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A Lys^{27}-to-Glu^{27} mutation in the human insulin-like growth factor-1 prevents disulfide linked dimerization and allows secretion of BiP when expressed in yeast

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Recombinant human insulin-like growth factor-1 (IGF1) secreted from yeast contains only 10–15% of the active monomer. A majority of the IGF1-like molecules are disulfide bonded dimers. These dimers are not formed in an IGF1 mutant where Lys^{27} has been replaced by glutamic acid. However, increased levels of secreted BiP (the yeast *KAR2* gene product) are seen in cells expressing the mutant. These results imply that by preventing ionic interactions between two IGF1 molecules, intermolecular disulfide bonds do not form in yeast, and that in the mutant there is a structural change which induces BiP, allowing its secretion.

Insulin-like growth factor 1; Dimerization; Intermolecular disulfide bond; Folding/malfolding in vivo; BiP secretion; *Saccharomyces cerevisiae*

1. INTRODUCTION

Human IGF1 (hIGF1) is a 70 amino acid polypeptide with 3 intramolecular disulfide bonds involving all 6 cysteine residues. The IGF1 polypeptide is highly homologous to the insulin molecule. The amino acid residues in the B and A chains are 50% identical [1]. Due to its similarity, the three-dimensional structure of hIGF1 can reasonably be modelled on the basis of the known structure of porcine insulin [2,3].

Human proinsulin and insulin oligomerize in vivo and in vitro to form dimers [4]. The dimerization has been proposed to be a result of the close approach of a charged amino acid of one molecule to an oppositely charged amino acid of another. Monomeric insulin has been obtained by substituting Pro^{28} with Asp, the Asp^{28} of one mutant molecule preventing close proximity to the Glu^{21} of another molecule [5].

In the expression of hIGF1 in the yeast *Saccharomyces cerevisiae*, we have observed that molecules secreted into the culture medium are approximately 50% dimers (data not shown). Unlike insulin, these dimers are covalently connected by disulfide bonds. We have investigated the possibility that the dimerization of IGF1 in yeast may be due to an initial ionic interaction between 2 molecules of IGF1, the positive charge of Lys^{27} interacting with the negative charge of Asp^{20} in another. A substitution (Lys^{27}-to-Glu^{27}) was made, in a similar position in the IGF1 molecule to the one made previously in insulin [5] with the hope that introduction of a negatively charged amino acid in place of a positively charged one would prevent 2 molecules of IGF1 coming close together, and thus prevent intermolecular disulfide bond formation and dimerization.

We have also examined the result of the change in the charge of an amino acid on the surface of the IGF1 molecule on the levels of the yeast *KAR2* gene product (BiP), a 78 kDa protein [6,7]. BiP is known to be induced by the improper folding of proteins [8–10]. The mutant and the wild type (wt) molecules have been used to gauge whether BiP recognizes them differently. Both intracellular and extracellular levels of BiP have been analysed after expression in yeast of both the mutant and wt IGF1.

Abbreviations: BiP, heavy chain binding protein; BPTI, bovine pancreatic trypsin inhibitor; DTT, dithiothreitol; ER, endoplasmic reticulum; hIGF1, human insulin-like growth factor-1; HPLC, high performance liquid chromatography; PMSF, phenyl-methane-sulfonyl-fluoride; RNase A, ribonucleaseA; TCA, trichloroacetic acid; wt, wild type.

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2. MATERIALS AND METHODS

2.1. *Plasmid constructions*

Plasmid BC1, containing the wt IGF1 gene in an expression cassette subcloned in pDP34, was constructed as previously described [11]. The mutant was created using 2 oligonucleotide primers and a M13mp8 DNA template containing the positive DNA strand of the IGF1 expression cassette [12]. The mutation Lys^{27}-to-Glu^{27} was introduced using the mutagenic primer 5′-GTAACCGGTTGGttcGTTGAAG-TAG-3′, the lower case letters indicating the changed codon. After checking the correctness of the mutated DNA sequence, the mutant coding DNA was re-introduced at the *Bam*HI site of pDP34 [11] yielding the plasmid pBC2.

