

Yeast aspartic protease 3 (Yap3) prefers substrates with basic residues in the P₂, P₁ and P₂' positions

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Abstract The yeast aspartic protease Yap3 is localised to the secretory pathway and correctly cleaves pro- α -mating factor at its dibasic sites. We determined the specificity of Yap3 for mono-, di- and multi-basic cleavage sites in the context of 15 residue synthetic proalbumin peptides. Yap3 cleaved after dibasic ArgArg and LysArg sites but not after monobasic Arg sites even when there was an additional arginine at –6 and/or –4. Yap3 did not cleave a tetra-arginine site and tribasic sites (RRR and RRR) were poor substrates. Cleavage always occurred C-terminal to the last arginine in the di- or tri-basic sequence. The optimal cleavage site sequence was RR↓DR and this substrate was cleaved 8–9-fold faster than the normal RR↓DA sequence. In contrast to Kex2, Yap3 did not remove the propeptide from normal proalbumin or a range of natural or recombinant proalbumin variants. However at pH 4.0 Yap3 slowly cleaved proalbumin and albumin between domains 2 and 3.

Key words: Proalbumin processing; Yeast; Aspartic protease; Yap3

1. Introduction

The yeast *Saccharomyces cerevisiae* is a well established eukaryotic model for studying limited endoproteolysis at sites marked by pairs of basic amino acid residues. In *S. cerevisiae* the *KEX2* gene encodes a serine protease which processes pro- α factor and pro-killer toxin during their transit through the secretory pathway (for reviews see [1–3]). A second non-essential gene encodes another potential processing enzyme, Yap3 (yeast aspartic protease 3), a 569 residue protein capable of processing pro- α -mating factor when the propheromone is overexpressed in *KEX2* deficient *S. cerevisiae* [4]. Recently a third novel proprotein processing aspartic protease, MKC7, has been cloned from *S. cerevisiae* [5]. Antibodies directed against Yap3 recognise an aspartic protease in bovine pituitary intermediate lobe secretory granules [6]. Thus, just as Kex2 was the first eukaryotic subtilisin-like convertase, it is likely that Yap3 is the prototype of a family of mammalian aspartic proprotein processing endoproteases.

A truncated form of Yap3 has been overexpressed in *S. cerevisiae* and the resulting 68000 Da soluble glycoprotein purified and characterised by its ability to cleave mouse pro-opiomelanocortin (POMC) and anglerfish prosomatostatin I and II (aPSS-I and aPSS-II) in vitro. Yap3 specifically cleaved between and after paired basic residues in POMC to generate adrenocorticotropin, β -endorphin and γ -melanocyte

stimulating hormone [7]. Anglerfish PSS-I and -II each have a dibasic and a monobasic site and cleavage at these sites results in the production of somatostatin-14 (SS-14) and somatostatin-28 (SS-28) respectively. Truncated Yap3 cleaved at the dibasic ArgLys site in aPSS-I releasing SS-14 and Lys⁻¹SS-14, and at the monobasic Arg in aPSS-II releasing SS-28 [8]. However a membrane associated Yap3, overexpressed in *S. cerevisiae*, cleaved solely C-terminal to arginines to produce Lys⁻¹SS-14 from aPSS-I and SS-28 from both aPSS-I and -II [9].

To date the in vitro analyses of Yap3 have not completely defined its specificity, in particular it is unclear whether Yap3 has a preference for paired- or mono-basic sites, and whether it is able to recognise a similar range of sequence motifs to the members of the Kex2/furin family. We have previously shown that the albumin propeptide RGVFRR is specifically cleaved from the N-terminal of proalbumin by Kex2 in vivo and in vitro [10–12]. In this study we investigate the specificity of Yap3 in vitro using a series of synthetic proalbumin peptides and natural and recombinant proalbumin variants.

2. Materials and methods

2.1. Expression and partial purification of Yap3

Yap3 was overexpressed in *Saccharomyces cerevisiae* strain BJ3501 and purified from the culture medium as previously described [8]. Briefly the secreted Yap3 activity was partially purified by concanavalin A affinity chromatography. The Con A column eluate was desalted on a PD-10 column and concentrated. The concentration was determined by quantitative Western blot analysis to be 1.9 pmol/ μ l and the enzyme was stored at –80°C.

