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Cleavage of proalbumin peptides by furin reveals unexpected restrictions at the P_2 and P'_1 sites

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Abstract

Proalbumin is the principal substrate of the in situ hepatic convertase. Here we investigated the specificity of furin using synthetic peptides based on the N-terminal sequence of human proalbumin. The propeptide was rapidly cleaved from the normal ((−6)RGVFRR(−1)DAHKSEAVW(+9)) peptide but as expected, there was no cleavage of the proalbumin Lille analogue with a −2 His (−2H). Surprisingly, the effect of this substitution could not be corrected by introducing a −4 Arg (−4R−2H). In contrast, the peptide −4R−2A was an excellent substrate being cleaved five times faster than normal, indicaabting that His is not allowed as an P_2 residue. Replacement of the −4 Val by Glu supported the expected importance of a positive charge at P_4 as the cleavage rate dropped to 10% of normal after this substitution. The −6 Arg makes a small contribution to cleavage, its replacement by Ala decreased the cleavage rate to 60% of normal. The Lys-Arg propeptide was almost as good a substrate as the normal Arg-Arg peptide, but the introduction of a Lys at P'_1 totally abolished processing. The exclusion of P'_1 positive charges would be an important requirement for preventing aberrant cleavage in the middle of tetrabasic sequences.

Key words: Convertase; Furin specificity; Proalbumin peptide

1. Introduction

Since the discovery of the first mammalian KEX2-like protease in hepatic secretory vesicles [1] a series of cDNA homologs (furin PC2, PC1/3, PC4, PC6 and PACE4) of this yeast convertase has now been identified in different mammalian tissues [2–10]. Members of this emerging family of Ca^{2+}-dependent serine proteases vary in their site of expression and in the basic sequence that they recognise.

Although furin mRNA is expressed ubiquitously, it reaches particularly high levels in the liver, making it a likely candidate as an endogenous hepatic convertase. Although some 23 different proproteins are constitutively cleaved before export from the liver, quantitatively the amount of proalbumin processed far exceeds that of all other proproteins combined.

We have recently shown that a purified recombinant form of furin correctly cleaved the propeptide (Arg-Gly-Val-Phe-Arg-Arg) from human proalbumin but failed to cleave unprocessed natural variants with mutation of −2Arg→His, −1Arg→Gln, +1Asp→Val and −2Arg→Cys, giving this protease the correct credentials as an in situ convertase [11].

In order to further define the substrate requirements of furin and to capitalise on the insights already gained from the natural variants, we examined its ability to cleave a series of synthetic peptides based on the N-terminal of human proalbumin.

2. Materials and methods

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 [12]. The major 81/83 kDa doublet form of the protease was used in this investigation and it had a specific activity of 178 U/mg where 1 U is defined as the amount which hydrolyses 1 nmol Boc-Arg-Val-Arg-Arg-MCA per min at pH 7.0 and 37°C [12]. 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.

Synthetic peptide substrates were based on the N-terminal sequence of human proalbumin and were obtained from Chiron Mimotopes, Vic., Australia. All peptides were 15 residues long and had blocked N-(acetyl) and C- (amide) terminals. Trp rather than the normal His was incorporated at position +9 to facilitate the rapid identification of the N- and C-terminal cleavage fragments. The purity and sequence fidelity of the peptides were determined/verified by the manufacturer using ion spray/triple quadrapole mass spectrometry.

The reaction mixture for each assay contained 1 nmol of proalbumin peptide in 2.5 μl 50 mM MES, 1 mM $CaCl_2$, pH 5.5 containing 1 mg/ml α globin. To this was added 0.35 μl (4.5 mU) of furin and the solution incubated at 30°C for specified times between 0.5 and 8 h. The reaction was stopped by the addition of 50 μl of buffer A (below) and after microfuging 40 μl was injected onto a 3μ Nova Pac C-18 column and the extent of hydrolysis determined by HPLC. The solvent system consisted of: solvent A, 49 mM phosphate buffer, pH 2.9; B, equal mixture of solvent A and acetonitrile [13]. The flow rate was 1 ml/min and the gradient went from 18 to 46% B over 14 min. The absorbance was monitored at both 215 and 254 nm. In order to negate the effects of any slight variation in injection volume between runs the reaction rate was calculated as percentage conversion/min. This was expressed as peak height of the C-terminal product divided by height of the C-terminal product + height of the parent peptide peak.