2.2. *Strains and growth conditions*

S. cerevisiae AB110 (*Mat α, his4-580, leu2, ura3-52, pep4-3, [cir°]*) has been used for yeast transformation with plasmids pBC1 and pBC2. Three transformants from each of the 2 transformations were grown up in a rich medium for expression of the mutant and wt IGF1 [11].

2.3. *Immunoblot analysis*

Preparation of cell lysates, and samples for SDS-PAGE followed by immunoblot analysis were performed according to previously published protocols [11]. Rabbit polyclonal IGF1 anti-serum was generated using purified IGF1 monomer. The polyclonal anti-Kar2 antibodies were raised in rabbits against an 11-amino acid peptide belonging to the yeast protein BiP, using a published procedure [7]. The antibodies were kindly provided by K. Einsle and A. Hinnen.

3. RESULTS

To examine the consequence of the Lys^{27}-to-Glu^{27} mutation, 6 individual yeast transformants, 1–3 bearing the wt gene and 4–6 bearing the mutant gene, were grown in a complex medium for 72 h. The nature of the secreted IGF1 molecules was compared for the 2 series of transformants by Western blots under non-reducing conditions (Fig. 1A). The mutant clearly shows no dimer formation. The dimer formed by the wild type molecule can be reduced with DTT yielding the monomer (Fig. 1B). This indicates that the dimers are held together by intermolecular disulfide bonds.

To address the question that the folding of the mutant IGF1 might not be the same as the wt molecule, we have looked at the possibility of BiP induction by the mutant molecule. No clear difference between levels of intracellular BiP induced by the wt and the mutant was observed (Fig. 2A). However, we found that expression of the mutant gene in yeast causes BiP to be secreted much more than in the case of the unmutated IGF1 gene (Fig. 2B).

From these results we can conclude that introduction of a glutamic acid residue in place of lysine prevents formation of disulfide bonded dimers in IGF1. We can also infer from the increased levels of BiP secreted that BiP identifies the mutant and the wt molecules differently.

4. DISCUSSION

The in vitro formation of disulfide bonds in denatured, scrambled RNase A and BPTI has been extensively studied in order to understand protein folding pathways [13,14]. We have attempted to elucidate a structural determinant which might be involved in in vivo intermolecular disulfide bond formation between 2 protein molecules. It appears that the proximity of 2 molecules of IGF1 through charged interactions leads to intermolecular disulfide bonds. Keeping them apart by altering the polarity of a specific amino acid prevents this dimerization. From molecular modelling, it is apparent that the projected IGF1 dimer conformation, on the basis of the insulin structure, does not allow interaction of the cysteine residues in one molecule with the cysteines in the other (Fig. 3A,B). It is possible that the 2 extra domains in IGF1 (the C and D chains, which are absent in mature insulin) [1,2] do not allow the final tertiary structure of IGF1 to be identical to that of the insulin molecule. We propose that IGF1 acquires a conformation which exposes at least one cysteine residue in such a way that close proximity between 2 molecules allows intermolecular disulfide bonds to form. Elimination of a specific charged interaction between two IGF1 molecules prevents this intermolecular disulfide bond formation.

