A C-terminal domain, which prevents secretion of the neuroendocrine protein 7B2 in *Saccharomyces cerevisiae*, inhibits Kex2 yet is processed by the Yap3 protease

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Abstract **Recent reports reveal that the C-terminal half of the neuroendocrine polypeptide 7B2 selectively inhibits and binds PC2, a mammalian prohormone converting enzyme that is homologous to the yeast pro-α-factor processing protease Kex2. During attempted secretion of the 185 amino-acid human 7B2 in *Saccharomyces cerevisiae*, we observe that the protein is mostly retained inside the cell. However a mutant polypeptide (7B2Δ1), where the C-terminal 48 amino acids of 7B2 are deleted, is efficiently secreted. Two shorter C-terminal truncations either permit poor secretion or no secretion at all. Surprisingly, full-length 7B2 but not 7B2Δ1 abolishes the catalytic activity of Kex2, indicating that C-terminal residues of 7B2 might also be important for inhibition of the yeast protease. When the *KEX2* gene is disrupted, yeast cells unexpectedly secrete a 7B2 variant similar in size to 7B2Δ1, suggesting involvement of the alternate yeast prohormone convertase Yap3 in processing. Secretion is enhanced by overexpression of Yap3 and by the presence of a Lys-Arg residue at the processing site of precursor 7B2. These results purport that, in neuroendocrine cells too, secretion of 7B2 could be mediated by a homologue of Yap3.**

Key words: 7B2; Kex2 inhibition; PC2 convertase; Processing by Yap3; *Saccharomyces cerevisiae*; Secretion

1. Introduction

The 7B2 protein is present selectively in the regulated secretory pathway of neurons and endocrine cells [1–2]. It is produced in all hormone-synthesizing cells of the pituitary gland and is distributed widely in different regions of the brain [2]. Within the cell, 7B2 is stored in the secretory granules suggesting that it may partake in the secretion of peptide hormones. Release of hormones from the cell is triggered by specific external stimulii, events typical of secretion in the regulated secretory pathway. Before actual secretion, prohormones are first converted to active hormones by specialized enzymes known as the prohormone convertases [3]. It appears that 7B2 itself behaves like a prohormone and is often exported into the extracellular medium after due processing [4].

All the known 7B2 cDNA sequences from different species encode signal peptides [5] which normally allow entry of nascent proteins into the secretory pathway. Comparison of the various mature polypeptides reveals that 7B2 is a highly conserved protein. Although its physiological importance has long been suspected, it was only recently that 7B2 was found to play a major role in preventing premature activation of the precursor form of the prohormone convertase PC2 [6]. In vitro experiments suggest that the C-terminal half of 7B2 may be an endogenous inhibitor of mature PC2 [7].

Besides the overall similarity, the amino-acid sequences of 7B2 from different species exhibit conservation in a set of basic residues, Arg-Xaa-Lys/Arg-Arg (at position 148/149 of the polypeptide sequences). Although this motif is generally not used as a substrate for processing in the regulated secretory pathway, mammalian 7B2 proteins are cleaved at this site. Cleavage is concomitant with secretion of a 21-kDa truncated molecule [8–9]. However, processing in the intermediate lobe of the *Xenopus laevis* (the South African clawed toad) pituitary glands, where 7B2 is co-expressed with the prohormone proopiomelanocortin (POMC), is different [5]. It occurs at a Lys^{138}-Lys^{139} pair of basic residues, which is N-terminal to the Arg-Xaa-Lys/Arg-Arg motif, and leads to the secretion of a 18-kDa derivative of 7B2 [5]. Although the variations in processing are inexplicable, the available data makes it abundantly clear that full-length 7B2 is never secreted in mammals or in the *Xenopus*.

In contrast to the complexities of the regulated secretory pathway of neuroendocrine cells, the yeast *Saccharomyces cerevisiae* offers a simple model system in which processing and secretion of 7B2 could be studied in the constitutive secretory pathway. In yeast, two unique prohormone convertases have been unambiguously identified: (i) Kex2, a member of the subtilisin family of serine proteases, functionally and structurally related to its mammalian counterparts PC1/PC3 and PC2 [9] and (ii) Yap3, an aspartic protease, a putative homologue of the POMC converting enzyme [10]. The *YAP3* gene product has the ability to suppress the phenotype of a *kex2* null mutant [11].

We have inquired if the complete human 7B2 polypeptide would be secreted in yeast when allowed entry into its secretory pathway. It appears that, in this unicellular eukaryote too, a C-terminal domain of 7B2 needs to be cleaved for secretion to occur. The enzyme which accomplishes this cleavage is not Kex2 but another endoprotease which, in all likelihood, is Yap3. The C-terminal domain of 7B2 appears to be instrumen-

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Abbreviations: 7B2, the neuroendocrine protein 7B2; human 7B2, full-length 7B2 (1–185 amino acids), excluding the signal peptide; 7B2Δ1, human 7B2(1–137); 7B2Δ2, human 7B2(1–149); 7B2Δ3, human 7B2 (1–170); *7B2ss*, signal sequence of the human *7B2* gene; *CYP1p*, the promoter of the *S. cerevisiae* cytoplasmic cyclophilin gene; ER, endoplasmic reticulum; *GAPDHp*, the promoter of the *S. cerevisiae* glyceraldehyde-3-phosphate dehydrogenase gene; IGF1, human insulin-like growth factor-1; *Invss*, the yeast *SUC2* signal sequence; PCR, polymerase chain reaction; *PHO5t*, transcription terminator of the yeast *PHO5* gene; POMC, proopiomelanocortin.

