Specificity of Yeast KEX2 Protease for Variant Human Proalbumins Is Identical to the in vivo Specificity of the Hepatic Proalbumin Convertase*

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Yeast KEX2 protease was examined as a potential model for a human proprotein convertase and, in all respects, mimicked the predicted properties of a proalbumin convertase. The enzyme rapidly cleaved the propeptide Arg-Gly-Val-Phe-Arg-Arg from the NH₂-terminal end of proalbumin but, unlike trypsin, failed to cleave physiologically unprocessed human proalbumin variants. There was little or no cleavage of proalbumin Lille (Arg⁻² → His) or Christchurch (Arg⁻¹ → Glu), and there was negligible cleavage of proalbumin Blenheim (Asp¹ → Val), despite the fact that it retains the dibasic processing signal. Proalbumin Kaikoura (Arg⁻² → Cys), which appears to be partially processed in vivo, was cleaved at about half the rate of normal proalbumin despite the absence of a diarginylation sequence. Restoration of a dibasic site in the proalbumin processing signal, Proalbumin Blenheim (Asp⁺ + Val), yielded the predicted properties of a human proalbumin, except that it retains the dibasic processing signal. Proalbumin Kaikoura (Arg⁻² → His), and proalbumin Lille (Arg⁻² → His) were totally unprocessed in vivo. The KEX2-catalyzed cleavage of normal proalbumin was found to be independent of pH between pH 6.0 and 8.0. Antitrypsin Pittsburgh (Met³⁵₈ → Arg), in a boy with a fatal phenotype, was acting as an intracellular inhibitor of proalbumin convertase which is specific for the diarginylation site of proalbumin convertase. The similarity between these proteases is further exemplified by the observation that the proalbumin convertase correctly cleaves human proalbumin. Their relevance as physiological convertases is verified by the fact that neither cleaves proalbumin Christchurch (Arg⁻¹ → Glu) and that both are specifically inhibited by α₁-antitrypsin Pittsburgh (Met³⁵₈ → Arg) but not by normal human antitrypsin with an inhibitory site methionine (residue 358) (6, 7).

The yeast KEX2 protease is tantalizingly similar to these two mammalian convertases in terms of its microsomal location, inhibition by class-directed inhibitors, Ca²⁺ dependence, detergent solubility, and inhibition by antitrypsin Pittsburgh. Also, we have previously shown that KEX2 correctly cleaves the NH₂-terminal hexapeptide Arg-Gly-Val-Phe-Arg-Arg from human proalbumin but that it does not cleave the physiologically unprocessed variant proalbumin Christchurch (Arg⁻¹ → Glu) (8). In this numbering system, the NH₂-terminus (Asp) of mature serum albumin is designated as residue 1 and the propeptide residues are assigned negative numbers from −1 to −6.

In this investigation, we examine the relationship between KEX2 and the predicted properties of the human proalbumin convertase by testing its ability to process other circulatory forms of human proalbumin with mutations in or adjacent to the dibasic sequence, viz. proalbumin Lille (Arg⁻² → His) (9), proalbumin Kaikoura (Arg⁻² → Cys) (10), and proalbumin Blenheim (Asp¹ → Val) (11).

Proalbumin Christchurch (Arg⁻¹ → Glu) and proalbumin Lille (Arg⁻² → His) are totally unprocessed in vivo as the proalbumin forms 50% of the serum albumin in carriers. Although proalbumin Lille and Kaikoura each have mutations involving the Arg⁻², phenotypically they appear quite different; proalbumin Kaikoura represents only 3–5% of the albumin in heterozygotes. Proalbumin Blenheim (Asp¹ → Val) provides another refined way of probing the similarities between KEX2 and the proalbumin convertase. Although it retains the diarginylation site, proalbumin Blenheim is not a substrate for the hepatic convertase (11). We further examine the dibasic specificity of KEX2 by determining its effect on the aminooethylated derivative of proalbumin Kaikoura.