Amino acid analysis of isolated cleavage products was performed

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using the PITC derivatisation procedure, and sequence analysis was performed on an Applied Biosystems 471A instrument.

3. Results

Table 1 shows the sequences of the variant proalbumin peptides that were used in this study and their retention times in the HPLC assay system. The peptides were greater than 90% pure as judged by HPLC (not shown) and this was consistent with the manufacturer's assessment using ion spray mass spectrometry. The normal sequence has been designated peptide A and the numbering system of serum albumin has been retained, with residues −1 to −6 representing the propeptide and 1 to 9 the mature N-terminal sequence.

Incubation of the normal peptide A with furin (Fig. 1) resulted in its progressive hydrolysis and the concomitant appearance of two more polar peaks, the second of which contained the Trp residue, as indicated by the 254 nm absorbance trace (not shown). The identity of both products was confirmed by composition/sequence analysis as RGVFRR and DAHKSEVAW, respectively, and the hydrolysis rate was 0.75 nmol/min/U enzyme.

As neither furin nor the in situ hepatic convertase cleave the natural circulating variant proalbumin Lille (−2Arg→His), it was not surprising to find that the proalbumin peptide (−2H) with the corresponding substitution was not hydrolysed even after 8 h incubation (Fig. 2).

We reasoned, however, that because of the known preference of furin for paired arginines in either a −2−1 or −4−1 configuration, the introduction of a −4R in the Lille peptide analogue would convert it to a substrate that might be cleaved faster than the normal peptide. This was not the case since, despite its apparently ideal −6−4−1 triplet Arg sequence there was no cleavage of the −4R−2H peptide even after 8 h (Fig. 2). This suggests that furin's sequence requirements disallow a P_2 His under any circumstances.

Table 1
Sequences of proalbumin peptides

	-5			-1	1				5							Cleavage rate*	Relative rate#	Retention time (min)
A	Ac-R	G	V	F	R	R	D	A	H	K	S	E	V	A	W-NH_2	0.75	100	12
-2H					H											0.00	0	12
-4R-2H			R		H											0.00	0	8.5
-4E			E													0.06	8	8.5
-2K					K											0.51	72	12
-6A	A															0.17	22	15
+1K						K										0.00	0	11
-4R-2A			R		A											3.55	474	10.5
Proalbumin	R	G	V	F	R	R	D	A	H	K	S	E	V	A	H			

* nmol/min/U enzyme.
#Cleavage rate relative to normal peptide (100).

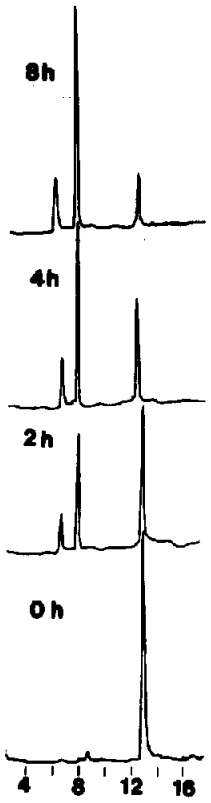

Fig. 1. Reverse-phase HPLC profile showing furin catalysed cleavage of proalbumin peptide A (normal). 1 nmol samples of peptide were analyzed after 0, 2, 4 and 8 h incubation with furin (0.25 fM); 3μ C-18 column, detection at 215 nm; other details in text.