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tal in the inhibition of Kex2 but not of the alternate enzyme. These observations not only unveil similarities in the biochemical conversion of precursor 7B2 in organisms which are quite distantly related in evolution, but also hint that an enzyme resembling Yap3/POMC convertase may be responsible for processing 7B2 in neuroendocrine cells.

2. Materials and methods

2.1. DNA constructions of human 7B2, linked to signal sequences from human 7B2 and yeast SUC2 genes, for secretion in yeast

An *EcoRI–SphI* fragment of the *7B2* coding sequence, linked at the N-terminus to a 54 bp human 7B2 signal sequence (*7B2ss*; see Fig. 1), was amplified by the polymerase chain reaction (PCR) [12] using human *7B2* cDNA as template (kindly provided by Dr. G.J.M. Martens, University of Nijmegen, The Netherlands) [13]. The primers used were

5′-ATGAATTCATGCTATCTGGCCTACTG-3′

and

5′-TAGCATGCTTACTCTGGATCCTTATC-3′

The last two amino-acid residues encoded by the PCR fragment are Pro^{184} and Glu^{185} and are followed by a stop codon. The *EcoRI–SphI* fragment was subcloned in pBluescriptKS+ (Stratagene) and the resulting plasmid was named pBlue/*EcoRI–SphI*/7B2ss-7B2.

A fusion between the *S. cerevisiae SUC2* signal sequence (*Invss*) [14] and the human *7B2* gene [13] was created as follows (see Fig. 1). A ~465 bp *BamHI–XhoI* (5′ to 3′) fragment from pUC19/*BamHI–XhoI*/GAPDH-Invss, containing the 400 bp promoter region of the *S. cerevisiae* glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene [15] and 57 bp of the chemically synthesized *Invss* (the promoter and *Invss* are linked by an *EcoRI* site), was flushed at the 3′-end and ligated to *NcoI* linkers. Similarly, a ~270 bp *EcoRI–SacI* fragment from the 5′-end of the human 7B2 cDNA (see Fig. 1; the *EcoRI* site of which is flushed and converted to *NcoI* by linker ligation), was isolated. The resulting *BamHI–NcoI* and the *NcoI–SacI* fragments were subcloned in pUC19 yielding the template (pUC19/GAPDHp-Invss-7B2) for PCR-mediated deletion mutagenesis. The three primers used for the construction of an exact fusion between the *Invss* and *7B2* DNA sequences were:

5′-GCAGCCAAAATATCTGCATACAGCCCCCGGACCCCT-3′ (spans the 3′-end of the coding strand of *Invss* and 5′-end of the *7B2*)

5′-ATGAGCTCCACCTTCAATGC-3′ (spans the *SacI* site of *7B2* and belongs to the non-coding strand of the *7B2* gene)

and

5′-TAGAATTCATGCTTTTGCAAG-3′ (spans the *EcoRI* site and the 5′-end of the coding sequence of *Invss*)

The ~220 bp *EcoRI–SacI* product of the PCR reactions, encompassing the complete *Invss* and the DNA encoding the first 52 amino-acid residues of 7B2 (see Fig. 1), was confirmed by DNA sequencing. This *EcoRI-SacI* segment of DNA and the ~400 bp *SacI-SphI* fragment from pBlue/*EcoRI-SphI*/7B2ss-7B2 were subcloned in pBluescriptKS+ to yield the plasmid pBlue/*EcoRI-SphI*/Invss-7B2 (1–185).

2.2. DNA construction of the 7B2 variants 7B2Δ1, 7B2Δ2 and 7B2Δ3 fused to Invss

The genes for 7B2Δ1, 7B2Δ2 and 7B2Δ3 (see Fig. 1), already fused to *7B2ss*, were obtained by deleting 3′-end regions from the wild type human *7B2* gene [13]. The three mutant genes (either as *EcoRI–SphI* or as *EcoRI–HindIII* fragments) encode the N-terminal 137, 149 and 170 amino acids of human 7B2. The primers used for constructing the deletions are depicted in Table 1. The authenticity of the gene constructs was confirmed by sequencing.

The *EcoRI–SacI* segment, containing *Invss* fused to an N-terminal part of *7B2* (see Fig. 1), and a *SacI–SphI* fragment from the 7B2Δ1 gene were subcloned in pBluescriptKS+ to obtain pBlue/*EcoRI–SphI*/Invss-7B2Δ1. The plasmids pBlue/*EcoRI–HindIII*/Invss-7B2Δ2 and pBlue/*EcoRI–HindIII*/Invss-7B2Δ3 were obtained similarly (see Fig. 1).