The association of circulating proalbumin (with normal structure) with the presence of a variant protease inhibitor, α₁-antitrypsin Pittsburgh (Met³⁵₈ → Arg), in a boy with a fatal bleeding disorder led to the prediction that the new inhibitor was acting as an intracellular inhibitor of proalbumin conver-
sion in the hepatocyte (12). It was not until several years later
that it was shown that antitrypsin Pittsburgh was a specific inhibitor of a convertase, the yeast KEX2 protease (8). It is only comparatively recently that antitrypsin Pittsburgh was shown to be a specific inhibitor of the proalbumin and proinsulin Ca\(^{2+}\)-dependent convertases (6, 7). In this investigation, we further investigate the nature of this inhibitory complex formed between antitrypsin Pittsburgh and KEX2.

MATERIALS AND METHODS

Human proalbumin substrates, proalbumin Christchurch (Arg\(^-1\) \rightarrow Gln), proalbumin Lille (Arg\(^-\) \rightarrow His), proalbumin Kaikoura (Arg\(^-\) \rightarrow Cys), and proalbumin Blenheim (Asp\(^-1\) \rightarrow Val), were purified from the plasma of heterozygous carriers by chromatography on DEAE-Sephadex using a pH gradient from pH 5.2 to 4.5 (11). Minor contaminating amounts of a2-macroglobulin were removed by gel filtration on Ultragel Aca34. The plasma containing proalbumin Lille was kindly provided by J. M. Fine, Centre National de Transfusion Sanguine, Paris, France. a1-Antitrypsin Pittsburgh (Met\(^{16}\) \rightarrow Arg) and normal human proalbumin were purified from plasma of the afflicted child as described earlier (12).

Bovine threonin was further purified from Parke-Davis Thrombost by ion-exchange chromatography on CM-Sephadex in 0.1 M phosphate buffer, pH 8.6, with a NaCl gradient from 0 to 0.9 M.

The KEX2 protease was obtained as a membrane extract from Saccharomyces cerevisiae strain AB100 overproducing the protease from a multicopy plasmid pAB230 (14, 15). The yeast was grown to midlog phase, and the cells were harvested by centrifugation and broken by glass bead lysis in 50 mM Hepes/KOH, pH 7.6. The cell debris was removed by centrifugation at 5,000 \(\times\) g for 15 min at 4°C and the membranes were pelleted by centrifugation at 100,000 \(\times\) g for 50 min at 4°C. These membranes were resuspended in 50 mM Hepes/Tria, pH 7.6, containing 1% Triton X100 and stored at −80°C.

Proalbumin Kaikoura was aminooxethylated under denaturing conditions. Thiol groups were dissolved in 200 \(\mu\)l of 50 mM Tris/HCl, pH 8.0, containing 150 nmol of diithiothreitol. After an overnight reduction at 37°C, 200 \(\mu\)l of 1 M Tris/HCl, pH 9.1, was added together with 1.5 \(\mu\)l of ethyleneimine: a 120-fold molar excess over total thiol groups present. After 4 h, the solution was dialyzed and the protein was freeze-dried.

Conversion of proalbumin to albumin was assessed by electrophoresis in a 1% agarose gel in Tris-barbiturate buffer (38 mM Tris, 46 mM sodium barbiturate, 16 mM diethyl barbituric acid), pH 8.6.

RESULTS

The yeast KEX2 protease specifically cleaves the prepeptide Arg-Gly-Val-Phe-Arg-Arg from normal human proalbumin to produce mature serum albumin of N\(\text{H}_{2}\)-terminal sequence Asp-Ala-His. The loss of the three positive charges is clearly seen by the increased anodal mobility on agarose gel electrophoresis, and authentic cleavage is confirmed by the ability of the product to bind \(^{3}\)H\(^{2}\)N\(^{2}\) binding is a free \(\alpha\)-amino group on residue 1 and \(\alpha\)-antitrypsin Pittsburgh was reacted with KEX2 extracts (data not shown).

Gln-albumin. It also cleaves proalbumin Blenheim (Asp\(^-1\) \rightarrow Val) at both arginines to give two products, Arg albumin Blenheim (80%) and albumin Blenheim (20%) (11).

