Endoproteolytic processing of recombinant proalbumin variants by the yeast Kex2 protease

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The yeast Kex2 protease is regarded as the prototype of the eukaryotic family of subtilisin-like serine proteases involved in processing after dibasic amino acid sequences. Here we investigate the specificity of Kex2 using recombinant human proalbumin variants. Proalbumins with the processing site sequences Arg-Arg and Lys-Arg were cleaved after the dibasic sequence at approximately the same rate by Kex2 *in vitro*, and yeast expressing either of these sequences secreted mature albumin into the culture medium. As expected, the Arg-Gly-Val-Phe-His-Arg-albumin (proalbumin Lille) was not a substrate for Kex2 and neither was the Arg-Gly-Arg-Phe-His-Arg-albumin.

In contrast to the mammalian endoproteases furin and the hepatic proalbumin convertase, the Kex2 protease was adversely affected by a P4 arginine. There was an 85% decrease in the cleavage of Arg-Gly-Arg-Phe-Arg-Arg-albumin compared with normal; also chicken proalbumin with an Arg-Phe-Ala-Arg processing site sequence was not a substrate for Kex2. A P1' arginine had a marked negative effect on processing and N-terminal sequence analysis confirmed that cleavage was occurring at the P1–P1' bond. The sequence context surrounding the classical dibasic site is critical in determining susceptibility to cleavage by the Kex2 protease.

INTRODUCTION

Endoproteolytic cleavage of precursor polypeptides targeted to the secretory pathway is a common post-translational event. Cleavage on the carboxyl side of pairs of basic residues occurs in a diverse range of precursors including plasma proteins, peptide hormones, neuropeptides, growth factors and receptors. The *Saccharomyces cerevisiae* Kex2 protease (kexin) (EC 3.4.21.61) was the first enzyme proven by biochemical and genetic means to cleave proproteins at dibasic sites [1,2]. The Kex2 gene sequence [3] showed that the predicted protease was related to the subtilisin family of serine proteases and that it encoded an 814-amino-acid type-I membrane protein with six structural domains. The transcript is a Ca²⁺-dependent serine protease and in the yeast is involved in the proteolytic maturation of killer toxin and α -mating factor by cleaving after -Arg-Arg-, -Lys-Arg- and possibly -Pro-Arg- sequences [1].

There are striking similarities between the processing of mammalian proproteins and the endogenous substrates of Kex2. The discovery over the last five years of mammalian homologues of Kex2 (e.g. furin, PC2, PC1/3, PACE4, etc) suggests conservation of this enzymic mechanism throughout eukaryotic evolution (for recent reviews see [4–6]). There is 40–65% identity between the catalytic domains of members of this superfamily. However, the sequence similarity between Kex2 and the mammalian convertases finishes at the end of the P-domain which is also essential for activity [7]. Further evidence for the similarity of the yeast and mammalian proteases has come from the demonstration that Kex2 can correctly cleave a number of mammalian proproteins including human proinsulin [8], human proalbumin [9], mouse pro-opiomelanocortin [10], human protein C precursor [11], human pro-von Willebrand Factor [12], rat proinsulin I [13] and human parainfluenza virus type-3 F protein

[14]. This confirms that Kex2 is an appropriate model for the mammalian dibasic-directed proprotein convertases.

Proalbumin is cleaved by a Ca²⁺-dependent Kex2-like membrane-bound protease in hepatocyte Golgi membranes [15]. We have previously shown that this activity and the Kex2 protease have similar, but not identical, specificities for human proalbumin and a number of naturally occurring proalbumin variants [9,16], thus demonstrating that Kex2 mimics the properties of a human proalbumin convertase.

