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Calcium-dependent KEX2-like protease found in hepatic secretory vesicles converts proalbumin to albumin

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The yeast KEX2 protease is the only enzyme that has a proven role in the activation of polypeptide hormones through cleavage at pairs of basic residues. The enzyme that fulfils this role in higher eukaryotes has yet to be unequivocally identified. In this investigation, a KEX2-like calcium-dependent protease has been identified in rat hepatic microsomes. The enzyme is membrane-bound, has a pH optimum of 5–6 and converts proalbumin to albumin. More importantly, like the KEX2 protease, it meets two other exacting criteria defined by specific mutations in humans. Namely, it does not process proalbumin Christchurch (–1 Arg→Gln) which lacks one of the requisite basic residues and, whilst not itself a serine protease, it is inhibited by the reactive center variant, α_1-antitrypsin Pittsburgh (358 Met→Arg) but not by normal α_1-antitrypsin.

Proalbumin convertase; α_1-Antritrypsin Pittsburgh; Proalbumin Christchurch; (Liver microsome)

1. INTRODUCTION

Proteolytic processing at pairs of basic residues is a critical event in the activation of peptide hormones, neuropeptides and some plasma proteins [1,2]. Because of the central importance of the cleavage products in man, much attention has focused on the identity of the cleaving enzyme. Not surprisingly, therefore, a number of different proteases have been implicated. The difficulty is to decide which of these enzymes is of physiological importance. This question has been resolved in the case of the yeast (*Saccharomyces cerevisiae*) where mutations in the *KEX2* gene, which encodes a proposed calcium-dependent Golgi enzyme, prevent processing at Lys-Arg sequences in the secreted α-factor and killer toxin precursor peptides [3]. Reintroduction of the gene into deficient strains restores the missing proteolytic activity and, hence, the killer expression phenotype [4]. This unique protease may be a prototype of the mammalian enzyme as it correctly processes a number of purified human proproteins and transfection of mammalian cells with the mouse proopiomelanocortin gene together with the *KEX2* gene results in the production of the expected hormones through cleavage at the double basic sequences [5].

While persuasive, these experiments do not in themselves prove that a KEX2-like protease is involved in the in vivo cleavage of mammalian proproteins. Just as *Kex2* mutants facilitated the identification of the converting enzyme in yeasts, mutations in humans that block proalbumin processing closely define the proalbumin-converting enzyme. The peptide Arg-Gly-Val-Phe-Arg-Arg is cleaved from proalbumin just before secretion from the hepatocyte [6].

The existence of the circulating variants, proalbumin Christchurch (–1 Arg→Gln) [7], proalbumin Lille (–2 Arg→His) [8], and proalbumin Takefu (–1 Arg→Pro) [9] establish a critical requirement for a pair of basic residues at the

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Abbreviations: TLCK, tosyl-L-lysine chloromethyl ketone; PMSF, phenylmethylsulfonyl fluoride; PCMB, *p*-chloromercuribenzoate

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cleavage site. Proalbumin with this requisite dibasic sequence has also been observed in circulation. This was in a child with a fatal bleeding disorder caused by the presence of a variant form of α_1-antitrypsin, α_1-antitrypsin Pittsburgh (358 Met → Arg) [10]. Here the reactive center, or 'bait', of the inhibitor had mutated to Arg, thereby directing it against arginyl-specific proteases, demonstrably targeting it against thrombin and, by implication, against the proalbumin-cleaving enzyme [11]. This was a reasonable deduction based on the case history and the fact that α_1-antitrypsin and proalbumin are exported from the same secretory vesicles in the hepatocyte [12].

Based on these criteria, the yeast KEX2 enzyme is indeed an appropriate model for a human convertase; it processes normal human proalbumin but not proalbumin Christchurch, and it is inhibited by α_1-antitrypsin Pittsburgh but not by normal α_1-antitrypsin [13]. We report here the identification of a KEX2-like protease, with these same critical properties, present in rat hepatic microsome preparations.

2. MATERIALS AND METHODS

Human proalbumin and α_1-antitrypsin Pittsburgh were isolated from the plasma of the affected child [10,11]. Proalbumin Christchurch was isolated from the plasma of a heterozygous carrier [14] and normal α_1-antitrypsin was isolated from the plasma of an individual with the MM phenotype as described in [15].

