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A modified Kex2 enzyme retained in the endoplasmic reticulum prevents disulfide-linked dimerisation of recombinant human insulin-like growth factor-1 secreted from yeast

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The majority of the recombinant human insulin-like growth factor-1 (IGF1) molecules, secreted from yeast using the prepro sequence of the prepro-α-factor, are not active monomers but inactive, disulfide-linked dimers. The prepro sequence of the prepro-α-factor, usually referred to as the α-factor leader (αFL), consists of a pre or signal sequence and a proregion. After signal sequence removal during translocation into the endoplasmic reticulum (ER) the proregion is still attached to IGF1 when it folds to acquire a tertiary structure. Mature IGF1 is released only in a late Golgi compartment by the membrane-bound endoprotease Kex2p. We find that co-expression of a novel ER-retained Kex2p variant, soluble Kex2pHDEL, can prevent intermolecular disulfide bond formation between two IGF1 molecules, implying that the presence of the proregion during the folding of IGF1 in the ER could be a reason for disulfide-linked dimerisation. This result indicates that the proregion of the αFL may have a role in the folding of some heterologous proteins in yeast, and that the ER-retained Kex2p mutant could be used as a convenient tool to study the cellular function of the proregions present naturally in various eucaryotic precursor proteins.

Protein-folding in vivo; Insulin-like growth factor-1; Intermolecular disulfide-bonded dimer; Kex2 endoprotease; *Saccharomyces cerevisiae*; Yeast ER-retention signal HDEL

1. INTRODUCTION

Heterologous proteins have been targeted to the secretion pathway [1] of the yeast, *Saccharomyces cerevisiae*, using signal sequences [2] fused to the NH_2-terminus of a protein. Classical signal peptides are usually 15–30 amino acid segments, containing a central hydrophobic core, which mediate translocation of a nascent polypeptide into the endoplasmic reticulum (ER) [3]. The signal peptide is usually cleaved during translocation, after which the polypeptide is free to acquire its native conformation. Folding of a polypeptide chain destined for secretion is thought to occur in the ER [4].

The prepro sequence of the yeast prepro-α-factor [5] is a signal sequence which has been widely used for the secretion of foreign proteins from *S. cerevisiae* [6]. This α-factor leader (αFL) is an unusually long polypeptide consisting of a 19 amino acid N-terminal signal sequence and a 66 amino acid segment, termed the proregion. The function of the proregion in the secretion of the 13 amino acid mating pheromone α-factor [5] is not clearly understood. A construction where the proregion to the αFL has been deleted permits translocation of α-factor across yeast microsomal membranes in vitro, though poorly [7]. In the context of the secretion of foreign proteins it has been reported that the three heterologous proteins, aminoglycoside phosphotransferase encoded by Tn903 that directs kanamycin resistance, human granulocyte-macrophage colony stimulating factor and interleukin-1β, do secrete from yeast using only the pre or signal sequence of the αFL [8].

However, the pre sequence of the αFL alone does not allow any secretion of human insulin-like growth factor-1 (IGF1) into the yeast culture medium [9]. It appears that the proregion (proαFL) of the αFL is essential for the secretion of IGF1 from yeast. Human IGF1 [10] is a 70 amino acid polypeptide with three intramolecular disulfide bonds involving all six cysteine residues. IGF1 is one of the two insulin-like growth factors which is structurally related to insulin.

It has been observed that only 10–20% of the IGF1 molecules secreted from yeast are active monomers [11]. The majority of the IGF1-like molecules are dimeric. Unlike insulin these dimers are not merely physical aggregates [12] but are covalently linked through disulfide bonds [11,13]. We have suggested earlier that formation of dimers could be the result of a specific, charged interaction between two IGF1 molecules during the process of folding in the cell [13]. It is also possible that dimer-

Abbreviations: αFL, α-factor leader; D, aspartic acid; DTT, dithiothreitol; E, glutamic acid; ELISA, enzyme-linked immunoabsorbant assay; ER, endoplasmic reticulum; H, histidine; HPLC, high-performance liquid chromatography; IGF1, human insulin-like growth factor-1; K, lysine; L, leucine; proαFL, proregion of the αFL; wt, wild-type.