To further explore the requirement for substrates with a positive residue in the P_4 position, we examined the hydrolysis of a peptide (−4E) where the natural Val was replaced by a negatively charged Glu residue. In this case, the cleavage rate was substantially reduced with a decrease to 8% of the normal peptide (Figs. 2 and 3). Note that in this case only the C-terminal product is seen as the increased polarity of the propeptide causes it to emerge in the injection peak.

The positive contribution to the cleavage rate of a −4Arg residue was further established by examining the hydrolysis of peptide −4R−2A. This contained a −4, instead of a −2 Arg residue. Not only did cleavage take place, but the rate was 5-times faster than that of the normal peptide (Figs. 2 and 3). This result, taken together with the complete failure to cleave the corresponding −4R−2H peptide, confirms that histidine must be excluded for the −2 position.

Peptide −2K was investigated to establish if a Lys-Arg sequence was as effective as an Arg-Arg as a permitted processing site. Good cleavage was observed (Fig. 2) with the rate (0.51 nmol/min/U enzyme) only sightly less

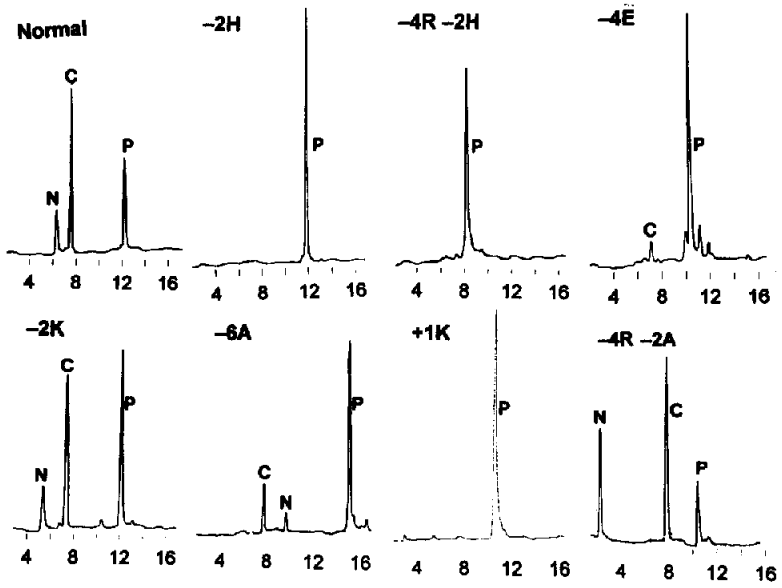

Fig. 2. Cleavage of variant proalbumin peptides by furin. Top left panel: normal peptide (RGVFRRDAHKSEVAW); subsequent panels show variants with the designated substitutions. The data shown is for a 4 h incubation except in the case of − 4R− 2A where the 1 h incubation is shown. P, parent peptide; N, N-terminal fragment; C, C-terminal fragment. HPLC conditions as described in section 2.

than that (0.75 nmol/min/U enzyme) for the normal Arg-Arg peptide.

It has been suggested that the lack of a −4Arg in the sequence of human proalbumin might be partially offset by the presence of Arg in the −6 position. We found that the −6Arg does make a significant contribution to cleavage since its replacement by Ala in peptide −6A resulted in a four-fold reduction in the hydrolysis rate of the peptide (Figs. 2 and 3).

There has been little examination of the contribution of the P'_1 site to peptide bond cleavage. This is partly because there are no suitable chromogenic/fluorogenic substrates available, since the reporting group itself occupies the P'_1 site, and because comparison of the sequence in this area in a series of proproteins (Table 2) does not reveal any obvious trend that might be exploited by site-directed mutagenesis. If furin is the in situ hepatic convertase, however, then it must be able to cleave after tri- and tetrabasic sequences (e.g. RRKR or RRRR) without cleavage after the first dibasic pair (RR). This could be achieved if the enzyme's specificity excluded positive charges in the P'_1 position. To test this possibility, we examined the cleavage of peptide + 1K where the P'_1 Asp was replaced by Lys, and confirmed that this change totally blocked processing at the Arg- Arg site

4. Discussion

The findings here provide new insights into the primary sequences that are recognised by furin, and strengthen furin's suggested role as an in situ hepatic convertase [11].