2.3. Construction of yeast 2μ-plasmids that encode cassettes for expression of 7B2, 7B2Δ1, 7B2Δ2, 7B2Δ3, and for co-expression of 7B2/IGF1, 7B2Δ1/IGF1, 7B2/Yap3

The ~545 bp *BamHI–EcoRI* promoter fragment from the yeast cytoplasmic cyclophilin gene (*CYP1*) [16] was isolated by PCR, using genomic DNA from the yeast strain S288C as template and the two primers

5′-ATATGGATCCTCTAGAACCTTTCATCATCT-3′,

and

5′-TAATGAATTCGGTAGTATTAGCGGTTGAGT-3′

The *BamHI–EcoRI CYP1* promoter (*CYP1p*), the *EcoRI–SphI* or *EcoRI–HindIII* signal sequence-7B2 fusions (obtained from plasmids which encode 7B2ss-7B2, Invss-7B2, Invss-7B2Δ1, Invss-7B2Δ2 or Invss-7B2Δ3) and a *SphI-SalI* or *HindIII-SalI* transcription terminator fragment from the yeast *PHO5* gene (*PHO5t*) [15] were subcloned in the 2μ-plasmid pDP34 (at its *BamHI* and *SalI* sites) to yield the plasmids listed in Table 2. The vector pDP34 encodes the complete yeast 2μ sequence and the yeast selection marker *URA3* [15].

For construction of plasmids for co-expression of two genes of interest, a linker containing *SacI*, *XhoI* and *BglII* sites were first introduced in the the unique *SacI* site of pDP34 [15] to yield the vector pDP34Xho. After subcloning the *Invss-7B2* and *Invss-7B2Δ1* genes (under the control of *CYP1p*) in the *BamHI*, *SalI* sites of pDP34Xho, an *XhoI* fragment of the IGF1 expression cassette (under the control of *GAPDHp*) is ligated to the unique *XhoI* site of the vector. The resulting plasmids, pHB15 and pHB16, are listed in Table 2.

A *BamHI–SacI YAP3* [11] expression cassette was assembled from a *BamHI–XbaI* fragment which contains the promoter and the coding sequence of *YAP3* and a *XbaI–SacI YAP3* transcription terminator fragment. The fragments were obtained by PCR using genomic DNA from the strain S288C as template, American Type Culture Collection #26108, and the pairs of primers:

5′-ATGGATCCCCGTTTTCTTTTCGTAAAAA-3′,
5′-ATTCTAGAATGTGGTAGGTAATATTATA-3′

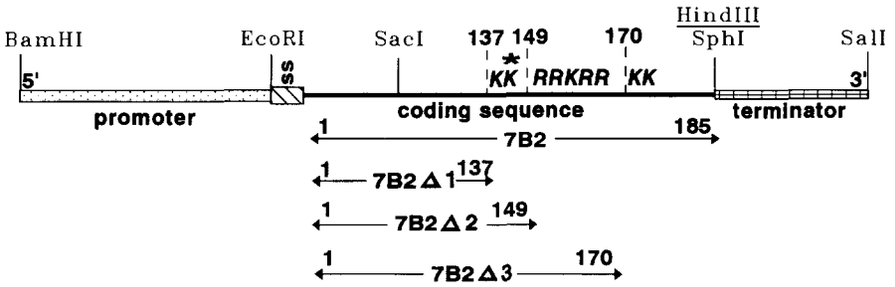


Fig. 1. The cassette used for expressing human 7B2 protein and its variants. All constructions are under the control of the constitutive *CYP1* promoter [16], the transcription terminator being *PHO5t*, and have been subcloned as *BamHI–SalI* fragments in the 2μ-vector pDP34 [15]. The restriction sites used for constructing the different expression cassettes are shown. 'ss' stands for either of the two signal sequences, *Invss* or the *7B2ss*. The three motifs (*KK*, *RRKRR* and *KK*), containing basic amino acid residues, are putative processing sites for precursor 7B2 (see section 3). The lengths of wild type and mutant 7B2 polypeptides are also indicated.

Table 1
Primers used for the construction of *7B2* variants fused to *7B2ss*

Gene	Fragment	Primers used
7B2ss-7B2Δ1	*Eco*RI–*Sph*I	(i) 5′-ATGAATTCATGCTATCTGGCCTACTG-3′ (ii) 5′-TAGCATGCTTAGTTCCACTTGCCCAAGCC-3′
7B2ss-7B2Δ2	*Eco*RI–*Hin*dIII	(i) 5′-ATGAATTCATGCTATCTGGCCTACTG-3′ (ii) 5′-ATAAGCTTTTACTCTCCTCCCTTCATCTT-3′
7B2ss-7B2Δ3	*Eco*RI–*Hin*dIII	(i) 5′-ATGAATTCATGCTATCTGGCCTACTG-3′ (ii) 5′-ATAAGCTTTTATGCAACAACATTATCCAG-3′

and

5′-ATTCTAGAATGAAACTGAAAACTGTAAG-3′,

5′-ATGAGCTCTTATTAGAATATTAAAGCCT-3′.

The expression cassettes for *YAP3* and *Invss-7B2* were subcloned in pDP34Xho to yield the plasmid pHB17 (see Table 2).