BiP is an endoplasmic reticulum (ER) retained heat

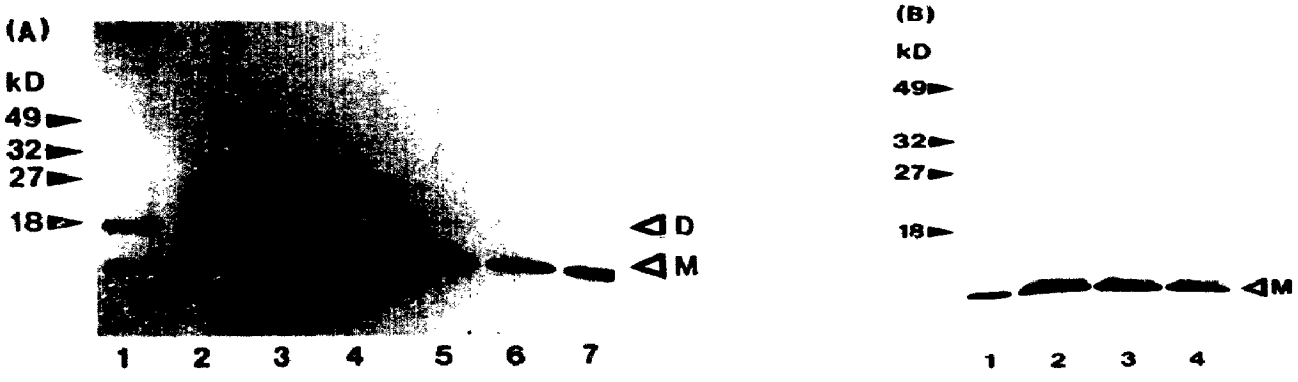

Fig. 1. Western blot analysis of secreted wt IGF1 and the mutant, using 15% SDS-PAGE and IGF1 anti-serum. Protein was blotted to polyvinylidene difluoride membrane (Millipore) on a semi-dry Sartorious blotter at 0.8 mA/cm² of membrane, for 1 h. Yeast transformants of strain AB110, harbouring the plasmids pBC1 (lanes 1–3) and pBC2 (lanes 4–6), were analysed under non-reducing conditions (A). DTT was added to supernatants from strains bearing pBC1 (lanes 2–4) (B). The amounts of mutant proteins loaded in lanes 4–6 were 3 times the amounts of wt IGF1 loaded in lanes 1–3 (A). It was estimated from HPLC analysis that, per unit volume, the amount of secreted mutant protein (viz. 5 μg/ml) was less than half of wt IGF1 (11 μg/ml). The total protein secreted by all strains was similar (130 μg/ml; Bio-Rad protein assay). Secreted mutant protein in the supernatant was concentrated 6-fold using CENTRICON-3 concentrators (Amicon). 10 μl of unconcentrated supernatants (lanes 1–3) and concentrated supernatants (lanes 4–6) were loaded. Lane 7, purified IGF1 monomer, M, monomer; D, dimer.