The rat hepatic converting enzyme was previously shown to have a preference for chicken proalbumin with a processing sequence of RFAR over human proalbumin (VFRR). It was suggested that the hepatic vesicle activity was principally directed towards an RXYR or XYRR sequence with a 5- to 10-fold preference for the former [14]. We find that purified furin has a similar specificity, cleaving the RFAR proalbumin peptide five times faster than the normal VFRR peptide.

The finding that furin cleaved the KR site of the −2K peptide almost as efficiently as it cleaved the RR peptide was unexpected since the hepatic vesicle activity fails to cleave at the PQKR site of proinsulin [14]. However, if furin is 'the' hepatic convertase, it would be necessary for it to be capable of cleaving at some simple KR sites since the processing of proprotein C involves cleavage at both an IRKR and a HLKR site (Table 2). Co-expression experiments are also consistent with this having shown that furin can cleave at some, e.g. mutant, pro Von Willebrand factor (ASKR) [10], but not all, e.g. prorenin (FTKR) [15], KR sites. Elucidating the precise features that make some KR sites favourable will need further investigation.

That these propeptide analogues correctly mimic their natural equivalents is well established by the processing of the normal peptide and lack of processing of the proalbumin Lille analogue with the −2H. We have previously shown that neither the hepatic activity nor purified furin cleave proalbumin Lille (−2Arg ›His) [11], and Matsuo [16] has demonstrated that a synthetic Lille peptide is not cleaved by hepatic vesicle extracts.

A novel restriction to furin's specificity was discovered in its failure to cleave the −4R−2H peptide. The positive

effects of the highly favoured −4−1 diarginyl sequence that was observed for the RFAR sequence were totally abolished if the −2 residue was a His. This is particularly surprising since at the pH used (5.5) the peptide would have positive centers at the −2 as well as at the −4 and −1 sites. This finding does, however, strengthen furin's role as an in vivo hepatic convertase since of the 23 different proprotein sites that are cleaved during the export from the mammalian liver, none has a P_1 His (Table 2). Indeed, when mammalian species variation is taken into account, this number increases to 47 out of 47 sites that lack a −2 His.

The 78% decrease in cleavage rate observed when the −6R of proalbumin was replaced by Ala, points to the enhancing effect of this residue on processing at XYRR sites, and is reflected by the presence of a −6R residue in other hepatic substrates with a simple XYRR site such as the protein C junction (Table 2). The in situ cleavage at this site is still, however, suboptimal since 10–20% of circulating protein C is in the single chain form [17].

Many hepatic substrates have consecutive tri- or tetrabasic sequences, e.g. RRRR (Table 2) and since furin is capable of cleavage after RR sites, a potential problem arises where there might be aberrant cleavage after the first paired sequence. This could be avoided if the protease had a specificity restriction to exclude P'_1 basic residues. Furin appears to have just this specificity; there was no cleavage of the + 1Lys peptide. This finding adds to the restrictions imposed on this site that exclude large alkyl sidechains (Val-Leu-Ile) that was first highlighted by the discovery of the unprocessed variant proalbumin Blenheim (+1Asp→Val) [18] and expanded by site-directed mutagenesis of proalbumin [19].

