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A novel type of FKBP in the secretory pathway of *Neurospora crassa*[1]

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Abstract FKBPs define a subfamily of peptidyl-prolyl *cis/trans* isomerases (PPIases). PPIases are known to play roles in cellular protein folding, protein interactions and signal transduction. Here we describe NcFKBP22 from *Neurospora crassa*, a novel type of FKBP. NcFKBP22 is synthesized as a precursor protein with a cleavable signal sequence. In addition to a typical FKBP domain in the amino-terminal part mature NcFKBP22 contains a novel second domain which is unique amongst all known FKBPs. The amino acid composition of this carboxy-terminal domain is highly biased. Secondary structure predictions suggest that this domain may form an amphipathic α-helix. The carboxy-terminus of NcFKBP22 is –HNEL, a potential endoplasmic reticulum (ER) retention signal, suggesting that NcFKBP22 is a resident protein of the ER.

Key words: Prolyl isomerase; FKBP; Endoplasmic reticulum; Late embryogenesis abundant protein; Protein folding

1. Introduction

Peptidyl-prolyl *cis/trans* isomerases (PPIases; EC 5.2.1.8.) are enzymes able to catalyze the *cis/trans* isomerization of Xaa–Pro bonds in oligopeptides and proteins (for reviews see [1–5]). PPIases are ubiquitous and abundant proteins and have been found in bacteria, fungi, plants, and mammals. They belong to three structurally unrelated families, namely the cyclophilins (CyPs), the FKBPs, and the parvulin-like proteins. CyPs and FKBPs are characterized by their ability to bind the immunosuppressant drugs cyclosporin A or FK506/rapamycin, respectively. Due to their enzymatic activity, PPIases are able to accelerate slow refolding steps in certain proteins in vitro (see [2]). Mitochondrial CyPs from *Neurospora crassa* [6,7] and yeast [8] have been shown to be part of the protein folding machinery of the organelle involving molecular chaperones [6,7].

Whether FKBPs are also involved in eukaryotic cellular protein folding is not yet clear. Known functions of eukaryotic FKBPs (e.g. the abundant FKBP12) are functional interactions with other proteins, such as the rhyanodine receptor of the endoplasmic reticulum (ER) membrane, the inositol-1,4,5-trisphosphate receptor or the TGF receptors of the plasma membrane (see [9]).

Here we describe the identification and characterization of a novel type of FKBP in the ER of *N. crassa*, which in addition to a functional FKBP domain contains a second, novel domain, which is not present in any other characterized FKBP.

2. Materials and methods

2.1. Growth of N. crassa and preparation of protein extract

Cultures of *N. crassa* wild type 74A and *fkr-2* [10] were grown as described [11]. Hyphae from 10 l cultures were harvested. Per 1 g hyphae, 1.5 g quartz sand and 1 ml extraction buffer (10 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 5 mM DTT, 1 mM PMSF, 1% Triton X-100, pH 7.4) were added, cells broken and total extract prepared as described [11].

2.2. Affinity purification of N. crassa FKBPs

1 ml affinity beads (ascomycin bound to Sepharose beads; gift from Dr. U. Krieg) was equilibrated with 10 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, pH 7.4 (buffer H) in a column. 100–500 mg total protein was applied to the column. After extensive washes with buffer H and buffer H without Triton X-100, specifically bound proteins were eluted with 20 mM glycine, pH 2.5. 1 ml fractions were neutralized with Tris, precipitated using TCA and proteins run on SDS–PAGE [12].

Antibodies in rabbits were prepared as described [13]. Amino-terminal sequencing was done according to [15].

2.3. Cloning of a full-length cDNA encoding NcFKBP22

A λgt11 library of *N. crassa* [13,14] was used for screening. One positive clone contained a short cDNA insert encoding a carboxy-terminal part of NcFKBP22. Reverse primers from this region were used in combination with degenerate primers directed against the amino-terminus to isolated a specific NcFKBP PCR product, which was finally used in plaque hybridizations to isolate a full-length cDNA (EMBL submission number AJ006297.1).