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isation occurs because of an aberrant folding of IGF1 due to the presence of the extraneous proαFL sequence during folding of IGF1 in the ER. The proregion of the αFL is not cleaved during translocation. It is removed only later, either in the *trans*-Golgi or in the secretory vesicles by the KEX2 gene product [14] which recognizes a pair of exposed dibasic amino acids linking the N-terminal proαFL to the C-terminal IGF1. After cleavage by Kex2p mature IGF1 is obtained.

In order to investigate whether proαFL is really involved in the folding pathway of IGF1 dimerisation we have examined the effect of a processing enzyme, soluble Kex2pHDEL (sKex2pHDEL) [15], which could remove the proregion from the precursor protein in the lumen of the ER rather than in the late Golgi. An early processing would negate any effect that the proregion of the αFL would have on the folding of IGF1, and enable the mature IGF1 molecule to fold independently.

2. MATERIALS AND METHODS

2.1. *Strains and transformations*

All newly constructed plasmids were transformed in *E. coli* HB101. Yeast transformations [16] were performed in *S. cerevisiae* strains AB110 (*his4-580, leu2, ura3-52, pep4-3*, [*cir*^{0}]) [16] and AB110*kex2* [15]. The chromosomal copy of the *KEX2* gene in AB110 was disrupted by transformation with the linearised plasmid pUC19/*kex2*::LEU2 where a 2.9 kb *Bgl*II fragment containing the functional copy of the *LEU2* gene has been inserted at the *Bgl*II site of the *KEX2* gene [17].

2.2. *DNA construction of Kex2p variants*

The expression cassette for the soluble form of the Kex2p (sKex2p) [18] was obtained by deleting from the *KEX2* gene [17], the DNA encoding the C-terminal 200 amino acids. The gene construct for sKex2pHDEL was made by adding linkers, encoding the His-Asp-Glu-Leu (HDEL) peptide sequence and two stop codons, to the 3' end of the gene encoding sKex2p. Details of the construction have been reported earlier [15].

2.3. *Construction of plasmids*

A unique *Bgl*II site was created at the *Sac*I site of the *E. coli–S. cerevisiae* shuttle vector pDP34A [16] containing the complete 2 μm sequence of *S. cerevisiae* [19]. The resulting vector was named pDP34B. A *Bam*HI fragment containing the IGF1 expression cassette [16] was ligated in the unique *Bam*HI site of pDP34B yielding the plasmid pBC23. The IGF1 expression cassette consists of a 400 bp promoter fragment from the yeast glyceraldehyde-3-phosphate dehydrogenase gene [16], then the leader sequence (αFL) of the *MATα* gene [5,16] encoding the first 85 amino acids of the preproα-factor (the last four amino acids of the αFL being, Leu^{82}-Asp^{83}-Lys^{84}-Arg^{85}), directly followed by the chemically synthesized IGF1 gene [16] and the 275 bp α-factor terminator (αFT) [5,16].

The expression cassettes containing the genes encoding for Kex2p [17] and the mutants, sKex2p [18] and sKex2pHDEL [15], were isolated as *Bam*HI fragments and subcloned in the unique *Bgl*II site of pBC23 (the subcloned IGF1 expression cassette does not have a *Bgl*II site) to yield plasmids pBC24, pBC25 and pBC26, respectively. The promoter and the transcriptional terminator used for these constructions were from the *KEX2* gene. Clones, which had on the plasmid the expression cassettes for IGF1 and the Kex2p variants in the opposite orientation, were chosen for further expression of IGF1.

2.4. *Expression of IGF1*

pBC23 was transformed in the yeast strain AB110 and AB110*kex2*. pBC24, pBC25 and pBC26 were transformed only in the strain AB110*kex2*. Three transformants from each of the five transformations were grown for 72 h in a uracil-selective medium for expression of IGF1 following a procedure which was reported earlier [16].