Golgi vesicle fractions were prepared as described by Quinn and Judah [16]; a critical difference, however, was that no EDTA was incorporated in the various sucrose density solutions. Briefly, livers were homogenized in ice-cold 0.25 M sucrose; nuclei, mitochondria and cell debris were removed by centrifugation (9500 × g_{av}, 15 min). The supernatant containing the microsomes was layered over 1.3 M sucrose and centrifuged (SW28 rotor, 85 000 × g_{av}, 90 min). Membranes from the interface were diluted with 2 M sucrose and successively overlayered with 1.1, 0.85 and 0.25 M sucrose and centrifuged (TI60 rotor, 200 000 × g_{av}, 120 min). Membranes from the 0.85/0.25 M and 1.1/0.85 M sucrose interfaces were recovered, suspended in 0.25 M sucrose and pelleted (TI60 rotor, 200 000 × g_{av}, 30 min). Recovered fractions, designated light and heavy membranes respectively, were resuspended in approx. 2 vols of 0.1 M sucrose. Both fractions appeared similar on electron microscopy [17] and contained similar amounts of proalbumin-converting activity.

Washed, pelleted microsomal fractions were lysed in 1.5% Triton X-100 and ghosts removed by centrifugation prior to assay. Assay conditions are detailed in the figure legends.

Protein electrophoresis was carried out in 1% agarose gels using 0.1 M Tris/barbitone buffer, pH 8.6, and ^{63}Ni and ^{125}I autoradiography was carried out as in [11].

3. RESULTS AND DISCUSSION

The conversion of the proalbumin to albumin was assessed by an increase in electrophoretic mobility and concomitant ability to bind ^{63}Ni, which results from conversion to the mature albumin N-terminal sequences of Asp-Ala-His [14]. Both light and heavy membrane fractions converted normal human proalbumin to albumin (fig.1, lanes 3,10). Proalbumin Christchurch (−1 Arg → Gln), which is readily cleaved by trypsin-like enzymes, however, was unaffected by the vesicle protease (lane 7). The presence of normal α_1-antitrypsin (lane 4), or α_2-macroglobulin (lane 8) had no effect on proalbumin conversion, however, conversion was selectively inhibited by the reactive center variant α_1-antitrypsin Pittsburgh (358 Met → Arg) (lane 5). Like the KEX2 protease, conversion was also inhibited by EDTA (lane 9).

The hepatic enzyme has other features in common with its yeast counterpart (fig.2A) [18]. It is membrane-bound and its converting activity pelleted with the membranes on simple freeze-thawing but was released on treatment with 1.5% Triton X-100 (fig.2A, lane 2); the washed ghosts lacked activity (lane 1). The serine protease inhibitor PMSF (lane 3) had no effect on conversion, neither did the aspartyl protease inhibitor pepstatin (lane 8), nor the thiol protease inhibitor iodoacetamide (lane 7). Neither TLCK (lane 4) nor β-mercaptoethanol (lane 11) gave any inhibition. However, both PCMB (lane 5) and Hg^{2+} were potent inhibitors, suggesting the presence of a critical thiol group not directly involved in catalysis. The inhibition by EDTA (lane 10) was reversed by the addition of Ca^{2+} but not by Mg^{2+} (not shown). Zn^{2+} caused partial inhibition (lane 6), the extent of which was dependent on the Ca^{2+} concentration (not shown). The most striking difference between the yeast and hepatic enzymes is their pH optimum, pH 7.2 in the case of yeast [18] and pH 5–6 for the hepatic enzyme (fig.2B). The pH required for optimal proalbumin conversion, however, corresponds well to the pH of 5.5 found in secretory vesicles [19].