2.4. PPIase assays

Native NcFKBP22 was purified to homogeneity from *N. crassa* (manuscript in preparation). To determine PPIase activity, the isomer-specific protease assay [16] was used. The assay contained 35 mM HEPES–KOH, pH 7.5, 7.6 nM NcFKBP22, 800 μg/ml chymotrypsin and 80 μM succinyl-Phe-Pro-Phe-*p*-nitroanilide (Succ-Phe-Pro-Phe-pNA). For determination of the sensitivity of NcFKBP22 towards FK506 and rapamycin, 1 mg/ml BSA was added to the reaction mix and NcFKBP22 was preincubated for 15 min with various concentrations of the inhibitors. Reaction temperature was 7°C.

3. Results

3.1. Affinity purification of N. crassa FKBPs

The only known FKBP in *N. crassa* to date is NcFKBP13 [14]. NcFKBP13 is the receptor mediating the antifungal action of FK506 in *N. crassa* [10]. NcFKBP13 has a dual location in cytosol and mitochondria, both forms being encoded by a single nuclear gene, most probably *fkr-2* (manuscript in preparation; see [17]).

In order to identify novel FKBPs, we used affinity chroma-

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[1] The cDNA sequence for NcFKBP22 has been deposited under EMBL accession number AJ006297.1. The protein accession number is CAA06962.

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tography using the FK506 homolog ascomycin (see [9]) coupled to Sepharose beads.

Briefly, *Neurospora* lysates were prepared in the presence of 1% Triton X-100 (to include soluble organellar proteins for the analysis) and loaded onto an ascomycin-affinity resin. After extensive washes, specifically bound proteins were eluted by a shift of the pH from 7.4 to 2.5 and analyzed by SDS–PAGE (see also Section 2).

Two proteins running with apparent molecular weights of 29 and 13 kDa could be routinely seen in *N. crassa* wild type lysates (Fig. 1, lane 1/WT), the 13 kDa protein being more prominent.

We suggested that affinity-purified NcFKBP13 might be identical to NcFKBP13 already described (see above; [14]). Indeed, amino-terminal protein sequencing of the affinity-purified FKBP13 (Fig. 1, lane 1/WT) yielded the sequence TIPQLDGLQIEVQQEG (see Fig. 2A), which is identical to the published sequence of NcFKBP13 [14], with the exception that the initiator methionine is missing; this is obviously removed post-translationally from the cytosolic protein. In the case of the mitochondrial precursor protein, the presequence is cleaved after methionine corresponding to methionine +1 in the cytosolic protein (manuscript in preparation). This results in identical NcFKBP13 proteins in both cytosol and mitochondria.

One class of FK506-resistant *N. crassa* mutants (*fkr-2*) has lost immunodetectable NcFKBP13 [10]. This result is confirmed by ascomycin affinity chromatography of *fkr-2* lysates. No NcFKBP13 can be seen *fkr-2* lysates (Fig. 1, lane 2/*fkr-2*).

However, in addition to NcFKBP13, a second FKBP appears in lysates of the wild type strain, which runs with an apparent molecular weight of 29 kDa in SDS–PAGE (for reasons mentioned below, we named it NcFKBP22). From *fkr-2* lysates, NcFKBP22 can be isolated as the only apparent ascomycin-binding protein (Fig. 1, lane 2/*fkr-2*).