2.5. *Quantitative determination of IGF1 in the yeast culture media*

Correctly folded, active, monomeric IGF1 was quantified by reversed-phase HPLC and the total amount of secreted IGF1-like molecules was estimated by ELISA [16].

2.6. *Immunoblot analysis*

Samples for SDS-PAGE followed by immunoblot analysis were performed according to previously published protocols [16]. Rabbit polyclonal IGF1 anti-serum was generated using purified IGF1 monomer. The antibody was kindly provided by K. Einsle.

3. RESULTS AND DISCUSSION

In proteins, intramolecular disulfide bonds (-S–S-) form via redox reactions which involve thiol (-SH) functional groups present in the cysteine amino acid residues. Enzymes like thioredoxin, glutaredoxin [4] and protein disulfide isomerase [4] are thought to participate in these redox processes. In analogy to intramolecular disulfide bond formation it can be surmised that it would be thermodynamically feasible for intermolecular disulfide bonds to form if cysteines of two molecules are in close proximity to each other.

We have addressed the possibility that the presence of the proregion of the αFL during the folding of IGF1 could be a factor determining disulfide bond formation. In *S. cerevisiae* the maturation of the α-factor pheromone [14], or heterologous proteins [6] fused to the αFL, occurs when the Ca^{2+}-dependent serine protease Kex2p removes the proregion from precursor molecules by cleaving at a Lys-Arg residue [14]. It is evident [15] that the yeast Golgi membrane-bound endoprotease Kex2p [20] can function in the ER if the ER-retention signal HDEL [21] is attached to the C-terminus of the soluble form of the Kex2p (sKex2p) [18]. The availability of the ER-retained novel sKex2pHDEL [15] enzyme has allowed us to examine the role of the proregion of the αFL in the formation of intermolecular disulfide-linked dimers.

The gene encoding for sKex2pHDEL has been co-expressed with a polypeptide where the proregion of the αFL (proαFL) remains fused to IGF1 (i.e. proαFL-IGF1) after translocation [16]. The yeast strain (AB110*kex2*) used for expression would not normally allow any processing of proαFL-IGF1 because it lacks a functional copy of the *KEX2* gene. A plasmid containing only the IGF1 expression cassette (pBC23), when transformed in the strain AB110*kex2*, does not secrete any mature IGF1, as observed on a Western blot (Fig. 1); only diffuse disulfide-linked multimeric molecules, which can be reduced by dithiothreitol (DTT), are seen. When the wild-type (wt Kex2p) [17] is co-expressed with proαFL-IGF1 (pBC24) in the *kex2*$^-$ strain Western blot analysis of the secreted IGF1-like proteins fractionated