2.4. Introduction of a Lys^{139}Arg mutation in 7B2 by PCR-mediated site-directed mutagenesis followed by construction of 2μ-plasmids for expression of 7B2_K139R *and co-expression of* 7B2_K139R/sKex2pHDEL

In order to replace Arg^{139} in human 7B2 by Lys, PCR-mediated site-directed mutagenesis was performed using the plasmid pHB5 (encoding the *Invss-7B2* gene) as template and the three primers, 5′-AAGTGGAACAAGAgACTCCTTTACGAG-3′ (the lower case letter indicating the site where the Arg codon is changed), 5′-TAGCATGCTTACTCTGGATCCTTATC-3′ (the 3′-end non-coding strand of 7B2 containing a *Sph*I site) and 5′-TAGAATTCATGCTTTTGCAAG-3′ (the 5′-end coding strand of Invss containing an *Eco*RI site). The mutation in the *Eco*RI–*Sph*I DNA fragment was confirmed by DNA sequencing. The fragments containing the *CYP1p* (*Bam*HI–*Eco*RI), the *Invss-7B2_K139R* (*Eco*RI–*Sph*I) and the *PHO5t* (*Sph*I–*Sal*I) were subcloned in pDP34 [15] and the resulting plasmid is referred to as pHB13. To obtain a vector which would co-express Invss-7B2_K139R and sKex2pHDEL [18], the expression cassette of the latter was ligated to the unique *Xho*I site of pDP34Xho which already contained the *Invss-7B2_K139R* gene in its *Bam*HI–*Sal*I sites. The resulting plasmid is known as pHB14 (see Table 2).

2.5. Recombinant DNA techniques

Yeast transformations [19] (see Table 2) were performed in *S. cerevisiae* strains AB110 (*Matα* his4-580 leu2 ura3-52 pep4-3 [cir^0]) [17] and AB110*kex2* (*Matα* his4-580 kex2::LEU2 ura3-52 pep4-3 [cir^0]) [18]. Manipulation of DNA was carried out by using standard procedures [19]. *Escherichia coli* HB101 was used for the construction and propagation of plasmids. PCR was performed using Pfu (Stratagene) DNA polymerase. All newly constructed DNA was sequenced by the dideoxynucleotide chain-termination method, using the Applied Bio-Systems 370A automated DNA sequencer.

2.6. Expression of 7B2 and immunoblot analysis

Proteins were expressed in YPD (1% bacto-yeast extract (Difco), 2% bacto-peptone (Difco), 2% glucose and 0.05% bovine serum albumin (Sigma Cat. No. A-7638); pH6.5).

100 A_{600} cells (from 48 h cultures) were centrifuged and the supernatants were separated from the cell pellets. 2 ml supernatants were concentrated 5–20-fold using Centricon-3 mini-concentrators (Amicon). For PAGE analysis, 0.5 vol sample buffer (6% SDS, 0.15 M Tris, pH 6.8, 6mM EDTA, 30% glycerol, 0.05% Bromophenol blue) was added to the concentrated cell supernatants. The cell pellets were mechanically lysed using 0.2 g glassbeads (0.3 mm diameter) and 200 μl sample buffer. When cellular proteins were required for endoglycosidase F (Boehringer) treatment, cells were lysed in 200 μl 0.1 M sodium phosphate (pH 7) [18].

Immunoblot analyses of cell supernatants and lysates were performed according to previously published protocols [17]. The monoclonal 7B2 anti-serum MON-102 is specific for the residues 128–135 and was a gift from Dr. W.J.M. van de Ven of the University of Leuven, Belgium [20]. Rabbit polyclonal anti-serum was generated using purified monomeric IGF1 [17].

3. Results and discussion

In order to explore if human 7B2 would secrete from yeast, two signal sequences (the heterologous human *7B2ss* and the homologous yeast *Invss*) [13–14] have been used. Signal peptides usually allow the first step in secretion, that is, permit translocation of nascent proteins across the membrane of the endoplasmic reticulum (ER). The constructions *7B2ss-7B2*

Table 2
Plasmids and corresponding strains used for expression of 7B2, and co-expression of 7B2/IGF1, 7B2/sKex2pHDEL and 7B2/Yap3 in the presence and absence of Kex2 protease [a]

Plasmid name	Encodes the gene(s) for	Parent plasmid	Yeast strain(s) used for transformation	Corresponding yeast strain(s)
pHB-C	–	pDP34	AB110	yHB-C
pHB1	7B2ss-7B2	pDP34	AB110	yHB1
pHB5	Invss-7B2	pDP34	AB110 & AB110*kex2*	yHB5a & yHB5b
pHB6	Invss-7B2Δ1	pDP34	AB110 & AB110*kex2*	yHB6a & yHB6b
pHB7	Invss-7B2Δ2	pDP34	AB110	yHB7
pHB8	Invss-7B2Δ3	pDP34	AB110	yHB8
pHB13	Invss-7B2_K139R	pDP34	AB110 & AB110*kex2*	yHB13a & yhHB13b
pHB14	Invss-7B2_K139R & sKex2pHDEL	pDP34Xho	AB110*kex2*	yHB14
pHB15	Invss-7B2 & αFL-IGF1	pDP34Xho	AB110	yHB15
pHB16	Invss-7B2Δ1 & αFL-IGF1	pDP34Xho	AB110	yHB16
pHB17	Invss-7B2 & Yap3	pDP34Xho	AB110 & AB110*kex2*	yHB17a & yHB17b
pBC1	αFL-IGF1	pDP34	AB110 & AB110*kex2*	yBC1a & yBC1b

[a] The promoters used for the expression of 7B2, IGF1 [17], sKex2pHDEL [18] and Yap3 [11] are *CYP1* [16], *GAPDHp* [15], *KEX2p* [21] and *YAP3* [11], respectively.