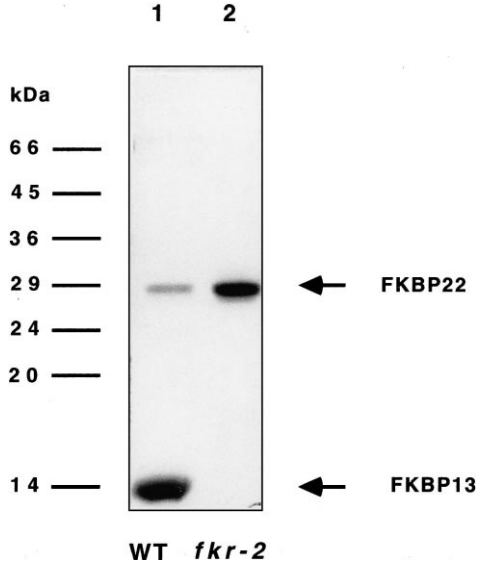

Fig. 1. Ascomycin binding proteins of *N. crassa*. Lysates from wild type *N. crassa* (WT; lane 1) and the FK506-resistant mutant *fkr-2* (lane 2) were prepared as described in Section 2. 100 mg protein was bound to the ascomycin matrix, washed extensively and bound proteins eluted by a shift of the pH to 2.5. Eluted 1 ml fractions were neutralized, precipitated by TCA, run on SDS–PAGE and stained with Coomassie blue.

A

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1   A----EELGIDVIVPV?CDR 16   FKBP22
1   TIPQLDGLQIEVQQEGQGTR 20   FKBP13

17  KTRKGDKINVHYR?TLQSN 35    FKBP22
21  ETRRGDNVDVHYKGVL-TS 39    FKBP13
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B

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1   MKSIFLSLSLLASAIVGVLAAEELGIDVIVPVECDRKTRK  40
41  GDKINVHYRGILQSNGQQFDASYDRGTPFSFKLGGGQVIK  80
81  GWDEGLVDMCIGEKRTLIVPPSYGYGQRSIGPIPAGSTLI  120
121 FETELIGIDGVPKPESIVYKQAAEKAEEAASAVEEKVAEA  160
161 TDKAGGKIADATKKVEEKAEEASANVVEKVASVVSGAAEA  200
201 VKTVVADTDDVQEHNEL                         217
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C

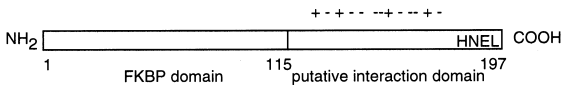

Fig. 2. NcFKBP22 is synthesized as an ER precursor protein; the mature protein contains a novel domain. A: Comparison of amino-terminal sequences of NcFKBP22 and NcFKBP13, respectively. B: Amino acid sequence of NcFKBP22 precursor derived from the cDNA sequence; the cleavage site of signal peptidase is indicated by an arrow; a putative ER retention signal (–HNEL) is indicated in bold print. C: Domain organization of mature NcFKBP22.

In order to characterize the protein, affinity-purified NcFKBP22 was used for amino-terminal sequencing and for the production of rabbit antisera.

3.2. Determination of the amino-terminal sequence of NcFKBP22

Affinity-purified FKBP22 was blotted onto PVDF membranes and subjected to automatic amino-terminal Edman degradation [15]. 34 out of 36 amino acids could be identified unambiguously. Comparison of the amino-termini of NcFKBP22 with that of NcFKBP13 [14] revealed that we had indeed identified a novel FKBP in *N. crassa* (see Fig. 2A). Out of 33 identified amino acids of NcFKBP22, 12 are identical to FKBP13 (shown in bold in Fig. 2A).

3.3. Molecular cloning of a cDNA encoding NcFKBP22

A *N. crassa* cDNA library [13,14] cloned into expression vector λgt11 was screened using NcFKBP22 specific antisera. One clone proved to be NcFKBP22-specific, but contained only the carboxy-terminal region (data not shown). PCR reactions, making use of the known amino-terminal sequence (see Fig. 2A) finally yielded a full-length cDNA.

The deduced amino acid sequence of NcFKBP22 is shown in Fig. 2B.

Obviously, NcFKBP22 is made as a precursor protein with 20 additional amino acids at the amino-terminus. The precursor protein is 217 amino acids in length. The additional sequence is a typical presequence for entry of the precursor protein into the ER [18]. A basic amino acid at the amino-