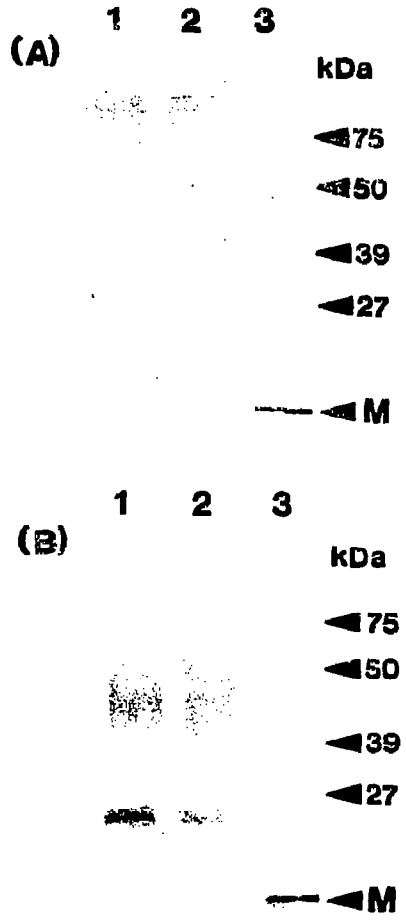

Fig. 1. Western blot analyses of IGF1-like proteins secreted from two yeast transformants of strain AB110*kex2* harbouring the plasmid pBC23 (bearing only the IGF1 expression cassette). 2 ml of cells (A_{600}=30) were harvested from 72 h yeast cultures. The supernatants were concentrated 4-fold using Centricon-3 concentrators (Amicon). 5 μl of cell supernatant were electrophoresed on a 15% SDS-polyacrylamide gel. After blotting with polyvinylidene difluoride membrane (Millipore), proteins were visualised using IGF1 anti-serum and the Bio-Rad immune assay kit. (A) Lanes 1 and 2, supernatants in the absence of any reducing agent; lane 3, 150 ng of purified IGF1 monomer (M). (B) Same as in (A) but the supernatants were treated with DTT. The 27, 39, 50 and 75 kDa bands used as markers belong to the pre-stained low-range standard proteins (Bio-Rad).

by SDS-PAGE under non-reducing conditions shows a mixture of molecules (Fig. 2A). The band with the faster gel mobility (~8 kDa) corresponds to the previously characterised authentic monomer [16]. Comparison of the mol.wt. of the second IGF1-like protein band to molecular weight markers is indicative of a dimer (~16 kDa). The ~16 kDa band can be reduced by DTT to the ~8 kDa band (Fig. 2B), confirming that the dimers formed are disulfide linked. The monomeric and dimeric molecules secreted are identical in mol.wt. to those obtained from strains where AB110, with the single genomic copy of *KEX2* intact, has been transformed with pBC23 (Fig. 2A). This implies that co-expression of Kex2p on a multi-copy yeast plasmid, pDP34B, does not have a profound effect on the nature of the secreted IGF1 molecules. Formation of reducible dimeric molecules (Fig. 2B) is also observed when sKex2p [18] is co-expressed with proαFL-IGF1 (pBC25). sKex2p is a mutant form of Kex2p from which the membrane-spanning region and C-terminal tail have been removed [18,20]. In contrast to Kex2p, sKex2p is secreted in considerable amounts and still retains protease activity [18].

In order to direct sKex2p to the ER, the *S. cerevisiae* ER-retention signal (HDEL) [21] has been attached to the C-terminal end of sKex2p. The only difference between the coding region of sKex2p and sKex2pHDEL is the extra 12 bp DNA sequence encoding the last four amino acids in sKex2pHDEL. Surprisingly, dimer formation is hardly seen (Fig. 2A) in strains co-expressing sKex2pHDEL and proαFL-IGF1 (pBC26).

It has been shown earlier that HPLC is a sensitive and reliable assay for quantifying active, monomeric IGF1 [16]. A comparison of chromatograms of yeast culture supernatants makes it evident that the monomeric molecules generated by sKex2pHDEL, sKex2p and wtKex2p are alike (Fig. 3). The HPLC system detects genuine human IGF1 with correct intramolecular disulfide bonds (i.e. Cys^{6}-Cys^{48}, Cys^{18}-Cys^{61}, Cys^{47}-Cys^{52}; retention time ~17.6 min) and also monomeric IGF1 species (retention time ~16.8 min) where two disulfide bonds are interchanged (i.e. Cys^{6}-Cys^{47}, Cys^{48}-Cys^{52}, Cys^{18}-Cys^{61}) [16]. Like the dimeric molecules this positional isomer of IGF1 is biologically inactive. The dimers are so hydrophobic that they cannot be eluted from the C4 reverse-phase columns used for the HPLC analysis (unpublished results) and therefore cannot be seen on the chromatograms.

ELISA, however, can estimate the total amount of IGF1-like molecules (i.e. it assays for the sum of the active monomers and inactive monomers/dimers) present in the supernatants. A comparison of HPLC titres and ELISA vaues reveals that secretion of inactive IGF1 molecules from the strains expressing sKex2pHDEL are remarkably reduced (Table I). Though sKex2pHDEL removes dimeric IGF1 molecules from the supernatant (Fig. 2) it is obvious that species other than the authentic IGF1 monomer also secrete into the culture medium (Fig. 3). The inactive monomeric forms cannot be distinguished from the active monomers on the gel system used for Western blot analysis [16]. The results tabulated in Table I also show that the amounts of secreted monomeric IGF1 using sKex2p and sKex2pHDEL are appreciably lower than that obtained when membrane integrated wtKex2p is used. This could indicate that the propensity for sKex2p and sKex2pHDEL to cleave the substrate (proαFL-IGF1) in the cel might not be the same as for wtKex2p. It is possible that substrate-specificity is facilitated because wtKex2p is immobilised on the membrane.

