cleaves aminoethylated proalbumin Kaikoura at the same rate as normal proablumin [28].

Since neither furin, the hepatic Golgi vesicle converting activity [24], nor co-transfection of COS cells [25] produce any significant cleavage of proalbumin Blenheim it raises the question why does plasma contain 10% proalbumin and 40% mature albumin Blenheim? The most reasonable explanation in that, like proalbumin Lille or Christchurch which form 50% of the circulating albumin in heterozygotes, it is initially exported unprocessed, but undergoes specific proteolysis in circulation. Once activated, the coagulation zymogens cleave their targets at specific arginyl sites, which ae frequently followed by a hydrophobic residue (e.g. factor X_a, XII_a and thrombin activate factor VII by cleavage at a Gly Arg Ile site and factor XII_a cleaves factor XI at a Pro Arg Ile site). We have shown (unpublished) that prolonged incubation with thrombin leads to cleavage of proalbumin Blenheim (-RRV-) but not proalbumin Lille (-HRD-) supporting the notion that, physiologically, proalbumin Blenheim is specifically cleaved in circulation by activated coagulation factors.

The only instance where normal proalbumin, with an intact processing site sequence, has been found in circulation, was in association with a mutation at the P_1 inhibitory site of α_1-antitrypsin Pittsburgh. This 358 Met→Arg mutation abolished the inhibitor's ability to inhibit its physiological substrate, leukocytic elastase, and instead, targeted it against serine proteases that cleaved at Arg residue [32]. This provided an explanation for the circulatory proalbumin: the conversion was being blocked by the new inhibitor [19] which is co-secreted with proalbumin from hepatic secretory vesicles [33]. This explanation was supported by co-expression experiments [34] and the recent finding of circulatory proalbumin in association with a second de novo Pittsburgh mutation [29] validates the proposal that antitrypsin Pittsburgh is an in situ inhibition of liver conversion and the corollary to this, that neither normal antitrypsin or its P_1 Arg homolog antithrombin (minus heparin) are such inhibitors. The demonstration that antitrypsin Pittsburgh specifically inhibited the yeast-KEX2 protease helped validate KEX2 as a model mammalian convertase and indeed, was the first substantial indication that the KEX2 enzyme was a serine protease [35].

The recent finding that recombinant antitrypsin Pittsburgh, when present at 0.5 mg/ml, did not inhibit the furin catalyzed cleavage of anthrax toxin protective anti-

gen [17] cast doubt on both the Pittsburgh prediction and furin's potential role as an in vivo convertase. This finding, however, is at variance with the demonstration that furin's cleavage of proC3 reached 50% inhibition at antitrypsin Pittsburgh concentrations of 0.1 mg/l but was unaffected by the highest concentration of normal antitrypsin M used (0.4 mg/l) [36].

The results presented here clearly show that furin's serpin inhibitory profile precisely matches that of the in situ hepatic convertase, with no inhibition by antitrypsin M or antithrombin even at concentrations of 2.8 mg/ml, but with 50% inhibition by antitrypsin Pittsburgh at 0.14 mg/ml. These results contrast markedly with the effects of these inhibitors on trypsin, which is inhibited, not only by both forms of antitrypsin, but also by antithrombin in the absence of heparin. To be an effective inhibitor of coagulation, antithrombin requires activation by heparin which is located on endothelial surfaces. The need to produce antithrombin in a latent form, however, might be unrelated to its role as an anticoagulant; it may be to prevent it acting as an inhibitor of proprotein conversion during antithrombin's secretion for the hepatocyte. Certainly, activated antithrombin is a good inhibitor of furin ($K^1/_2$, 0.5 mg/ml).

Previous reports using fluorogenic peptide substrates placed furin's pH optimum that just over pH 7 [16,17], casting doubt on its potential role in the acidic environment of the secretory vesicle. The findings here, however, show that with a more physiologically relevant substrate, its activity is an order of magnitude greater between pH 5.5 and 6.0 than it is at pH 7.0. This parallels the findings with a recombinant mouse prorenin substrate ($M2R^{-4}$) where the pH optimum for cleavage was reported to be 6.0 [37].

This lower pH optimum has important implications; it indicates that the protease could be involved in post Golgi cleavage. This, together with the proalbumin specificity and serpin inhibitory properties as the in situ convertase, give furin the hallmarks of an authentic hepatic convertase.

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