Specificity studies using fluorogenic peptides and overexpression with potential substrates have indicated that the primary specificity determinants for Kex2 are the P1 and P2 residues. It has, however, been shown that overexpression can give a false impression of an enzyme's substrate specificity [17], and the cleavage of small peptide substrates may not be fully representative of native proproteins. To circumvent these difficulties we have investigated the *in vivo* and *in vitro* specificity of the Kex2 protease by expressing various human proalbumin variants in *kex2+* and *kex2-* *S. cerevisiae* strains. We further define the contribution of -1, -2 and -1, -4 dibasic configurations to processing efficiency by determining the relative cleavage rates of human proalbumin (Arg-Gly-Val-Phe-Arg-Arg-alb) and chicken proalbumin (Arg-Asp-Leu-Gln-Arg-Phe-Ala-Arg-alb) *in vitro*.

MATERIALS AND METHODS

Mutagenesis

A human preproalbumin cDNA in the yeast expression vector pAB23 [18] (Figure 1) was supplied by Chiron Corporation (Emeryville, CA, U.S.A.). A *Bam*H1 fragment containing the entire preproalbumin coding sequence and the glyceraldehyde-3-

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Figure 1 Structure of plasmid pAB23.alb

Human preproalbumin cDNA was cloned into the *Bgl*II site of pAB23 and is flanked by the glyceraldehyde-3-phosphate dehydrogenase promoter and terminator (pGAP and tGAP). *bla*, β -lactamase gene; URA3, yeast selectable marker; 2 μ , yeast 2 μ circle sequences.

Table 1 Sequences of oligonucleotide primers used for mutagenesis (mismatches underlined)

Mutant	Oligonucleotide sequence
–2Lys	5'-TCC AGG GGT GTG TTT <u>AAG</u> CGA GAT GCA CAC AAG-3'
–2His	5'-GGG GTG TGT TTC ATC <u>GAG</u> ATG CAC A-3'
–2His –4Arg	5'-GCT TAT TCC AGG GGT <u>CGG</u> TTT CAT CGA GAT GCA CAC AA-3'
–4Arg	5'-GCT TAT TCC AGG GGT <u>CGG</u> TTT CGT CGA GAT GCA-3'
+1Arg	5'-GGT GTG TTT CGT CGA <u>CGT</u> GCA CAC AAG AGT GA-3'

phosphate dehydrogenase promoter and terminator was subcloned into pBLUESCRIPT II SK(–) (Stratagene). Oligonucleotide-directed mutagenesis was carried out on single-stranded uracil-containing templates [19] using primers (Table 1) synthesized on an Applied Biosystems (ABI) 391 DNA Synthesizer. All mutants were sequenced by the dideoxy-chain-termination technique (Sequenase 2.0, United States Biochemical) to verify each mutation and then subcloned back into pAB23 and the orientation determined by restriction enzyme digestion.

Yeast transformation and growth

Yeast strains AB110 (*Mat*, *leu2*, *trp1*, *ura3-52*, *prB1-1122*, *prC1-407*, [cir]) and AB118 (AB110+*kex2*) were transformed with pAB23.alb, pAB23.alb-2K, pAB23.alb-2H, pAB23.alb-4R, pAB23.alb-2H-4R and pAB23.alb + 1R by lithium acetate transformation [20]. Transformed yeast were selected by growth on *ura*[–] minimal plates. Starter cultures were grown in 0.67% Difco yeast nitrogen base without amino acids, 0.5% Difco casamino acids, 0.24 g/l tryptophan, 0.48 g/l adenine, 20 mg/l histidine, 2% D-glucose (YCGM) at 29 °C, 400 rev./min, and large-scale cultures were grown in 1% Difco yeast extract/2% Difco peptone/2% ethanol (YPE) at 29 °C, 400 rev./min to *A*₆₀₀ values of 10–12 for AB110 or 6–8 for AB118.