It has already been reported that sKex2pHDEL can

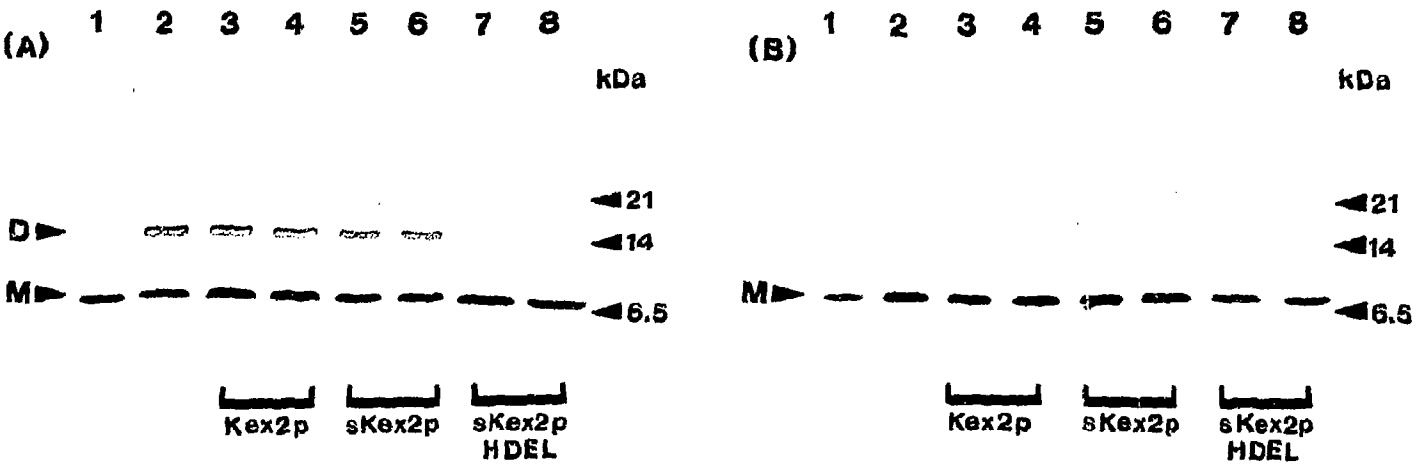

Fig. 2. A comparison of IGF1-like proteins secreted from AB110*kex2* transformants harbouring the plasmids, pBC24 (bearing IGF1 and Kex2p expression cassettes), pBC25 (bearing IGF1 and sKex2p expression cassettes) and pBC26 (bearing IGF1 and sKex2pHDEL expression cassettes), on a Western blot. Two transformants from each of the three transformations were used for analyses. An AB110 transformant of the plasmid pBC23 (bearing only the IGF1 expression cassette) was used as a control. 2 ml of supernatants were concentrated as in Fig. 1. Supernatants from pBC23 and pBC24 were concentrated 2-fold and the ones from pBC25 and pBC26 were concentrated 4-fold. Proteins from 5 μl of concentrated supernatants were analysed as in Fig. 1. (A) Lane 1, 150 ng of purified IGF1 monomer (M); lane 2, pBC23::AB110; lanes 3 and 4, pBC24::AB110*kex2*; lanes 5 and 6, pBC25::AB110*kex2*; lanes 7 and 8, pBC26::AB110*kex2*. (B) Same as in (A) but the supernatants were treated with DTT. Rainbow coloured (Amersham) protein markers (low mol. wt. range) were used as standards.