2.2. Assays with synthetic proalbumin peptides

Synthetic peptide substrates based on the N-terminal sequence of human proalbumin were obtained from Chiron Mimotopes, Victoria, Australia. The peptides were 15 residues long and had blocked N-(acetyl) and C-(amide) terminals. Tryptophan rather than the normal histidine was incorporated at position +9 to facilitate identification of the N- and C-terminal cleavage products. The reaction mixture contained 0.5 nmol proalbumin peptide in 0.1 M sodium citrate pH 4.0 with 95 or 19 fmol Yap3 in a total volume of 4.5 μ l. Reactions were incubated for specified times, of up to 8 h, at 37°C and stopped by the addition of 50 μ l buffer A (see below). For the determination of the pH optimum the following buffers were used: 50 mM sodium acetate, 1 mM Ca²⁺ pH 4.0, 4.5, 5.0 and 5.5; 50 mM 2-(N-morpholino)ethanesulfonic acid (MES), 1 mM Ca²⁺ pH 5.0, 5.5 and 6.0. After microfuging 40 μ l was injected on to a 3 μ m Nova Pac C-18 column and the extent of hydrolysis determined by HPLC. The solvent system was: buffer A, 49 mM potassium phosphate pH 2.9; B, equal mixture (1:1) of buffer A plus acetonitrile. The flow rate was 1 ml/min and the gradient went from 16% to 45% B over 14 min. Absorbance was monitored at 215 and 254 nm. To compensate for any slight variations in injection volumes between runs the reaction rate was calculated as % conversion/min. This was expressed as peak height of C-

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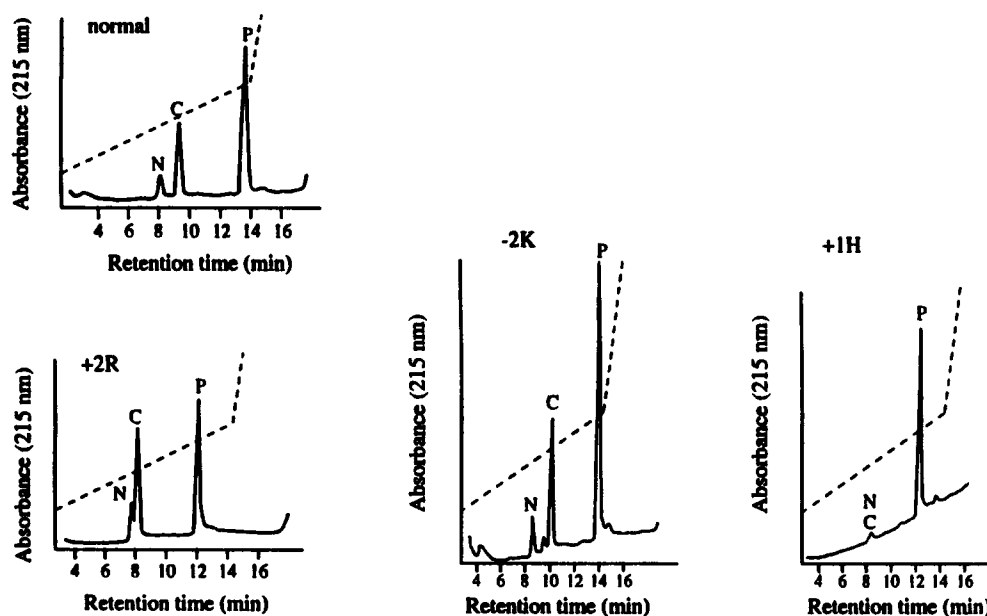


Fig. 1. Reverse phase HPLC showing cleavage of proalbumin peptides by Yap3. 0.5 nmol peptide was incubated with either 19 fmol (+2R peptide) or 95 fmol (normal, -2K and +1H peptides) Yap3 as described in section 2.2. The HPLC traces above show the 1 h time point for each labelled peptide. P, parent peptide; C, C-terminal fragment; N, N-terminal fragment.

terminal product divided by (height C-terminal product + height parent peptide). Amino acid analysis and PTH N-terminal sequence analysis were used to confirm the identity of the cleavage products.

