Cleavage of proalbumin peptides by furin reveals unexpected restrictions at the P₂ and P₁ 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, indicating that His is not allowed as an P₂ residue. Replacement of the -4 Val by Glu supported the expected importance of a positive charge at P₁ 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 P₁ totally abolished processing. The exclusion of P₁ 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 Ca²⁺-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/A704, expressing the A704 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 CaCl₂ 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 peptides were determined/verified by the manufacturer using ion spray/triple quadrupole mass spectrometry.

The reaction mixture for each assay contained 1 nmol of proalbumin peptide in 2.5 µl 50 mM MES, 1 mM CaCl₂, 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 3 µ 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 acetone (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
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 P2 His under any circumstances.

To further explore the requirement for substrates with a positive residue in the P4 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 slightly less

Table 1
Sequences of proalbumin peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Cleavage rate</th>
<th>Relative rate</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.75</td>
<td>100</td>
<td>12</td>
</tr>
<tr>
<td>-2H</td>
<td>0.00</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>-4R-2H</td>
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<td>0</td>
<td>8.5</td>
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<td>8</td>
<td>8.5</td>
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<td>0.51</td>
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<td>12</td>
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<td>15</td>
</tr>
<tr>
<td>+4K</td>
<td>0.00</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>-4R-2A</td>
<td>3.55</td>
<td>474</td>
<td>10.5</td>
</tr>
</tbody>
</table>

Proalbumin RGVFRRDAHKSEVAH

* 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); 3 μ C-18 column, detection at 215 nm; other details in text.
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.

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].
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 $P_2$ 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 tetra-basic 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 ($+1$Asp$\rightarrow$Val) [18] and expanded by site-directed mutagenesis of proalbumin [19].

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 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, indicates 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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References