These results show that this hepatic protease

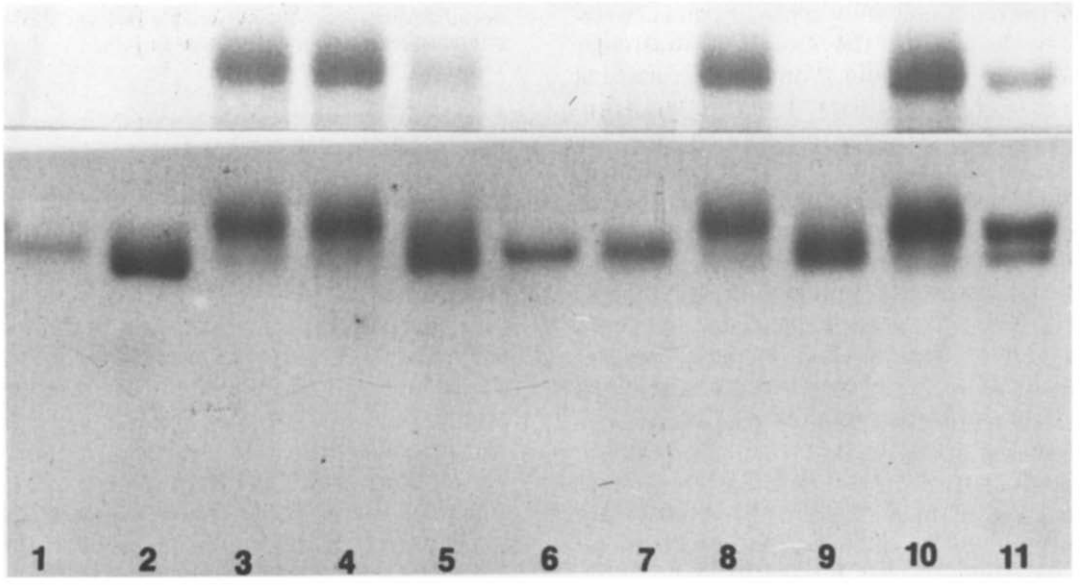

Fig.1. Agarose gel electrophoresis (pH 8.6) showing the specific conversion of human proalbumin to albumin. ^{63}Ni autoradiograph, confirming correct cleavage at the Arg-Arg site is offset above the Coomassie blue-stained protein electrophoresis pattern. Lanes: 1,6, proalbumin Christchurch; 2, proalbumin; 3,10, proalbumin + membrane extract; 4, proalbumin + α_1-antitrypsin M and membrane extract; 5, proalbumin + α_1-antitrypsin Pittsburgh and membrane extract; 7, proalbumin Christchurch + membrane extract; 8, proalbumin + α_2-macroglobulin and membrane extract; 9, proalbumin + EDTA and membrane extract; 11, markers of albumin plus proalbumin Christchurch. Incubations were performed for 3.5 h at 37°C in 10 μl of 50 mM Hepes-Tris (pH 6.0) containing 20 mM Ca^{2+}, 1.5% Triton X-100, 10 mM PMSF and 1 mM iodoacetamide. Where added, proalbumin and proalbumin Christchurch were present at 25 μg, α_1-antitrypsin, α_1-antitrypsin Pittsburgh and α_2-macroglobulin at 2.5 μg, and EDTA at 40 mM. Where added, 25 μg (protein) of microsomal membranes were used. Washed and pelleted membrane fractions were solubilized in 50 mM Hepes-Tris (pH 6.0) containing 20 mM $CaCl_2$ and 1.5% Triton X-100 and the membrane ghosts removed before incubation. Serum protein electrophoresis was performed on 5 μl of the assay solutions after addition of ^{63}Ni.