2.3. Determination of kinetic parameters

For four of the proalbumin peptides (normal, -2K, +2R and +1H) $V_{\max}$ and K_m were determined by the Lineweaver-Burk method [13]. The concentration of the peptide in the assay was varied from 4×10^{-4} to 2.2×10^{-5} M under conditions such that the formation of product was linear over time. K_{cat} was calculated as $V_{\max}/[E]_T$ where $[E]_T$ is the total enzyme concentration. As the total protein concentration rather than active enzyme concentration was used in this calculation the k_{cat} values may be underestimated although the comparisons will still be valid.

2.4. Assays with proalbumin and albumin

Normal human proalbumin was purified from the plasma of an individual with antitrypsin Pittsburgh as previously described [14]. Proalbumin (30 pmol) was incubated with Yap3 (0.19 pmol) in 0.1 M sodium citrate pH 4.0 in a total volume of 5 μ l at 37°C. The reactions were stopped after 16 h by the addition of pepstatin A to 50 μ g/ml. Proalbumin was incubated with Kex2 as previously de-

scribed [10]. Reactions were analysed in 1% agarose gels using Tris-barbital buffer pH 8.6 [14].

Normal human serum albumin was purified from plasma [15] and 75 pmol was incubated with Yap3 (0.38 pmol) in 0.1 M sodium citrate pH 4.0 in a total volume of 5 μ l at 37°C for 24h. The reaction was analysed by reducing SDS PAGE [16].

3. Results

Incubation of Yap3 with the synthetic peptide RGVFRRDAHKSEVAW representing the propeptide cleavage site within proalbumin resulted in its specific hydrolysis at the $R^{-1}-D^{+1}$ bond to generate the N- and C-terminal fragments shown in Fig. 1. The identity of these peaks as RGVFRR and DEAKSEVAW was established by amino acid analysis and PTH sequence analysis respectively. Complete cleavage at this site was attained after 4.5 h and there was no further hydrolysis of the products after this time. The initial cleavage rate, V_0 , for this peptide was 0.33 ± 0.02 pmol/

Table 1
Sequences and relative rates of cleavage of proalbumin peptides by Yap3

Peptide	Peptide sequence															Relative rate*
normal	Ac-R	G	V	F	R	R	D	A	H	K	S	E	V	A	W-NH ₂	100 $\pm$ 6
-2K	-	-	-	-	K	-	-	-	-	-	-	-	-	-	-	97 $\pm$ 7
+2R	-	-	-	-	-	-	-	R	-	-	-	-	-	-	-	855 $\pm$ 40
-6A	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	69 $\pm$ 3
-4E	-	-	E	-	-	-	-	-	-	-	-	-	-	-	-	50 $\pm$ 3
+2E	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-	58 $\pm$ 4
+1K	-	-	-	-	-	-	K	-	-	-	-	-	-	-	-	18 $\pm$ 1
+1H	-	-	-	-	-	-	H	-	-	-	-	-	-	-	-	12 $\pm$ 0.4
-3R	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	7 $\pm$ 0.2
-4R-3R	-	-	R	R	-	-	-	-	-	-	-	-	-	-	-	0
-2H	-	-	-	-	H	-	-	-	-	-	-	-	-	-	-	0
-4R-2H	-	-	R	-	H	-	-	-	-	-	-	-	-	-	-	0
-2A	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	0
-4R-2A	-	-	R	-	A	-	-	-	-	-	-	-	-	-	-	0
normal	Ac-R	G	V	F	R	R	D	A	H	K	S	E	V	A	W-NH ₂	100 $\pm$ 6
	-6	-5	-4	-3	-2	-1	+1	+2	+3	+4	+5	+6	+7	+8		

*Rate (%) $\pm$ 1 S.D. relative to cleavage of the normal peptide.

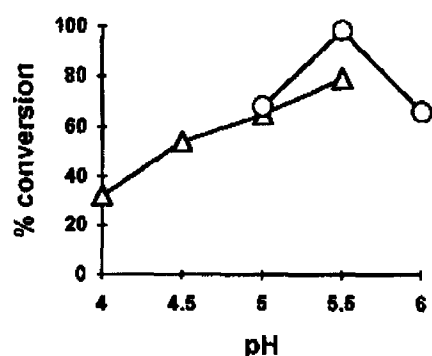


Fig. 2. pH dependence of Yap3. Yap3 (95 fmol) was assayed with the -2K peptide (0.5 nmol) for 3 h in Na acetate buffer (Δ) or MES buffer (○) at different pH values.