Purification of expressed proalbumins

Large-scale cultures (600 ml) from AB110 and AB118 transformed yeast were centrifuged and the supernatant precipitated

by addition of ammonium sulphate to 75% saturation. The precipitate was resuspended in a minimal volume of MilliQ water and each AB118 precipitate (containing proalbumin) was applied to a Sephacryl-300 SR gel-filtration column (80 cm × 3 cm) (Pharmacia) equilibrated in 50 mM ammonium bicarbonate. Albumin-containing fractions were pooled, lyophilized and resuspended in MilliQ water. The final purification was performed on a Q-Sepharose column (5 cm × 1 cm) (Pharmacia) equilibrated in 0.1 M Tris, pH 8.3/0.1 M NaCl and eluted with a gradient to 0.1 M Tris, pH 8.3/0.3 M NaCl. Fractions containing albumin were dialysed against water, lyophilized and stored at –20 °C.

Amino acid sequence analysis

After agarose gel electrophoresis proteins were transferred to a Pro-Blott membrane (ABI) and sequenced on an ABI 471A Protein Sequencer according to the manufacturer's protocols.

Source of Kex2 protease

The Kex2 protease was obtained as a membrane extract from *S. cerevisiae* strain AB100 overproducing the protease from a multicopy plasmid pAB230 [16]. Yeast were grown to mid-logarithmic phase and the cells harvested by centrifugation and broken by glass bead lysis in 50 mM Hepes/KOH (pH 7.6). Cell debris was removed by centrifugation at 5000 *g* for 15 min at 4 °C, and membranes were pelleted by centrifugation at 100000 *g* for 90 min at 4 °C. These membranes were resuspended in 50 mM Hepes/Tris (pH 7.6) containing 1% Triton X-100 and stored at –80 °C.

Purification and iodination of human and chicken proalbumins

Human proalbumin was isolated from the plasma of a patient with circulating proalbumin [21] and chicken proalbumin was isolated from liver microsomes as previously described [22]. Iodination was carried out by the method of Hunter [23].

Assay of Kex2 activity

Kex2-enriched membrane preparations (0.6 μ g of protein/assay) were incubated with 2 μ g of expressed proalbumin in a total volume of 5 μ l of 50 mM Mes, pH 6.5/1 mM Ca²⁺/1% Triton X-100 at 30 °C. Incubations were stopped after the specified times by the addition of EDTA to 10 mM.

When ¹²⁵I-labelled chicken or human proalbumin (100000 c.p.m.) were used as substrates, 1 μ g of protein/assay of Kex2-enriched membrane preparations was incubated in a total volume of 6.5 μ l of 0.1 M Hepes/Tris, pH 7.0/5 mM Ca²⁺/1% Triton X-100 at 20 °C. Incubations were stopped by the addition of EDTA to 10 mM.

Conversion of proalbumin to albumin was analysed by agarose gels (1%) electrophoresed in Tris/barbital buffer (38 mM Tris, 46 mM sodium barbital, 16 mM diethyl barbituric acid), pH 8.6. ¹²⁵I or ⁶³Ni autoradiography was carried out as previously described [21].

RESULTS

In vivo processing of proalbumin variants by *S. cerevisiae* strain AB110

Normal human proalbumin and the proalbumin variants listed in Table 2 were expressed in the *kex2*⁺ yeast strain AB110. Ammonium sulphate precipitates of the culture supernatants were analysed by agarose gel electrophoresis (Figure 2). This

Table 2 Sequences of normal and mutant proalbumins and yields from expression in AB110

Abbreviations: Alb, albumin; Proalb, proalbumin.

	P6 -6	P5 -5	P4 -4	P3 -3	P2 -2	P1 -1	P1' +1	P2' +2	P3' +3	Yield (mg/l)*	
										Alb	Proalb
WT	Arg	Gly	Val	Phe	Arg	Arg	Asp	Ala	His	3.0	—
-2Lys	Arg	Gly	Val	Phe	Lys	Arg	Asp	Ala	His	3.0	—
-2His	Arg	Gly	Val	Phe	His	Arg	Asp	Ala	His	—	0.1
-2His-4Arg	Arg	Gly	Arg	Phe	His	Arg	Asp	Ala	His	—	0.1
-4Arg	Arg	Gly	Arg	Phe	Arg	Arg	Asp	Ala	His	0.5	—
+1Arg	Arg	Gly	Val	Phe	Arg	Arg	Arg	Ala	His	3.0	—

* Approximate yield (mg/l) from AB110 culture supernatants.