KEX2 on the other hand does not cleave the -Arg-Gln-Asp-site of proalbumin Christchurch (Fig. 1, lanes 4–6), and there is negligible cleavage at the -His-Arg-Asp-site of proalbumin Lille (lanes 1–3). Despite the fact that proalbumin Blenheim retains a dibasic processing site sequence (-Arg-Val-), there is only very minor cleavage of this substrate; approximately 5% is cleaved in 4 h (lanes 15–16) compared with 95% cleavage for normal proalbumin. Surprisingly, there is distinct cleavage at the -Cys-Arg-Asp-site of proalbumin Kaikoura with approximately 50% conversion in 4 h (lanes 10–12).

The specificity of KEX2 for dibasic sites was further examined by testing its action on aminooxyethylation proalbumin Kaikoura. Proalbumin Kaikoura contains two free cysteine residues, one in the propeptide at residue -2 and one in the mature albumin sequence at residue 34. Successful derivatization of these 2 residues is evidenced by the alteration in charge seen on electrophoresis. Native proalbumin Kaikoura migrates 2 charges more cathodally than mature albumin (Fig. 1, lane 22), and the aminooxyethylated form comigrates with proalbumin Blenheim which has an excess of 4 positive charges over mature albumin (lanes 23 and 24). After a 4-h incubation with KEX2, most of the S-aminooxyethyl proalbumin was converted to S-aminooxyethyl albumin, running 1 charge more cathodal to albumin (lanes 16–18).

KEX2 has been recorded as having a pH optimum of 7.5 (16, 17), though one investigation reports an optimum of between pH 5.5 and 6.0 (18). The pH dependence in all cases was determined using synthetic dibasic substrates. In Fig. 2, we investigate the pH dependence of the cleavage of proalbumin by KEX2. While there is virtually no cleavage below pH 5.5, there is approximately equal cleavage of \(^{125}\)I-proalbumin at all pH values between pH 6.0 and 8.0 (Fig. 2).

While both mammalian convertases and the KEX2 protease are inhibited by some thiol reagents such as Hg\(^{2+}\) and \(p\)-chloromercuribenzoate, neither the proinsulin or proalbumin convertase is inhibited significantly by iodoacetamide. However, this reagent has been reported (in some investigations) to inhibit KEX2 (18, 19). Comparing lanes 7 and 10 in Fig. 2 shows that there is little inhibition of KEX2 by 1 mM iodoacetamide; so again the mammalian and KEX2 convertases appear to be very similar.

We have shown previously that KEX2 was specifically inhibited by the variant serine protease inhibitor \(\alpha\)-antitrypsin M (8); this inhibition can be seen in Fig. 3 (lanes 4 and 5, respectively). Antitrypsin and other homologous inhibitors (e.g. antithrombin, antichymotrypsin, and antiplasmin) form 1:1 covalent complexes with their target proteases (20). These complexes can be visualized as stable high molecular weight acyl complexes on sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

No such complexes were observed when \(^{125}\)I-antitrypsin Pittsburgh was reacted with KEX2 extracts (data not shown). It appeared, therefore, that it might be possible to reverse the inhibition of KEX2 by antitrypsin Pittsburgh using thrombin, which has a very high affinity for antitrypsin Pittsburgh (13). The arginyl-directed serine protease thrombin which rapidly cleaves the fluorogenic substrate (t-butyloxycarbonyl-Gln-Arg-Arg-7-amino-4-methylcoumarin) does not cleave the diarginyl site of proalbumin (Fig. 3, lane 1). Neither does thrombin interfere with the KEX2-catalyzed cleavage of proalbumin through proteolytic inactivation of KEX2 (Fig. 3, lane 22).
KEX2 Protease Cleavage of Human Proalbumin Variants

**DISCUSSION**

The results presented here show that KEX2 has all the hallmarks of a mammalian convertase. Evolutionary experiments of nature have generated a number of unprocessed variants of human proalbumin which provide a unique insight into the in vivo specificity of the hepatic proalbumin convertase. In all respects, the KEX2 protease mimics the physiological convertase. There is little or no processing of proalbumin Christchurch (Arg→Gln), proalbumin Lille (Arg2→His), or proalbumin Blenheim (Asp→Val). The fact that proalbumin Kaikoura (Arg2→Cys) is a moderately good substrate for KEX2 is also in accord with the physiological evidence; there appears to be some in vitro processing of proalbumin Kaikoura in carriers.