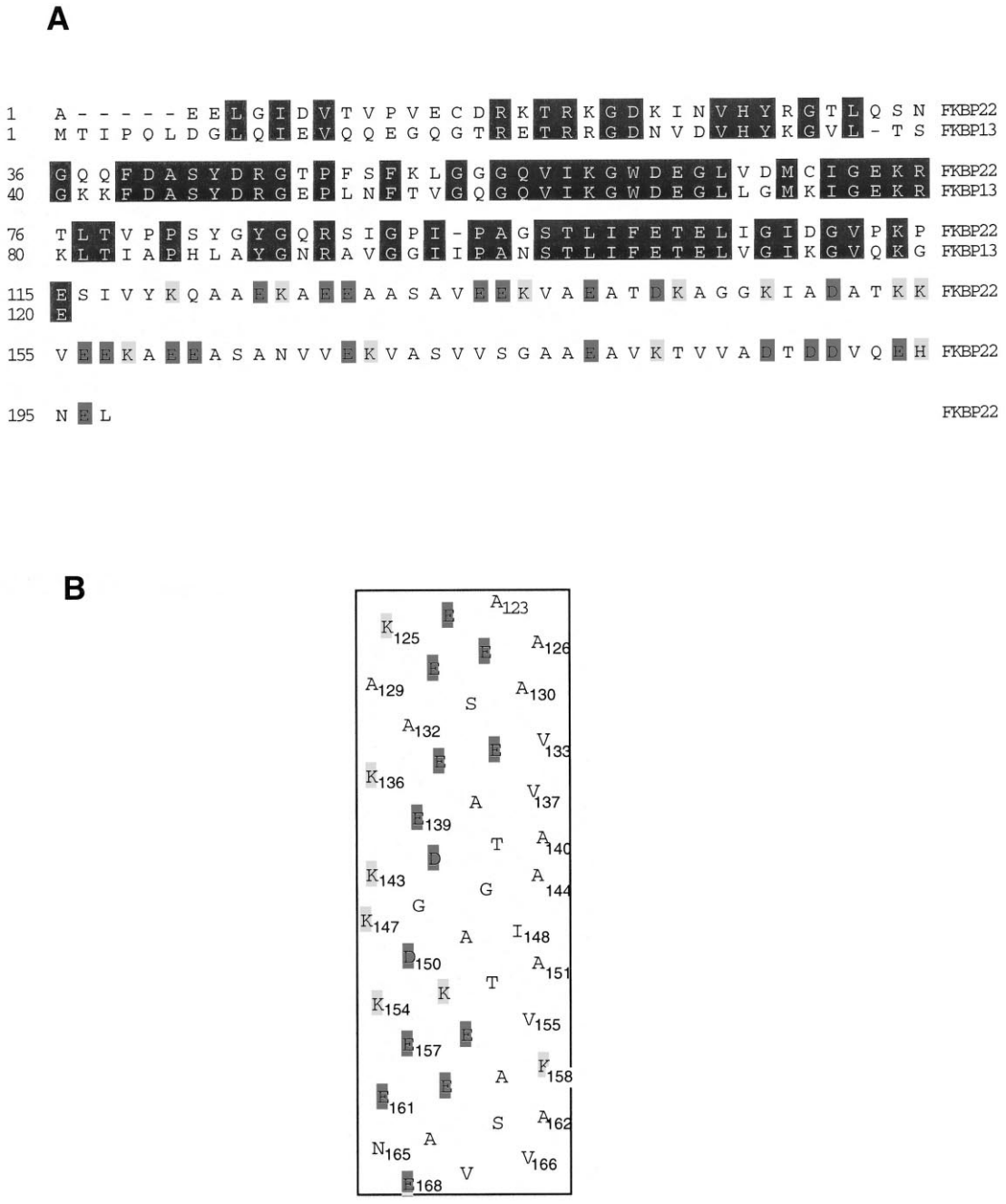

Fig. 3. NcFKBP22 contains a typical FKBP domain at the amino-terminal end and a novel carboxy-terminal domain capable of forming an amphipathic α-helix. A: Sequence comparison of *N. crassa* NcFKBP22 and NcFKBP13 [14]. Identical residues are heavy shaded; in the carboxy-terminal part of NcFKBP22, basic residues are lightly shaded and acidic residues are medium shaded. B: 'Helical net' diagram [22] of residues 123–168 of mature NcFKBP22. Acidic residues are medium shaded, basic residues are lightly shaded. For reasons of clarity, residues are not interconnected.

terminus (lysine at position 2) is followed by a stretch of hydrophobic residues. Cleavage by the signal peptidase obviously occurs after GVLA, AEEL being the amino-terminus of the mature protein (Fig. 2B; cleavage site is indicated by an arrow).