Table 2
Processing site sequences of human liver proproteins

Plasma proteins	P_6	P_5	P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3
Proalbumin	R	G	V	F	R	**R**	D	A	H
Proalbumin c	L	Q	**R**	F	A	**R**	D	A	E
Pro-apoA2	–	A	L	V	R	**R**	Q	A	K
Pro-Iα Ti 1	I	P	**R**	V	R	**R**	A	V	L
Pro-Iα Ti 2	N	R	**R**	Y	Q	**R**	S	L	P
Coagulation factors									
Fibrinogen α-chain	K	S	**R**	P	V	**R**	G	I	H
Prothrombin	L	Q	**R**	V	R	**R**	A	N	T
Profactor IX	L	N	**R**	P	K	**R**	Y	N	S
Profactor X	L	A	**R**	V	T	**R**	A	N	S
Factor X h/l chain	L	E	**R**	R	K	**R**	S	V	A
Profactor XII	L	H	**R**	R	R	**R**	A	N	A
Proprotein C	L	R	**I**	R	K	**R**	A	N	S
Protein C h/l chain	R	S	**H**	L	K	**R**	D	T	E
Proprotein S	L	V	**R**	K	R	**R**	A	N	S
Proprotein Z	L	V	**R**	W	K	**R**	A	G	S
Receptors									
Insulin proreceptor	P	S	**R**	K	R	**R**	S	L	G
IGF proreceptor	P	E	**R**	K	R	**R**	D	V	M
Pro-LDL RRP	S	N	**R**	H	R	**R**	Q	I	D
HGF receptor	E	K	**R**	K	K	**R**	S	T	K
Complement system									
C3 α/β junction	A	A	**R**	R	R	**R**	S	V	Q
C4 α/β junction	T	T	**R**	K	K	**R**	N	V	N
C4 α/γ junction	R	N	**R**	R	R	**R**	E	A	P
C5 α/β junction	I	L	**R**	P	R	**R**	T	L	Q
Convertases									
Profurin	K	R	**R**	T	K	**R**	D	V	Y

Pro-Iα Ti = prointer α-trypsin inhibitor, IGF = insulin-like growth factor, Pro-LDL RRP = pro LDL receptor-related protein, Chicken proalbumin (c) is included for comparison.

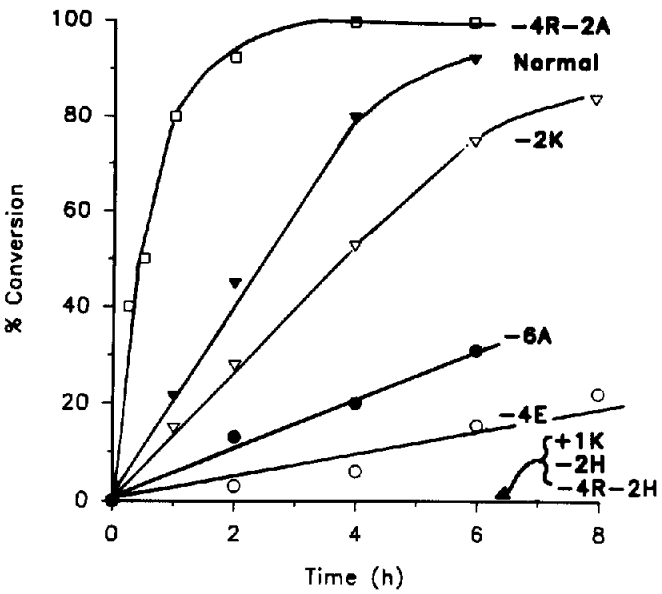

Fig. 3. Cleavage of designated synthetic proalbumin peptides by furin as a function of time.

The role that the furin homolog PACE4 may play in the liver is presently unclear but recent co-expression experiments have shown it to be proteolytically active and somewhat different to furin in its cleavage of mutated pro Von Willebrand factor (which is not produced in hepatocytes but endothelial cells). PACE4's lack of inhibition by α_1-antitrypsin Pittsburgh [20], however, makes it substantially different from furin and the in situ enzyme. The findings here, on the other hand, show that furin has a similar specificity to that expected of the in situ convertase. This, together with its inhibition by antitrypsin Pittsburgh, indicate that it could play a major role in liver processing. The substrate specificity of PACE4 should now be compared to the in vivo sequence and furin in order to assess its possible contribution to liver processing.

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