Proalbumin Kaikoura makes up only 3–5% of the serum albumin in heterozygous individuals; the rest of the albumin is made up of Arg-albumin (30%) and normal albumin A (63–65%). The new Cys introduces an alternative signal peptidease cleavage site (Phe-Cys)Arg in the propeptide so that the major protein production of the mutant gene is Arg-albumin. This is produced in the rough endoplasmic reticulum and exported without further processing. Cleavage at the still intact normal signal sequence, Tyr-Ser-Arg, gives rise to the variant proalbumin (Arg-Gly-Val-Phe-Cys-Arg-albumin). If this, like proalbumin Lille, was not a substrate for the convertase, it would be expected to pass through the Golgi vesicles to the circulation without further cleavage and form about 20% of the circulatory albumin. This is not the case, and the product of the variant gene (Arg-albumin + proalbumin) appears to account for only 35% of the circulatory albumin (10). If, however, proalbumin Kaikoura was partially processed in the Golgi, then some of the abnormal gene product would be cleaved to mature albumin and this would account for the observed plasma ratios of 5% proalbumin Kaikoura, 30% Arg-albumin, and 65% albumin A.

The data presented here show that the -Cys-Arg-Asp-sequence of proalbumin Kaikoura is a reasonably good substrate for KEX2 and that if a KEX2-like protease was responsible for the in vivo cleavage of proalbumin in the liver, then it would be capable of generating serum albumin components in the observed ratio.

We have recently found further evidence of partial in vitro processing of proalbumin Kaikoura, Golgi vesicle extracts from rat livers contain a Ca2+-dependent protease which cleaves this proalbumin at about a quarter of the rate of normal human proalbumin. The KEX2 protease cleavage of proalbumin variants by yeast KEX2 protease. Each proalbumin substrate was incubated with KEX2 extracts for 0, 2 and 4 h. Lanes 1–3, proalbumin Lille (Arg→Hia); lanes 4–6, proalbumin Christchurch (Arg→Gln); lanes 7–9, normal human proalbumin; lanes 10–12, proalbumin Kaikoura (Arg→Cys); lanes 13–15, proalbumin Blenheim (Asp→Val); lanes 16–18, S-aminoethyl proalbumin Kaikoura; lanes 19–21, serum albumin; lane 22, reference marker (mixture of proalbumin Kaikoura and serum albumin; lane 23, reference mixture of proalbumin Blenheim and serum albumin; lane 24, S-aminoethyl proalbumin Kaikoura and serum albumin. 10 µl of stock yeast membranes were suspended in 80 µl of 0.1 M Tris/Hepes, pH 6.5, containing 1% Triton X-100, 5 mM CaCl2, 1 mM pepstatin, 1 mM tosyl-lysine chloromethyl ketone, 1 mM tosylphenylethyl chloromethyl ketone, 5 mM phenylmethylsulfonyl fluoride.

For each lane, 5 µl of this membrane extract (1 µg of protein) was incubated at 20 °C with 10 µg of proalbumin substrate. Reaction was stopped by freezing at 0-, 2-, and 4-h intervals. Before electrophoresis, 0.2 µCi of 65Ni2+ were added to each sample. The 65Ni autoradiograph is shown above the Coomassie Blue-stained pattern.

**FIG. 1.** Agarose gel electrophoresis at pH 8.6 showing the processing of human proalbumin variants by yeast KEX2 protease. Each proalbumin substrate was incubated with KEX2 extracts for 0, 2 and 4 h. Lanes 1–3, proalbumin Lille (Arg→Hia); lanes 4–6, proalbumin Christchurch (Arg→Gln); lanes 7–9, normal human proalbumin; lanes 10–12, proalbumin Kaikoura (Arg→Cys); lanes 13–15, proalbumin Blenheim (Asp→Val); lanes 16–18, S-aminoethyl proalbumin Kaikoura; lanes 19–21, serum albumin; lane 22, reference marker (mixture of proalbumin Kaikoura and serum albumin; lane 23, reference mixture of proalbumin Blenheim and serum albumin; lane 24, S-aminoethyl proalbumin Kaikoura and serum albumin. 10 µl of stock yeast membranes were suspended in 80 µl of 0.1 M Tris/Hepes, pH 6.5, containing 1% Triton X-100, 5 mM CaCl2, 1 mM pepstatin, 1 mM tosyl-lysine chloromethyl ketone, 1 mM tosylphenylethyl chloromethyl ketone, 5 mM phenylmethylsulfonyl fluoride.