Figure 2 In vivo processing of proalbumins by *S. cerevisiae*

Agarose gel electrophoresis of ammonium sulphate precipitates of supernatants from AB110 cultures expressing proalbumin. Lane 2, normal proalbumin; lane 3, -2Lys proalbumin; lane 4, -2His proalbumin; lane 5, -2His-4Arg proalbumin; lane 6, -4Arg proalbumin; lane 7, +1Arg proalbumin. Each lane has an equivalent loading of culture supernatant (0.05%). Lanes 1 and 8, control plasma from heterozygote carrier of proalbumin Lille. Anode at top.

charge-based separation allows a clear distinction to be made between proalbumin and albumin since removal of the propeptide results in the loss of three positive charges. Both normal proalbumin (lane 2) and the -2Lys proalbumin (lane 3) were secreted in good yield (see Table 2) as processed mature albumin. However the -2His proalbumin (lane 4) and the -2His-4Arg proalbumin (lane 5) were secreted in the unprocessed form and in considerably lower amounts. Both these unprocessed variants had electrophoretic mobilities as expected from their net charge relative to mature albumin. When lysates of the pelleted yeast were analysed it was found that the normal and -2Lys variant were present in both unprocessed and mature forms. However, in the case of the -2His and -2His-4Arg variants only the unprocessed proalbumin was present inside the cells (not shown). With the -4Arg proalbumin mature albumin was secreted (lane 6), but the yield was 6-fold less than that obtained from normal proalbumin. The +1Arg proalbumin was also secreted in the processed form (lane 7) but in this case the yield was the same as from normal proalbumin. Both of these latter variants were found to be unprocessed intracellularly when the yeast pellets were examined. The +1Arg albumin ran on agarose gels two charges cathodally to normal albumin as expected. ⁶³Ni autoradiography (not shown) was used to confirm that cleavage was occurring at the correct site since the binding of Ni²⁺ to albumin requires a free α-amino group at residue 1 and a histidine at residue 3. Additionally, for the processed +1Arg albumin, amino

Figure 3 In vitro processing of recombinant human proalbumins by Kex2

Agarose gel electrophoresis of Kex2-enriched membranes incubated for 0, 4 and 8 h at 30 °C. Normal recombinant proalbumin (lanes 1–3), -2Lys proalbumin (lanes 4–6), -2His proalbumin (lanes 7–9), -4Arg proalbumin (lanes 13–15), -2His-4Arg proalbumin (lanes 10–12), and +1Arg proalbumin (lanes 16–18). Lane 19, normal albumin (alb). Anode at top.

acid sequence analysis showed that the processing site was the P1-P1' bond.

Is Kex2 responsible for *in vivo* proalbumin processing?

Each of the six constructs were also expressed in a *kex2*-deficient strain (AB118) to verify that the observed *in vivo* processing was due specifically to the Kex2 protease and not some other enzyme. Electrophoretic analysis of the culture supernatants indicated that all albumins, including the normal and the -2Lys variant, were present in the unprocessed state. This was confirmed by amino acid sequence analysis of all six proteins which clearly showed their expected proalbumin sequence. Yields of each were estimated at 3 mg/l and this provided a source of unprocessed proalbumins for more detailed *in vitro* experiments.