Calculated from the cDNA sequence, the mature protein (197 amino acids) has a molecular weight of 20 912 Da. Electrospray mass spectroscopy of affinity-purified protein, however yielded a mass of 22 043 Da (manuscript in preparation). Therefore, the protein was named NcFKBP22. The nature of the post-translational modification resulting in an increase of the molecular weight of 1131 Da is yet unknown (preliminary results suggest that NcFKBP22 might be glycosylated; see Section 4

The extreme carboxy-terminus of NcFKBP22 contains a putative *N. crassa* ER retention signal (–HNEL; shown in bold in Fig. 2B); we, therefore, propose that NcFKBP22 is a resident protein of the ER.

The amino-terminal part of NcFKBP22 (residues 1–115) is a typical FKBP domain; the highest amino acid identities of the core FKBP region are to cytosolic/mitochondrial *N. crassa* NcFKBP13 (60%; see Fig. 3A), *Vicia faber* FKBP15 (55%;

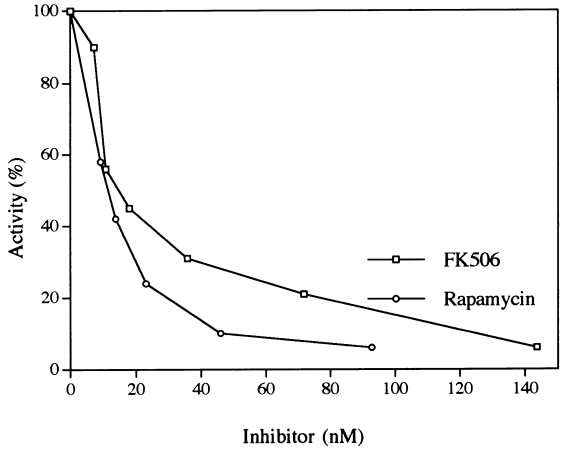

Fig. 4. PPIase activity of NcFKBP22 is inhibited by both FK506 and rapamycin. NcFKBP22 was preincubated with FK506 and rapamycin, respectively, and residual activity was measured as described in Section 2. K_i values were calculated according to [31].

see [19]) and ER-localized human FKBP13 (53%; see [21]) and yeast FKBP13 (50%; see [20]). All residues important for PPIase activity and FK506/rapamycin binding (see [9]) are present in the FKBP domain of NcFKBP22.

However, NcFKBP22 is unique amongst known FKBPs in containing an unusual carboxy-terminal domain (residues 116–197), which has no homology to a domain in any other known FKBP (see Fig. 3C). This carboxy-terminus (82 residues) contains an unusually high number of acidic residues (14 Glu and 5 Asp residues versus 10 Lys and no Arg residues).

Also, the amino acid composition of the carboxy-terminal domain of NcFKBP22 is clearly biased (see Table 1; comparing amino acid composition of the N-terminal FKBP domain (residues 1–115) to the C-terminal domain (residues 116–197)). Ala, Glu, Lys and Val are overrepresented, while there is a clear bias against Phe, Gly, Ile, Leu, Pro and Arg.

Secondary structure predictions according to Garnier-Robson predict that residues 116–197 form an amphipathic α-helix, with residues 166–189 being more hydrophobic than the rest. In a helical net diagram [22], residues 123 to 168 form a perfect amphipathic helix where hydrophobic, acidic and basic residues, respectively, are in distinct areas of the proposed α-helix (Fig. 3B).

Table 1
The C-terminal domain of NcFKBP22 has a biased amino acid composition

Amino acid	Amino-terminus	Carboxy-terminus
Ala	2.6	23.2
Glu	7.0	17.1
Phe	3.5	0
Gly	14.8	3.7
Ile	7.8	2.5
Lys	6.1	12.2
Leu	6.1	1.2
Pro	7.0	0
Arg	5.2	0
Val	7.0	15.9

Amino acid frequencies in % in the FKBP domain of NcFKBP22 (amino-terminus; residues 1–115 of the mature protein; see Fig. 3A) and the carboxy-terminal domain (residues 116–197 of the mature protein). Only those amino acids which differ by more than 3% are shown.