process the proregion of the αFL in the ER instead of in the Golgi [15]. It is probable that the HDEL tetrapeptide allows recycling of sKex2p to the ER from a compartment in between the ER and the Golgi via a receptor-mediated pathway [22]. In this communication we have attempted to establish that there is a correlation between the expression of sKex2pHDEL and dimer formation in IGF1. The results presented suggest that an earlier processing of the proregion of the αFL prevents intermolecular disulfide bond formation in molecules secreted from yeast. This points to the fact that proαFL may have a role in the in vivo folding of the intramolecular disulfide-linked molecule, IGF1. In the native conformation of IGF1 the 6 cysteines involved in the 3 *intra*molecular -S–S- bonds are masked [13]. At least 2 of the cysteines ought to be exposed if *inter*molecular

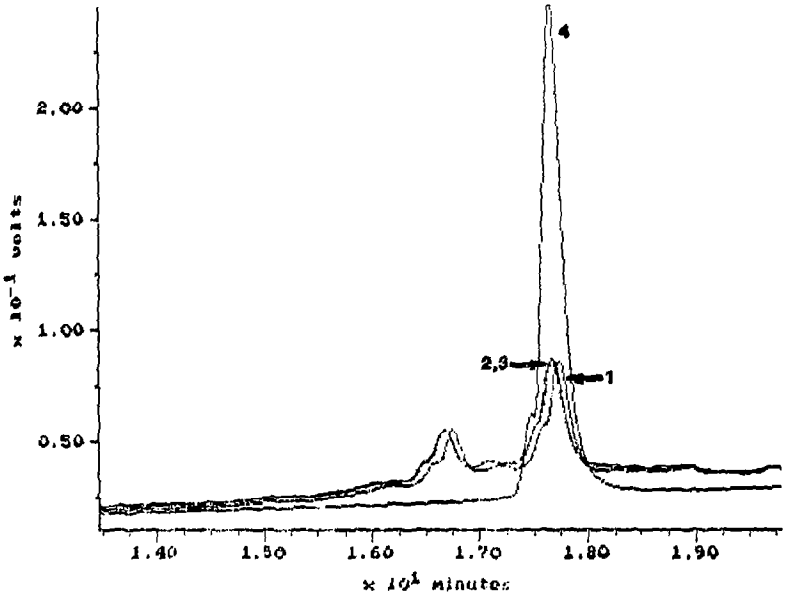

Fig. 3. HPLC chromatograms of monomeric IGF1 secreted from AB110*kex2* transformants bearing plasmids, pBC24, pBC25 and pBC26 (see Fig. 2). 2 ml of cells (A_{600}=30) from 72 h cultures were centrifuged. Different volumes of the supernatants were injected and analysed on a reverse-phase Delta Pak C4-30 nm column (Nihon Waters, Japan). A solvent gradient similar to the one described earlier was used [16]. (1) 60 μl supernatant from pBC24; (2) 120 μl from pBC25; (3) 120 μl from pBC26; (4) 100 μg of purified IGF1 monomer. Coincidentally chromatograms (2) and (3) superimpose on each other. Chromatograms have been drawn using the Maxima software (Waters).

Table I

An estimation of the percentage of monomeric IGF1 in the total amount of IGF1-like molecules secreted from strains expressing Kex2p, sKex2p and sKex2pHDEL

Plasmid	HPLC titres (μg/ml)[a]	ELISA values (μg/ml)[a]	Percentage of monomeric IGF1
pBC24	14	120	12
pBC25	6	55	11
pBC26	7	19	37

[a] These values are an average obtained from three individual transformants

-S–S- bond formation is to happen. It appears that when the proαFL-IGF1 polypeptide folds in the ER the folding pathway of nascent IGF1 is somehow compromised. However, it is not clear whether this event occurs because of misfolding or because proαFL merely slows down the rapid turnover of a folding intermediate [23] where a cysteine residue is momentarily exposed.

It is possible that proαFL can exert a similar effect on other heterologous polypeptides for which the αFL is essential for secretion from yeast. Studies of proregions of several proteins have shown that proregions could play the role of a molecular chaperone [24]. It is believed that proregions could have other cellular roles, too [25]. We anticipate that sKex2pHDEL and homologues, where the DDEL [26] and the KDEL [21] tetrapeptide are attached to the C-terminus of sKex2p [18], can be used as tools to study a variety of distinct cellular processes in different eukaryotic systems.

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