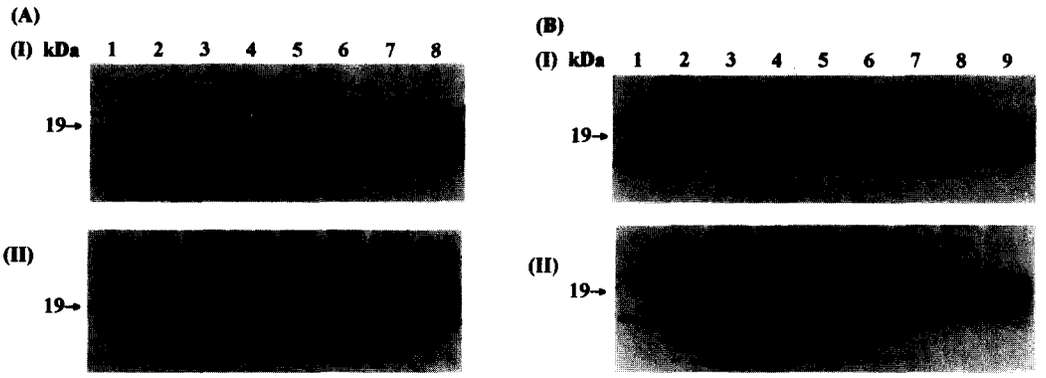


Fig. 2. Western blot analysis of intracellular and secreted 7B2-like proteins expressed in yeast from plasmids described in Table 2. Cell supernatants and lysates, from different transformants were analyzed by 15% SDS-PAGE. Fractionated proteins were blotted to Immobilon-P membranes (Millipore) on a Sartorius semi-dry blotter and were detected using the 7B2 anti-serum MON-102 [20]. (A) Full-length human 7B2 (a 185 amino-acid protein) does not secrete from yeast. (I) 10 μl supernatant from each culture (concentrated 20-fold; see section 2) was used. Lanes 1 and 2, yHB-C (negative control, bearing pDP34 with no expression cassette); lanes 3 and 4, yHB1 (7B2ss-7B2); lanes 5 and 6, yHB5a (Invss-7B2); lanes 7 and 8, yHB6a (Invss-7B2Δ1). (II) same as in (I) but for the use of 4 μl cell lysate in each lane. (B) 7B2 and 7B2Δ3 does not secrete from yeast, whereas 7B2Δ1 secretes better than 7B2Δ2. (I) Analysis of 10 μl supernatants (lanes 3–9). All samples were concentrated 20-fold except the supernatants from yHB6a which were concentrated only 5-fold. Lanes 1 and 2 have been used as controls. Lane 1, 200 ng recombinant 7B2 protein, expressed in *E. coli* (kindly provided by G. Martens, University of Nijmegen and used as positive control; the upper band resembles the full-length protein whereas the lower band probably is a degradation product obtained during purification); lane 2, yHB5a (Invss-7B2; 4μl cell lysate; the 7B2-like protein in the cell lysate has a larger molecular mass than *E. coli*-produced 7B2, either because the Invss has remained partially uncleaved or that 7B2 in yeast has undergone a type of post-translational modification which is not possible in *E. coli*); lane 3, yHB5a (Invss-7B2); lanes 4 and 5, yHB8 (Invss-7B2Δ3); lanes 6 and 7, yHB7 (Invss-7B2Δ2); lanes 8 and 9, yHB6a (Invss-7B2Δ1). (II) 4 μl cell lysate has been used in each lane. Lane 1, 200 ng recombinant 7B2 protein; lanes 2 and 3, yHB5a; lanes 4 and 5, yHB8; lanes 6 and 7, yHB7; lanes 8 and 9, yHB6a. Prestained low-molecular mass standard proteins (Bio-Rad) were used as markers.

(i.e. *7B2ss* fused to human *7B2* gene) and *Invss-7B2* (i.e. *Invss* fused to human *7B2* gene) are expressed under the control of the yeast *CYP1p*, a promoter element of the yeast cytoplasmic cyclophilin gene [16]. *CYP1p* promotes constitutive expression of heterologous proteins in a complex medium containing glucose as a carbon source (unpublished observations). The *CYP1p* is relatively weaker than the *GAPDHp*, known to be one of the strongest constitutive promoter in yeast [15]. The choice of the *CYP1* promoter was guided by our initial inability to express 7B2 from the promoter of the yeast *GAPDH* gene (unpublished observations).

Western blot examination of intracellular proteins from the yeast strains (see Table 2) yHB1 (harbouring 7B2ss-7B2) and yHB5a (harbouring Invss-7B2), using the 7B2-specific monoclonal antibody MON-102 [20], reveals that 7B2-like proteins are indeed expressed in yeast (Fig. 2A-II). However, there was no convincing evidence to indicate that 7B2 truly secretes (Fig. 2A-I). Even after 20-fold concentration of cell supernatants there was only a hint, barely visible on the immune-blot, that the strains yHB1 and yHB5a may have a propensity to secrete molecules which are smaller than full-length 7B2 (Fig. 2A-I, lanes 3–6).