BLAST searches [23] using the the carboxy-terminal domain (residues 116–197) reveal a low sequence similarity (approximately 30% sequence identity; data not shown) to short regions from late embryogenesis abundant (LEA) proteins from different plant species like wheat [23], barley, cotton or *Arabidopsis thaliana* (see [25,26]). During periods of water deficit, plants accumulate LEA proteins which are thought to protect cells from stress associated with dehydration. A tomato LEA protein confers salt and freezing tolerance when expressed in *Saccharomyces cerevisiae* [27]. The wheat LEA protein Em functions as an osmoprotective molecule in yeast [24]. Whether the carboxy-terminal domain of NcFKBP22 might have a similar function in the ER of *N. crassa* has to be determined.

3.4. PPIase activity of NcFKBP22

NcFKBP22 was purified to homogeneity from *N. crassa* hyphae using standard biochemical techniques (manuscript in preparation). PPIase activity of native NcFKBP22 was measured using the isomer specific protease assay [16]. As a substrate succinyl-Phe-Pro-Phe-pNA (see Section 2) was used. NcFKBP22 exhibits PPIase activity with a k_{cat}/K_M value of 6.94×10^5 M^{-1} s^{-1}. As expected (see Fig. 4), the enzymatic activity can be inhibited by FK506 ($K_i = 4.5$ nM) and rapamycin ($K_i = 6.2$ nM).

4. Discussion

We have identified a novel type of FKBP in *N. crassa*. NcFKBP22 is synthesized with a presequence of 20 amino acids which is cleaved upon entry into the ER. Indeed, mature NcFKBP22 can be isolated from luminal fractions of *N. crassa* microsomes (manuscript in preparation).

Mature NcFKBP22 is composed of an amino-terminal FKBP domain and a unique carboxy-terminal domain. As expected from the sequence [9], the FKBP domain is active as a FK506- and rapamycin-sensitive PPIase. The carboxy-terminal domain is unique and its amino acid composition is highly biased (Table 1). There is a preference for charged amino acids and for alanine. Based on secondary structure predictions, the carboxy-terminal domain of NcFKBP22 forms a perfect amphipathic α-helix (see Fig. 3B). No known FKBP contains a similar domain. There is some similarity, however, to region in proteins which occur in high amounts in plant LEAs. The functions of these proteins are thought to be maintenance of ion and water homeostasis [24].

NcFKBP22 is modified posttranslationally. The nature of this modification is still unclear. Experimental evidence for a possible glycosylation (data not shown) is that NcFKBP22 reacted positive in a PAS glycoprotein stain [28]. However, NcFNBP22 does not contain a consensus sequence for *N*-glycosylation and a variety of different lectins (GNA, SNA, MAA, DSA and PNA) did not bind to NcFKBP22; so the nature of the posttranslational modification remains to be determined.

What might be the function of NcFKBP22?

We suggest that it might be involved in folding and/or transport of certain proteins in the ER. The charged carboxy-terminus might interact with complementary regions in target proteins. Since FKBPs show a narrow substrate specif-

icity [29], the folding activity of NcFKBP22 might only reach a limited subset of ER proteins. yFKBP13, the product of the *FKB2* gene in *S. cerevisiae*, is regulated in response to accumulation of unfolded proteins in the ER [30]. There is some evidence that PPIases might be involved in protein folding in the ER (see [4]).

Since the carboxy-terminus of NcFKBP22 shares homology to LEAs in plants cells, the possibility exists that NcFKBP22 might also be part of the ER system which is responsible for ion and water homeostasis.