In vitro processing of proalbumin variants by Kex2

In order to determine the relative processing rates of the various proalbumins, the purified precursors were incubated with membranes isolated from yeast overexpressing the Kex2 protease. The processing of each proalbumin variant was followed by agarose gel electrophoresis over an 8 h incubation period (Figure 3). There was very similar processing of both the normal and -2Lys proalbumins over this time course, with approximately 90% processing of normal and 70% processing of the -2Lys proalbumin (lanes 1–3 and 4–6 respectively). There was no processing of either the -2His or the -2His-4Arg proalbumins (lanes 7–9 and 13–15 respectively) and only minor cleavage of both the -4Arg and +1Arg proalbumin variants, with the overall cleavage rate less than 5% of the rate observed for normal proalbumin (lanes 10–12 and 16–18). The variation in band intensity in lanes 10–12 was due to a loading artifact and other experiments confirmed the cleavage rate of this variant. The processing site for normal, -2Lys, -4Arg and +1Arg proalbumins was confirmed as being at the P1-P1' site by electrophoretic mobility and ⁶³Ni autoradiography (not shown).

Cleavage after -1, -4 dibasic sites

Since the presence of a -4 arginine had a significant deleterious effect on the ability of the Kex2 protease to cleave human proalbumin we further explored the apparent negative contribution of a P4 arginine by investigating the Kex2-catalysed processing of chicken proalbumin. Unlike human proalbumin, which has a classical Arg-Gly-Val-Phe-Arg-Arg dibasic sequence, chicken proalbumin has a -1, -4 dibasic site and a propeptide sequence of Arg-Asn-Leu-Gln-Arg-Phe-Ala-Arg. Figure 4 shows that human proalbumin underwent rapid hydrolysis with 50%

Figure 4 Processing of human proalbumin and chicken proalbumin by Kex2

Autoradiograph of agarose gel electrophoresis showing processing by Kex2-enriched membranes of ^{125}I -labelled chicken proalbumin (lanes 1–6) and ^{125}I -labelled human proalbumin (lanes 7–12) for 0, 0.5, 1, 2 and 4 h at 30 °C. Anode at top.

cleaved in 1 h and near 100% cleavage in 2 h (Figure 4, lanes 7–11). On the other hand there was no detectable cleavage of the chicken proalbumin even after 4 h incubation (Figure 4, lanes 1–5).

DISCUSSION

The expression of normal human proalbumin in Kex2-positive yeast leads to the secretion of correctly cleaved mature albumin into the culture medium. Changing the dibasic processing site from Arg-Arg to Lys-Arg had no effect on either the amount of albumin secreted or the extent to which it was processed. We had previously shown, *in vitro*, that the circulating variant proalbumin Lille (–2Arg→His) was not a substrate for the Kex2 protease [16]. When a recombinant proalbumin containing this mutation was expressed in strain AB110 (*kex2*+), analysis of the culture supernatant confirmed it was not processed, and also showed that the unprocessed proalbumin was poorly exported from these cells. An identical result was obtained with the corresponding double mutant –2His–4Arg (Arg-Phe-His-Arg). The –4Arg proalbumin with an Arg-Phe-Arg-Arg processing site sequence was present as mature albumin in the culture medium but its low yield implied it was poorly exported from the cells, perhaps because of inefficient intracellular cleavage.

We were surprised at the low level of secretion of the three unprocessed or poorly processed variants, especially since all the proalbumins were secreted equally well from the Kex2-deficient strain. One interpretation of this result is that if processing does not occur the unprocessed proproteins are partially prevented from being secreted; however, the mechanism by which this occurs is not known.

To obtain more precise data on the relative cleavage rates of the recombinant proalbumins, and to compare this with the known specificity of Kex2 for naturally occurring proalbumin variants, we expressed and purified uncleaved recombinant proalbumins from the supernatants of Kex2-deficient AB118 cultures. Indeed the complete failure of processing in this strain confirms that Kex2 and not another endoprotease such as YAP3 [24] is responsible for proalbumin cleavage.

As expected from their expression in AB110, normal proalbumin and the –2Lys proalbumin were processed at similar rates by the Kex2-enriched membranes. This is also consistent with our observation that Kex2-enriched membranes cleave aminoethylated proalbumin Kaikoura (AE-Cys-Arg), with a lysine analogue at P2, almost as efficiently as normal human proalbumin [16]. The lack of processing of the –2His proalbumin was in complete agreement with our earlier result with the

natural variant proalbumin Lille. The –2His–4Arg proalbumin with an Arg-Phe-His-Arg processing site sequence was not a substrate for Kex2. However, we have found that furin-catalysed cleavage of –1,–4 dibasic sites is inhibited by a P2 histidine [25].