7B2 is secreted from mammalian and *X. laevis* neuroendocrine cells only after the proteins have undergone processing at the C-terminal end of the molecule [5,8–9]. Since the full-length 185 amino-acid human 7B2 is incapable of being secreted from yeast, it could be that removal of a region from the C-terminus of the protein is necessary for secretion to occur in *S. cerevisiae*. In fact, the very small amounts of 7B2-like molecules that are obtained from yHB1 and yHB5a (Fig. 2A-I, lanes 3–6) are similar in size to the proteins expressed by the mutant 7B2Δ1 (Fig. 2A-I, lanes 7 and 8). The 7B2Δ1 gene should encode a 137 amino-acid polypeptide yet it appears that 7B2Δ1 is expressed as a mixture of at least two proteins. The polypeptide representing the lower band is probably a proteolytic product of the upper band. Since both proteins are recognized by the monoclonal antibody MON-102, specific for amino acids 128–135 of human 7B2 [20], it is probable that the upper band (representing the true C-terminal truncation 7B2Δ1) has undergone inadvertent N-terminal proteolysis.

In order to delineate the C-terminal domain which inherently prevents secretion of 7B2 in yeast, two more mutants were constructed (see Fig. 1). It was envisaged that smaller deletions, involving truncations at the 3′-end of the *7B2* gene and which remove 36 and 15 amino acids from the C-terminus of 7B2, would help to define this region. There are three sites in 7B2 which contain pairs of basic residues (in the human protein they are Lys^{138}-Lys^{139}, Arg^{150}-Arg-Lys-Arg-Arg^{154}, and Lys^{171}-Lys^{172}), any one of which could be a putative processing site for a secreted prohormone-like protein [5,8–9]. We observe that the 170 amino-acid 7B2Δ3 protein fails to secrete from yeast (Fig. 2B, lanes 4 and 5), whereas the 149 amino-acid 7B2Δ2 secretes poorly compared to the 137 amino-acid 7B2Δ1 (Fig. 2B; compare lanes 6 and 7 with 8–9). These results imply that processing at the Lys^{138}-Lys^{139} residue of human 7B2 may be potentially suited for secretion in yeast. If this happened naturally, one would expect that molecules similar to 7B2Δ1 would be spontaneously secreted from yeast. This event would not be so unlikely as there is precedence for similar processing at an identical site in the vertebrate *X. laevis* [5].

It can be argued that by providing for a Lys^{138}-Arg^{139} residue, instead of the Lys^{138}-Lys^{139} sequence already existing in the wild type protein, it might be possible to make 7B2 more amenable to processing by the yeast *KEX2* gene product [21]. The *KEX2* gene encodes an endopeptidase which plays an active part in the proteolytic processing of precursors of the α-factor phero-

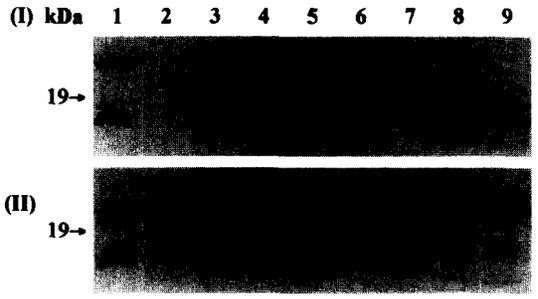


Fig. 3. Human 7B2, when expressed in a Kex2⁻ strain of yeast, secretes molecules similar in size to 7B2Δ1. Proteins from cell supernatants and lysates were analyzed by Western blotting, as described in Fig. 2. (I) 10 μl supernatant (concentrated 20-fold) was loaded in each lane, excepting lane 8 where a 5-fold concentrated supernatant was used. Lane 1, 200 ng recombinant 7B2 protein (as in Fig. 2); lane 2, yHB13a (Invss-7B2_K139R in AB110); lane 3, yHB5b (Invss-7B2 in AB110*kex2*); lanes 4 and 5, yHB13b (Invss-7B2_K139R in AB110*kex2*); lanes 6 and 7, yHB14 (Invss-7B2_K139R/sKex2pHDEL in AB110*kex2*); lane 8, yHB6a (Invss-7B2Δ1 in AB110); lane 9, yHB6b (Invss-7B2Δ1 in AB110*kex2*). (II) 4 μl cell lysate was loaded in each lane. Lane 1, 200 ng recombinant 7B2 protein; lane 2, yHB13a; lane 3, yHB5b; lane 4, yHB6a; lane 5, yHB6b; lanes 6 and 7, yHB13b; lanes 8 and 9, yHB14. The protein markers used were the same as in Fig. 2.

mone and killer toxin [22]. The Kex2 protein is a unique calcium-dependent serine-protease which has substrate specificity toward the carboxyl side of Lys-Arg, Arg-Arg and Pro-Arg sequences [23].

Surprisingly, secretion is not observed on expressing 7B2_K139R (which contains the Lys^{139} to Arg mutation) in the yeast strain AB110 (Fig. 3A, lane 2). It appears as if the newly introduced Lys^{138}-Arg^{139} substrate in 7B2 is not recognized by Kex2 so as to permit cleavage of the molecule. Could it be that Kex2-recognition is facilitated if cleavage was attempted during folding of 7B2? There is a possibility that the Lys^{138}-Arg^{139} motif would remain exposed before the polypeptide wholly acquires its native conformation. Since folding of secretory proteins is believed to occur in the ER [24], we endeavoured to use an ER-retained variant of *KEX2* (i.e. *sKEX2HDEL*) [18] in a strain where the *KEX2* gene was inactivated by targeted gene-disruption [18]. The results show that the action of the sKex2pHDEL protein on 7B2 may be of marginal help to the secretion of 7B2Δ1-like molecules (Fig. 3A, lanes 6 and 7).