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References

[1] Schreiber, S.L. (1992) Cell 70, 365–368.
[2] Fischer, G. (1994) Angew. Chem. Int. Ed. Engl. 33, 1415–1436.
[3] Galat, A. and Metcalfe, S.M. (1995) Prog. Biophys. Mol. Biol. 63, 67–118.
[4] Schmid, F.X. (1995) Curr. Biol. 5, 993–994.
[5] Dolinski, K. and Heitman, J. (1997) in: Guidebook to Molecular Chaperones and Protein Folding Catalysts (Gething, M.-J., Ed.), pp. 359–369, Sambrook and Tooze at Oxford University Press, Oxford.
[6] Rassow, J., Mohrs, K., Koidl, S., Barthelmess, I.B., Pfanner, N. and Tropschug, M. (1995) Mol. Cell. Biol. 55, 2654–2662.
[7] von Ahsen, O., Tropschug, M., Pfanner, N. and Rassow, J. (1997) EMBO J. 16, 4568–4578.
[8] Matouschek, A., Rospert, S., Schmid, K., Glick, B.S. and Schatz, G. (1995) Proc. Natl. Acad. Sci. USA 92, 6319–6323.
[9] Kay, J.E. (1996) Biochem. J. 314, 361–385.
[10] Barthelmess, I.B. and Tropschug, M. (1993) Curr. Genet. 23, 54–58.
[11] Stuart, R.A., Neupert, W. and Tropschug, M. (1987) EMBO J. 6, 2131–2137.
[12] Tropschug, M., Nicholson, D.W., Hartl, F.-U., Köhler, H., Pfanner, N., Wachter, E. and Neupert, W. (1988) J. Biol. Chem. 263, 14433–14440.
[13] Tropschug, M., Barthelmess, I.B. and Neupert, W. (1989) Nature 342, 953–955.
[14] Tropschug, M., Wachter, E., Mayer, S., Schönbrunner, E.R. and Schmid, F.X. (1990) Nature 346, 674–677.
[15] Eckerskorn, C. and Grimm, R. (1996) Electrophoresis 17, 899–906.
[16] Fischer, G., Bang, H. and Mech, C. (1984) Biochim. Biochim. Acta 43, 1101–1111.
[17] Tropschug, M. (1997) in: Guidebook to Molecular Chaperones and Protein Folding Catalysts (Gething, M.-J., Ed.), pp. 416–417, Sambrook and Tooze at Oxford University Press, Oxford.
[18] von Heijne, G. (1985) J. Mol. Biol. 184, 99–105.
[19] Luan, S., Kudla, J., Gruissem, W. and Schreiber, S.L. (1996) Proc. Natl. Acad. Sci. 93, 6964–6969.
[20] Partaledis, J.A., Fleming, M.A., Harding, M.W. and Berlin, V. (1992) Yeast 8, 673–680.
[21] Jin, Y.-J., Albers, M.W., Lane, W.S., Bierer, B.E., Schreiber, S.L. and Burakoff, S.J. (1991) Proc. Natl. Acad. Sci. USA 88, 6677–6681.
[22] Dunnill, P. (1968) Biophys. J. 8, 865–875.
[23] Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Nucleic Acids Res. 25, 3389–3402.
[24] Swire-Clark, G.A. and Marcotte Jr., W.R. (1999) Plant Mol. Biol. 39, 117–128.
[25] Baker, J., Steele, C. and Dure III, L. (1988) Plant Mol. Biol. 11, 277–291.
[26] Dure III, L., Crouch, M., Harada, J., Ho, T.-H.D., Mundy, J., Quatrano, R., Thomas, T. and Sung, Z.R. (1989) Plant Mol. Biol. 12, 475–486.
[27] Imai, R., Chang, L., Ohta, A., Bray, E.A. and Takagi, M. (1996) Gene 170, 243–248.
[28] Zacharius, R.M., Zell, T.E., Morrison, J.H. and Woodlock, J.J. (1969) Anal. Biochem. 30, 148–152.
[29] Harrison, R.K. and Stein, R.L. (1990) Biochemistry 29, 3813–3816.
[30] Partaledis, J.A. and Berlin, V. (1993) Proc. Natl. Acad. Sci. USA 90, 5450–5454.
[31] Morrison, J.F. (1969) Biochim. Biophys. Acta 185, 269–286.