μmol Yap3 and the reaction was completely inhibited by the presence of 50 μg/ml pepstatin A (not shown).

A further group of 13 synthetic proalbumin peptides with substitutions N- and C-terminal to the cleavage site (Table 1) were also tested as potential substrates for Yap3. In each case where there was hydrolysis, sequence analysis of the C-terminal product confirmed that cleavage had occurred after the six residue propeptide (not shown). Extended incubations of either 4 or 8 h did not result in any additional cleavage of either the parent peptides or the N- and C-terminal products. It was determined for each peptide in a series of time course experiments and Table 1 shows the relative initial cleavage rate of each variant peptide compared to the normal peptide.

To more definitively investigate the reasons for the differences in cleavage rates, kinetic parameters were determined for four of the peptides with average (normal and -2K), slow (+1H) and fast (+2R) relative cleavage rates (Table 2). The +2R peptide, which was the preferred Yap3 substrate in the relative rate experiments, had a higher K_m than the other substrates, but this was compensated for by a higher V_{max} . The slow rate of cleavage of the +1H peptide was entirely due to the much lower V_{max} for this substrate.

The pH optimum of Yap3 cleavage was determined using the -2K proalbumin peptide. Previous reports using either POMC or aPSS as substrates indicated an optimum of 4.0–4.5 [7,8], however in the present study we found the enzyme was optimally active at pH 5.5 (Fig. 2), with only 30% activity at pH 4.0. The assays in this study were however performed at pH 4.0 to allow direct comparison with the specificities reported for Yap3 using other substrates at pH 4.0.

The yeast Kex2 protease rapidly cleaves the RGVFRR propeptide from human proalbumin to generate the more anodal band of mature albumin (Fig. 3A, lane 3). However, while Yap3 cleaved the synthetic peptide containing the propeptide sequence, it did not recognise this site in full length normal human proalbumin (Fig. 3A, lane 2). This was despite the incubations with proalbumin having a 33-fold higher enzyme: substrate ratio compared to the assays with the synthetic

peptides. A number of natural proalbumin variants (-2Arg → His, -1Arg → Gln, -2Arg → Cys, +1Asp → Val) [17] and recombinant proalbumin variants (-4Val → Arg, -2Arg → Lys, -4Val → Arg-2Arg → His, +1Asp → Arg) [10] were also tested as substrates for Yap3 (not shown). Yap3 did not release the propeptide from any of these variants.

Interestingly Yap3 did cleave both albumin and proalbumin at a site distant from the propeptide to generate a more cathodal band (Fig. 3A, lane 2). This reaction was inhibited by pepstatin A and occurred at pH 4.0 but not at pH 5.5 (not shown). When analysed by reducing SDS PAGE (Fig. 3B, lane 2) this fragment was determined to have an M_r of 47 kDa, and it had the same N-terminal sequence as the starting material suggesting that cleavage was occurring between domains 2 and 3 of albumin. Faint lower molecular weight bands were visible at 24 kDa and 16 kDa but these did not account for the entire C-terminal product and we have not been able to determine the precise cleavage site. There was also minor cleavage of albumin incubated in the absence of Yap3 producing a 47 kDa band (Fig. 3B, lane 1). This is most likely due to partial acid hydrolysis at the single Asp-Pro bond in albumin [18,19].

4. Discussion

A diverse range of mammalian proproteins including neuropeptides, peptide hormones, plasma proteins and receptors are subjected to specific intracellular cleavage at basic sequences in order to generate biologically active forms. The characterisation of the yeast Kex2 protease and the gene that encodes it has led to the discovery of a family of homologous mammalian convertases [20,21]. Like Kex2 the Yap3 protease from *S. cerevisiae* is a membrane bound enzyme located in a post-ER compartment of the yeast secretory pathway [6,22] and as such has a potential role in precursor processing. Here, using a series of synthetic proalbumin peptides, we have analysed the specificity of a truncated soluble form of Yap3.