Amazingly however, the secretion of 7B2 occurs most efficiently when any form of Kex2 is totally absent from the cell (Fig. 3A, lanes 4 and 5), indicating that sKex2pHDEL may not have a real role in the secretion of 7B2. In the Kex2⁻ strain AB110*kex2*, full-length 7B2 clearly secretes as a 7B2Δ1-like molecule (Fig. 3A, lane 8). Although replacement of Lys by an Arg is perceptibly beneficial (Fig. 3A, compare lane 3 with lanes 4 and 5), secretion can be still perceived (Fig. 3A, lane 3) even if the residue at position 139 were Lys (as in the wild type human 7B2). These findings are reminiscent of the processing of pro-α-factor in a Kex2⁻ strain [11] and suggest that an alternate endoprotease, probably Yap3, might function in the processing of the precursor 7B2 allowing secretion of a truncated molecule. Apparently, Yap3 is induced in Kex2⁻ strains when pro-α-factor is overexpressed from a multi-copy plasmid [11], signifying that Yap3 induction is triggered when multiple copies of its substrate are expressed in strains which lack a functional *KEX2* gene.

In order to find out if Yap3 really does play a part in the processing of precursor 7B2 in yeast, the *YAP3* gene [11] was co-expressed with 7B2 from a 2μ-plasmid (pHB17). The levels of 7B2 secretion are appreciably more in strains which overexpress Yap3 (Fig. 4, lanes 5–8) than in strains which have only the genomic copy (Fig. 4, lanes 3 and 4). It was also reassuring that amounts of secreted 7B2Δ1 are similar in the strains AB110 and AB110*kex2* (Fig. 4, lanes 5 and 6, and 7 and 8), implying that the Yap3 and Kex2 enzymes may not have conflicting roles during the secretion of 7B2.

Studies with purified Yap3 have shown that it can cleave at both Lys-Lys and Lys-Arg residues [10]. Moreover, it has been proposed that Yap3 can cleave certain polypeptides at monobasic Arg residues [25–26]. The yeast aspartic protease Yap3 has been found to be functionally identical to the mammalian aspartic protease, POMC converting enzyme [10]. Interestingly, the secretion of POMC- and 7B2-derived peptides is probably intertwined and seems to occur in an extremely cooperative manner in *X. laevis* [5], where inhibition of secretion of POMC-derived molecules also prevents secretion of 7B2 derivatives. Therefore, it is likely that two similar enzymes (which could be even identical) process 7B2 and POMC before allowing secretion of their cleavage products.

In spite of the implied role of Yap3 in the secretion of 7B2, it was still difficult to understand that wild type Kex2 should fail to perform its normal function when co-expressed with 7B2. In order to investigate if Kex2 could be inactive in strains co-expressing 7B2, the fusion protein proαFL-IGF1 (proαFL represents the prosequence of pro-α-factor) [18, 27–28] was used as an in vivo substrate. We have shown earlier that, using this substrate, activity of Kex2 can be measured by the release of human insulin-like growth factor-1 (IGF1) into the extracellular medium [27]. It ought to be remembered that, in a Kex2⁻ strain (AB110*kex2*), proαFL-IGF1 is not processed and mature IGF1 remains undetected in the cell supernatant, whereas in a $Kex2^+$ strain (AB110) mature IGF1 is formed although some preproαFL-IGF1 (Fig. 5, bands marked with an asterisk) is also observed (presumably because the IGF1 precursor fails to undergo complete translocation into the ER) [28]. Analysis of intracellular proteins from AB110*kex2* show that the precur-

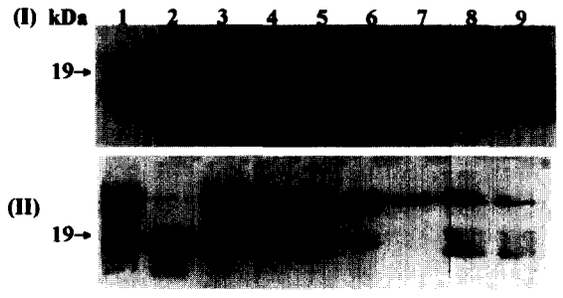


Fig. 4. 7B2 secretes as a truncated 7B2Δ1-like molecule when Yap3 [11] is overexpressed in AB110 and AB110*kex2*. Proteins were analyzed by Western blotting, as in Fig. 2. (I) 10 μl supernatant (concentrated 20-fold) was loaded in each lane. Lane 1, 200 ng recombinant 7B2 protein (as in Fig. 2); lane 2, yHB13a (Invss-7B2_K139R in AB110); lanes 3 and 4, yHB13b (Invss-7B2_K139R in AB110*kex2*); lanes 5 and 6, yHB17a (Invss-7B2/Yap3 in AB110); lanes 7 and 8, yHB17b (Invss-7B2/Yap3 in AB110*kex2*); lane 9, yHB6a (Invss-7B2Δ1 in AB110). (II) 4 μl cell lysate was loaded in each lane. Lane 1, yHB5a (Invss-7B2 in AB110, as a control); lane 2, yHB6a; lane 3, yHB13a; lanes 4 and 5, yHB13b; lanes 6 and 7, yHB17a; lanes 8 and 9, yHB17b. The protein markers used were the same as in Fig. 2.