Contrary to previous reports [11–13] we found that residues beyond P1 and P2 had a profound effect on the efficiency of proalbumin processing by Kex2. The recombinant proalbumin with the Arg-Phe-Arg-Arg processing site was a poor substrate for Kex2 *in vitro*, consistent with the low yield of mature albumin in the medium of strain AB110 yeast transformed with –4Arg proalbumin. This is in marked contrast to the beneficial effect of a P4 arginine on processing by furin [25] or the hepatic proalbumin convertase (E. C. Ledgerwood and S. O. Brennan, unpublished work). The predicted inability of Kex2 to cleave –1,–4 diarginyl sequences was confirmed by its inability to cleave native chicken proalbumin with a processing site of Arg-Phe-Ala-Arg, again in marked contrast to the hepatic proalbumin convertase which cleaves this substrate five times faster than human proalbumin [26]. In known endogenous Kex2 substrates there is a preference for aliphatic residues at P4 [27]. Possible explanations for the negative impact of a P4 arginine may be gained by comparing the putative substrate-binding region of Kex2 with that of furin which has been modelled on the crystal structures of thermolysin and subtilisin BPN' [28]. Although Kex2 and furin have the same total number of acidic residues in their proposed substrate-binding pockets, the locations differ, with Kex2 having one extra acidic residue in the S2 pocket and one less in the S4 pocket. When one of the P4 acidic residues in furin was mutated the affinity increased for a Lys-Arg site and decreased for an Arg-Xaa-Xaa-Arg site [29].

In vitro the +1Arg proalbumin was a poor substrate for Kex2, which was surprising given that processing occurred in the *kex2*+ strain *in vivo*. This highlights the conflicting results that can arise from *in vitro* versus *in vivo* observations. A possible explanation for the high yield of processed albumin by strain AB110 (*kex2*+) is that some other endoprotease cleaved between the P1 and P1' arginines and that the activity of this protease is *kex2*-dependent. Thus processing would not occur in the *kex2*- strain as was observed.

Clearly, however, a P1' arginine has an unfavourable effect on *in vitro* processing by Kex2. We have observed a similar effect with the mammalian endoprotease furin which will not cleave proalbumin peptides with a P1' lysine [25]. In the current study our prediction was that the processing site would shift to the P1'–P2' peptide bond since many mammalian proproteins have a multibasic processing site terminating in a C-terminal arginine. However, this did not occur and the limited processing occurred exclusively between the P1 and P1' arginine residues. The steric conformation of proalbumin may mean that the potential new site cannot fit correctly into the substrate-binding region of Kex2. Alternatively the new site would have a histidine at P2', and Hosaka et al. [30] have suggested that for furin a hydrophobic residue is preferred at P2'. This may also be true for Kex2, since on the basis of the putative model of furin's catalytic domain the important S2' residues are conserved in Kex2. Finally, Kex2 may not process when there is a P3 arginine. We have observed that P3 arginine is slightly unfavourable for processing by furin (S. O. Brennan and K. Nakayama, unpublished work).

As the first proven member of the eukaryotic family of subtilisin-like serine proteases, Kex2 has contributed significantly to our understanding of mammalian proprotein processing. The data presented here show that Kex2 has its own distinct substrate specificity. The principal criterion which distinguishes it from the archetypal mammalian enzymes furin and the proalbumin convertase is the negative effect of the P4 arginine on Kex2 processing

compared with its critical importance for rapid cleavage by these mammalian endoproteases.

We thank Chiron Corporation for providing us with yeast strains AB110 and AB118 and the expression vectors pAB23.alb and pAB230. This work was supported by a project grant from the Health Research Council of New Zealand.

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Received 26 September 1994/3 January 1995; accepted 11 January 1995