Both the ArgArg proalbumin peptide and the LysArg proalbumin peptide were good substrates for Yap3 and in each case cleavage occurred exclusively on the C-terminal side of the dibasic site. The peptides with the monobasic His-Arg and AlaArg sequences were not cleaved despite there being an arginine at -6 which has been suggested to be important for monobasic processing by Yap3 in aPSS-II [8]. In addition a -4 arginine did not enable processing of either of these monobasic sites. This is in stark contrast to furin which cleaves the -4R-2A peptide 80-fold faster than the -2A peptide (Ledgerwood, Brennan, Birch, Nakayama and George, submitted). Tribasic sequences were poor substrates, the ArgArgArg⁻¹ peptide being cleaved at only 7% the rate of normal and the ArgArg⁻¹Lys⁺¹ sequence at 18%. Interestingly in the former cleavage was after the third arginine and in the latter it was between the arginine and the lysine. The common mammalian tetrabasic motif (ArgArgArgArg⁻¹) was

Table 2
Kinetic parameters for processing by Yap3

	V_{max} (pmol s ⁻¹)	K_m (mol/l)	k_{cat} (s ⁻¹)	k_{cat}/K_m ((mol/l) ⁻¹ ·s ⁻¹)
normal	13.9	4.8×10^{-4}	7.4	1.5×10^4
-2K	2.1	1.1×10^{-4}	1.1	1.0×10^4
-2R	46	7.8×10^{-4}	24.4	3.1×10^4
+1H	0.15	0.5×10^{-4}	0.08	0.16×10^4

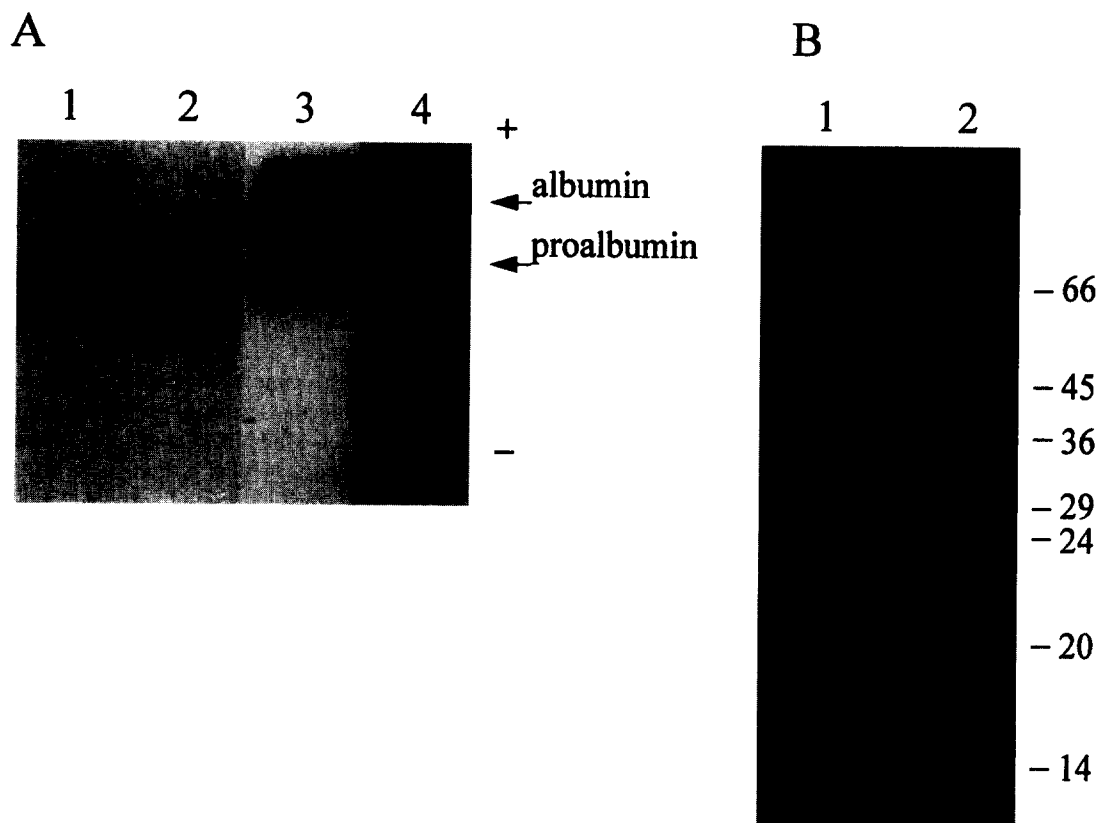


Fig. 3. Cleavage of human proalbumin and albumin by yeast proteases. A: Agarose gel electrophoresis. Proalbumin was incubated with either Yap3 (lane 2) or Kex2 (lane 3) as described in section 2.4. Lanes 1 and 4, proalbumin and albumin standards respectively. Anode at top. B: 12.5% reducing SDS PAGE. Albumin was incubated either alone (lane 1) or with Yap3 (lane 2) as described in section 2.4. Position of molecular weight markers (kDa) shown at right.