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**FIG. 2.** Autoradiograph of agarose gel electrophoresis showing pH dependence of proalbumin cleavage. 3H-Labeled human proalbumin was incubated with KEX2 membrane extracts for 2 h as described in the legend to Fig. 1; the buffer, however, was varied as follows: lanes 1–3, 50 mM sodium acetate buffer, pH 4.5, 5.0, and 5.5, respectively; for lanes 4–10, the buffer was 50 mM Tris/Hepes; lane 4, pH 5.5; lane 5, pH 6.0; lane 6, pH 6.5; lane 7, pH 7.0; lane 8, pH 7.5; lane 9, pH 8.0; lane 10, pH 7.0 (this buffer also contained 1 mM iodoacetamide).

**FIG. 3.** Autoradiograph showing the reversibility of antitrypsin Pittsburgh inhibition of KEX2-catalyzed 3H-proalbumin conversion. Lane 1, proalbumin + 5 µg of thrombin; lane 2, proalbumin + 5 µg of thrombin + KEX2; lane 3, proalbumin + KEX2 + 5 µg of antitrypsin Pittsburgh; lane 4, proalbumin + KEX2 + 5 µg of antitrypsin Pittsburgh; lane 5, proalbumin + KEX2 + 5 µg of normal antitrypsin. Reactions were carried out in a total volume of 22 µl in a buffer of 50 mM Tris/Hepes, pH 7.5, 0.5 mM CaCl2, 0.5% Triton X-100. Antitrypsin Pittsburgh was added to the KEX2 preparation, and after 5 min, the excess thrombin was added. The reaction was left for a further 2 h to permit possible dissociation of KEX2-antitrypsin complexes before addition of the proalbumin substrate. After addition of proalbumin, assays were conducted for 2 h and conversion was assessed by electrophoresis. lane 2). The subsequent addition of excess thrombin to antitrypsin Pittsburgh-inhibited KEX2 relieves the inhibition and partially restores proalbumin cleavage activity (lane 3), suggesting that covalent complexes are not formed between the inhibitor and KEX2.

*S. O. Brennan and R. J. Peach, unpublished results.*
Proalbumin Blenheim which retains an intact dibasic site (-Arg-Arg-Val-) is a very poor substrate for KEX2. This is an unusual finding, but again it is fully in accord with the predicted properties of the human proalbumin convertase and the measured specificity of the rat liver enzyme (11). A prediction arising from this result was that none of the propheromones of the yeast should have a valine residue immediately after the dibasic site sequence. This turns out to be the case; of the six Lys-Arg sequences flanking the α-factor repeats (from the MFα1 and MFα2 genes), none are followed by valine, nor is there a valine following the Lys-Arg or Pro-Arg cleavage sites in prokiller toxin (21, 22).

Indeed, the fact that KEX2 cleaves the monobasic Cys-Arg-Asp- sequence of proalbumin Kaikoura is indirect evidence that it may also be the enzyme responsible for the cleavage of the monobasic Pro-Arg-Glu of prokiller toxin.

The Ca²⁺-dependent proalbumin (5) and proinsulin convertases (6) have a pH optimum of 5.5, befitting the acidic compartments in which cleavage takes place, while most reports on KEX2 place its optimum at pH 7.5. This large difference in pH optima suggested that the mammalian and yeast convertases might be quite different. However, the results presented here show that KEX2 cleaves human proalbumin with similar efficiency at pH 7.5 and 6.0.

There are few mammalian proproteins more different than proalbumin and proinsulin in terms of structure, site of synthesis, and function of the product, yet the type I proinsulin convertase appears identical to the proalbumin convertase (5, 6). The data presented here show that the yeast KEX2 protease is also very similar to these two mammalian enzymes; it is likely that these three proteases will form a family of similar convertases involved in the cleavage/activation of a host of other prohormone and proprotein substrates. It is also a reasonable prediction that these three Ca²⁺-dependent convertases will have homologous amino acid and gene sequences and that a homologue of the KEX2 gene, such as the furin or PC2 or a similar gene, encodes the mammalian enzyme.
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