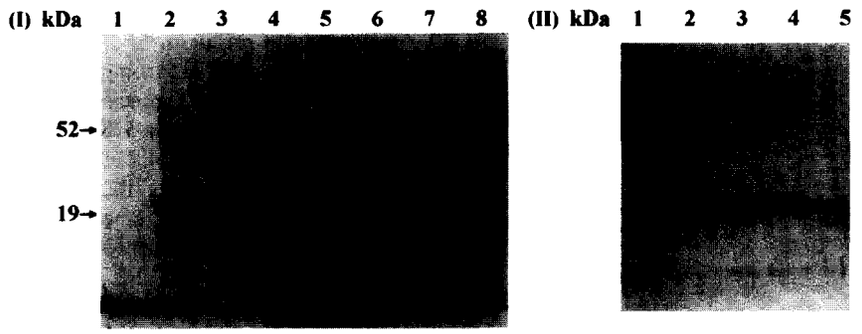


Fig. 5. Kex2 is unable to process the proαFL-IGF1 fusion protein when 7B2 is present in the yeast cell. All proteins were analyzed by Western blotting using the IGF1 polyclonal antibody, as in Fig. 2. The total proteins in the cells (obtained after lysis in 0.1 M sodium phosphate, pH 7) were roughly the same (280–300 μg/ml, using the Bradford protein-assay kit from Bio-Rad). Treatment of cell lysates with endoglycosidase F was performed following published procedures. (I) Lane 1, purified 100 ng IGF1 monomer [17]; lane 2, yBC1a (αFL-IGF1 in AB110) [28]; lanes 3 and 4, yBC1b (αFL-IGF1 in AB110*kex2*); lanes 5 and 6, yHB16 (Invss-7B2Δ1 and αFL-IGF1 in AB110); lanes 7 and 8, yHB15 (Invss-7B2 and αFL-IGF1 in AB110). (II) Samples after deglycosylation with endoglycosidase F [18]. Lane 1, 100 ng IGF1 monomer; lane 2, yHB16 (+endo F); lane 3, yBC1b (+endo F); lane 4, yHB15 (+endo F); lane 5, unglycosylated proαFL-IGF1 (as control) [28]. The bands marked with an asterisk (*) represent preproαFL-IGF1. The protein markers were the same as in Fig. 2.

sor proαFL-IGF1 is highly glycosylated (visible as diffuse bands on polyacrylamide gels; see Fig. 5A, lanes 3 and 4), which is typical of proteins that have traversed through the Golgi cisternae without being processed by the Kex2 enzyme [18, 27–28]. In marked contrast, the glycosylated intermediate proαFL-IGF1 is barely visible in AB110 (Fig. 5A, lane 2) because most of the molecules have undergone processing by the Golgi-resident Kex2 (cleavage occurring at a Lys-Arg residue which links proαFL to IGF1).

We find that co-expression of 7B2 and Kex2 drastically reduces the release of mature IGF1 into the medium (results not shown). An immune-blot of intracellular proteins depicts that Kex2 in the strain yHB5a (see Table 2) is nearly as inactive as the non-functional copy of Kex2 in the strain AB110*kex2* (Fig. 5A, compare lanes 3 and 4 with 7 and 8). Most of the heterogeneous mix of glycosylated proαFL-IGF1 molecules from yHB15 can be deglycosylated by treatment with endoglycosidase F (Fig. 5B, compare lanes 3 with 4). Significantly, co-expression of the truncated 7B2Δ1 protein does not have a palpable effect on the processing of proαFL-IGF1 (Fig. 5A, lanes 5 and 6). This emphasizes that Kex2 is functional in the presence of 7B2Δ1 and underlines the efficacy of the 48 amino-acid C-terminal region of human 7B2 in inhibiting Kex2. It is, therefore, likely that binding of this domain to Kex2 prevents secretion of the complete 7B2 molecule from yeast.

The inference that 7B2 has the capacity to inhibit Kex2 subtly reflects earlier findings involving the human 7B2 protein [7]. In vitro experiments have shown that complete 7B2, and not a molecule similar to 7B2Δ2, inhibits the prohormone convertase PC2 [7]. The action on PC2 is very specific since a related enzyme PC1/PC2 appears to be unaffected by 7B2. Altogether, six mammalian processing enzymes (the prohormone convertases furin/PACE, PC1/PC3, PC2, PACE4, PC4 and PC5/PC6) have been discovered to exhibit significant similarities in primary sequence to Kex2, the yeast enzyme related to the bacterial subtilisins [3]. Therefore, it is not a mere coincidence that the C-terminal domain of 7B2 is meant to inhibit yeast's only Kex2 protease, the prototype of the eukaryotic family of prohormone convertases.

We believe that the results reported in this communication could facilitate the understanding of the molecular basis for inhibition of a specific class of serine proteases (i.e. the prohormone convertases). Moreover, the observation that 7B2 is processed by an aspartic protease should provide further insight into the anomalous secretion of 7B2 from a variety of neuroendocrine tumors. It has been suggested that the protein could be used as an efficient diagnostic marker for these tumors since they secrete 7B2 in very high amounts. [29–30]. If aberrant secretion of 7B2 is mediated by the action of a Yap3-like convertase, it is possible that inhibition of such an enzyme could deprive certain tumor cells of its tumorogenic potential. Hence, the yeast *S. cerevisiae* also furnishes an excellent opportunity to identify the processing enzymes involved in the biogenesis of the secreted form of 7B2.

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