not cleaved by Yap3. Yap3 was also sensitive to changes that did not involve the paired basic residues. Acidic residues were unfavourable at either -4 or $+2$, decreasing the processing rate by about half. Replacing the aspartic acid at $+1$ with histidine had a similar effect to the substitution of lysine at this position with slow processing occurring between the arginine and the histidine. Although the -6 arginine did not enable Yap3 to cleave at a monobasic site it did contribute to dibasic processing since replacing it with alanine decreased the cleavage rate by 30%.

The most striking feature of this enzyme's specificity was the nine-fold enhancement in hydrolysis that occurred when the alanine at $+2$ was replaced with an arginine. This preference for substrates of sequence ArgArg↓XArg is in marked contrast to furin and PC3 where a $+2$ arginine completely prevents hydrolysis (Ledgerwood, Brennan, Birch, Nakayama and George, submitted). Cleavage of the $+2$ R peptide occurred at a catalytic efficiency similar to that observed for cleavage of the proinsulin B/C junction and cholecystokinin by Yap3 [23]. In agreement with Bourbonnais et al. [9] our results indicate that Yap3 has a preference for cleaving on the C-terminal side of the last arginine in di- or tri-basic sequences. However in ACTH¹⁻³⁹ efficient cleavage occurred between the lysines in the LysLysArgArg sequence generating ACTH¹⁻¹⁵ and CLIP¹⁶⁻³⁹ [7]. Interestingly in this configuration the LysLys bond has an arginine in the $+2$ position which may direct cleavage to the LysLys site.

The contrasting results obtained from the synthetic proalbumin peptide compared to full length proalbumin highlights

the dangers of extrapolating the results obtained with representative peptides to the full length proteins. This may explain the differences in the published results describing Yap3 processing of aPSS I and II since in one case full length proteins were used [8] whereas the other study used shorter synthetic peptides [9]. The results of the present study demonstrate that it is not only the primary sequence that determines specificity but the conformation at the processing site also effects the rate of reaction. It is likely that there are significant structural differences between a short peptide and a full length protein which explain the differential processing of the synthetic peptides compared to proalbumin.

The inability of Yap3 to remove the propeptide from human proalbumin in vitro is consistent with our previous finding that proalbumin is secreted unprocessed from a *kex2*-strain of *S. cerevisiae* [10]. Also there was no evidence of cleavage occurring between domains 2 and 3 when proalbumin was expressed in *S. cerevisiae*. This presumably is because Yap3 only cleaves at this site when albumin undergoes a pH dependent N→F conformational change. This only occurs below pH 4.5 and results in unfolding of the C-terminal domain making alternate sequences available for potential proteolysis [19].

The two yeast proteases Kex2 and Yap3 have different specificities and presumably complimentary functions in vivo. Although both are localised to the secretory pathway their different pH optima (5–5.5 for Yap3, 6–8 for Kex2 [11]) mean they are unlikely to be active in the same compartment. To date two aspartic proteases that are potentially involved in

proprotein processing have been identified in higher eukaryotes – in bovine pituitary intermediate and neural lobes and in anglerfish secretory granules [24,25]. One of these (the pituitary proopiomelanocortin converting enzyme) is immunologically related to Yap3 [6] suggesting Yap3 might be a prototype of a family of proprotein convertases. However as yet no mammalian homologues have been cloned.

To date it is not known whether there is a hepatic aspartic protease homologous to Yap3. The present study suggests that if there is such a protease, it will not be involved in proalbumin processing. It is also unlikely to cleave the majority of known liver proproteins which have unfavourable –1,–4 or –1,–2,–4 multibasic processing site sequences. Just as characterising the Kex2 protease provided the initial insights into the homologous family of mammalian enzymes, further study of Yap3, including identification of potential substrates, should provide important insights into the activity of this new family of proprotein processing endoproteases.

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