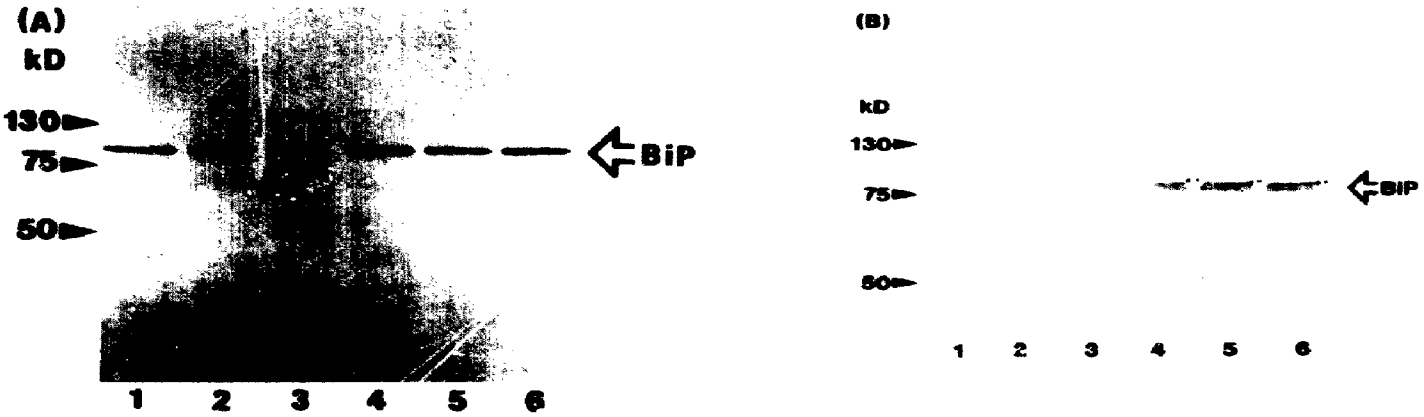

Fig. 2. Western blot of intracellular BiP (A) and secreted BiP (B), using 10% SDS-PAGE and BiP anti-serum. Protein was blotted as in Fig. 1 (A). Cells at 100 OD_{600} were mechanically lysed using 0.2 g glassbeads (0.5 mm diameter) with 200 μl of sample buffer (4% SDS, 0.1 M Tris pH 6.8, 4 mM EDTA) and 1.5 μl of the cell lysate was loaded in each slot. Lanes 1–3, transformants of AB110 harbouring pBC1; lanes 4–6, AB110 harbouring pBC2. (B) 1 ml of culture supernatant containing phenyl-methane-sulfonyl-fluoride (PMSF) (0.1 mM) was precipitated with trichloroacetic acid (TCA) (final concentration 25%). The pellet was resuspended in 20 μl of sample buffer. Lanes 1–3 and lanes 4–6, as in (A).

shock protein [15,16]. It has been shown that malfolding of mutant proteins causes an elevated level of intracellular BiP [9]. BiP binds to malfolded proteins permanently and prevents these malfolded structures leaving the ER [10]. In mammalian cells, degradation via an ER degradative pathway has been proposed for these terminally bound molecules [10].

Although we do not observe higher levels of intracellular BiP, we do see that BiP is secreted in the case of the mutant. It is conceivable that there is a limit to the number of molecules of BiP which can be retained in the ER of a yeast cell through the *ERD2* receptor-mediated pathway [17]. The *ERD2* gene product is thought to be in an intermediate compartment between the ER and the Golgi. The excess of BiP which is induced by the mutant cannot be retrieved in the ER because the receptor is saturated. This excess BiP, bound or unbound to the mutant molecules, is then free to be secreted, transport occurring by nonselective bulk flow. We propose that, in yeast, secreted levels of BiP could be a measure of its recognition of particular structures, often defined as malfolded. How BiP identifies malfolded molecules from folded ones remains an enigma [18,19]. Currently, we are analysing some peptides which might bind BiP in vivo and in turn induce it.

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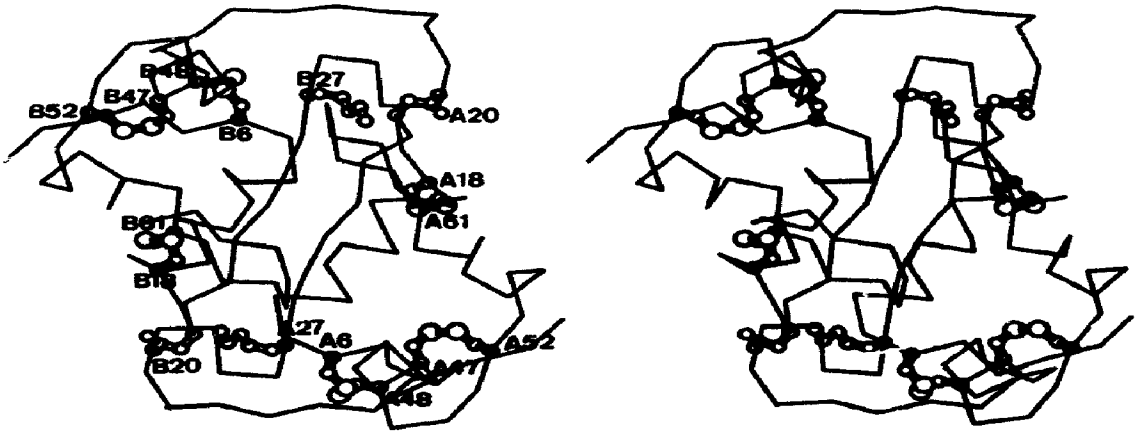

Fig. 3. Stereo view of the IGF1 dimer as modelled, based on the porcine insulin dimer. The disulfide bonds (6–48, 18–61, and 47–52, solid bonds) in both monomers (A and B) are highlighted as are residues Lys^{27} and Asp^{20} (open bonds).

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