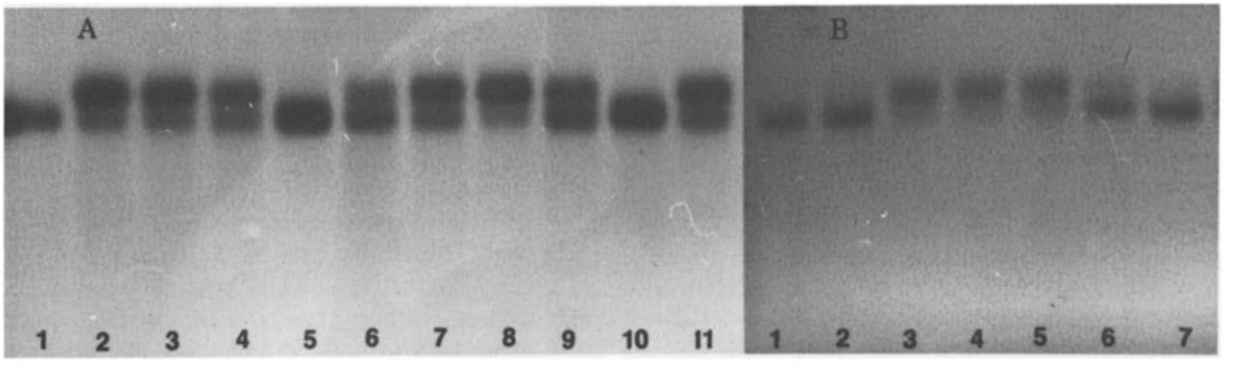

Fig.2. (A) Autoradiograph of agarose gel electrophoresis plate, showing conversion of ^{125}I-labeled human proalbumin to albumin by Triton X-100-solubilized Golgi membrane extracts (lanes 2–11) and washed ghosts (lane 1). Lanes: 1,2, no additions; additions: 3, 20 mM PMSF; 4, 1 mM TLCK; 5, 1 mM PCMB; 6, 5 mM $ZnCl_2$; 7, 2 mM iodoacetamide; 8, 1 mM pepstatin; 9, 1 mM leupeptin; 10, 30 mM EDTA; 11, 3 mM β-mercaptoethanol. Each assay contained 25 μg (protein) membrane extract in 10 μl of 50 mM Hepes-Tris (pH 6.0) containing 20 mM $CaCl_2$ and 1.5% Triton X-100 incubation was for 3 h at 37°C. (B) Effect of pH on proalbumin conversion. Conditions as for panel A except that the buffer was (lanes): 1, 0.1 M acetate, pH 3.7; 2, 0.1 M acetate, pH 4.5; 3, 0.1 M acetate, pH 5.5; 4, 0.1 M Hepes-Tris, pH 5.3; 5, 0.1 M Hepes-Tris, pH 6.0; 6, 0.1 M Hepes-Tris, pH 7.0; 7, 0.1 M Hepes-Tris, pH 8.0.

meets all the requirements of a proalbumin convertase. It is located in the Golgi and secretory vesicles, is membrane-bound, and is fully active at the pH of secretory vesicles. It correctly cleaves proalbumin at the Arg-Arg sequence but does not cleave the Arg-Gln sequence in proalbumin Christchurch which is very susceptible to tryptic cleavage [13]. Its most important claim to authenticity, however, is that while being unaffected by the classical serine protease inhibitors, including normal α_1-antitrypsin, it is specifically inhibited by the mutant inhibitor α_1-antitrypsin Pittsburgh. This confirms the prediction, from the clinical aspects of the Pittsburgh case, namely that the mutant antitrypsin was acting as an intracellular inhibitor of proalbumin conversion and that the unique observation of normal circulating proalbumin was consequential to the unique inhibitory site mutation in α_1-antitrypsin Pittsburgh [10,11].

The hepatic enzyme has another claim to authenticity in that it is similar to the KEX2 protease, which fulfils an analogous role in yeast. It is possible that similar proteases are involved in the processing of other mammalian proproteins. Very recently, Davidson et al. [20] identified a similar enzyme in insulin-secretory granules. This Ca^{2+}-dependent protease, which correctly processes proinsulin, is quite distinct from any previously proposed proinsulin convertase, but is remarkably similar to the proalbumin convertase described here in terms of its subcellular location, inhibitory properties and pH optimum (pH 5.0–6.0). Preliminary evidence (from our laboratory) indicates that microsomes from rat atria, the site of atrial natriuretic hormone production, contain a similar protease which is both Ca^{2+}-dependent and inhibitible by α_1-antitrypsin Pittsburgh. Cleavage of proatrial natriuretic hormone occurs at a Pro-Arg sequence [21], suggesting that the convertase for mono- or dibasic sites could be related. It is possible that a family of Ca^{2+}-dependent proteases may be involved in the processing of other mammalian proproteins.

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