RESEARCH COMMUNICATION

Induction of the FK506-binding protein, FKBP13, under conditions which misfold proteins in the endoplasmic reticulum

Kevin T. BUSH,* Barbara A. HENDRICKSON† and Sanjay K. NIGAM*‡

Harvard Medical School, *Department of Medicine (Renal Division), Brigham and Women's Hospital, and †Department of Genetics, Harvard Medical School and Division of Infectious Diseases, Children's Hospital, Boston, MA 02115, U.S.A.

In order to determine whether the endoplasmic reticulum (ER) luminal FK506-binding protein, FKBP13, shares properties of ER molecular chaperones, MDCK cells were treated with either tunicamycin or Ca^{2+} ionophores. By Northern-blot analysis, tunicamycin resulted in a 2-fold rise in FKBP13 mRNA, whereas ionophores (A23187 and ionomycin) caused a more impressive rise in FKBP13 mRNA (up to 5-fold with ionomycin). Actinomycin D chase experiments in ionomycin-treated cells revealed no change in the half-life of FKBP13 mRNA, indicating that the increase in FKBP13 mRNA observed was not due to greater message stability. Moreover, sequencing of the 5′ flanking region of the gene for murine FKBP13 revealed significant similarity to similar regions in human BiP (immunoglobulin-binding protein) and the human glucose-regulated protein grp94, including a 37 bp sequence in FKBP13 with ~ 50 % identity with the unfolded protein response element of the BiP gene. Together, these data suggest a role for FKBP13 in ER protein folding.

INTRODUCTION

Recently, we presented evidence that the 13 kDa FK506-binding protein (FKBP13), which has a high degree of homology to the cytoplasmic protein FKBP12, is localized to the lumen of the endoplasmic reticulum (ER) [1]. FKBP13, in addition to binding FK506, also binds the immunosuppressant drug rapamycin [2,3]. Since the lumen of the ER is the initial site of folding and assembly of secretory and transmembrane proteins, and since the highly conserved residues that comprise the peptidyl-prolyl-isomerase active site of FKBP12 are conserved in FKBP13 [3], the ER luminal localization of FKBP13 raises the intriguing possibility that FKBP13 could function as a molecular chaperone.

Molecular chaperones in the ER are believed to bind transiently to translocated proteins to catalyse the folding and/or multimeric assembly of transmembrane and secretory proteins [4]. Among the ER proteins thought to possess such a function are BiP (immunoglobulin-binding protein), the glucose-regulated protein grp94 and ERp72 [5–8]. The mRNAs for these proteins are increased by conditions associated with the accumulation of unfolded or misfolded proteins in the ER [5–7]. Furthermore, BiP and grp94 share similar regulatory sites in the 5′ flanking regions of their genes, including a 37 bp segment believed to be responsible for the induction of BiP in response to protein misfolding in the ER [9,10].

We have therefore determined FKBP13 mRNA expression under conditions which cause protein misfolding in the ER, and also analysed the promoter region of the gene for FKBP13. Our findings suggest co-ordinate induction at the transcriptional level of FKBP13 with known ER molecular chaperones and support the notion that FKBP13 may play a role in protein folding in the ER [1].

METHODS AND MATERIALS

Materials

cDNA probes were kindly provided by the following investigators: FKBP13 by Steven Burakoff (Harvard Medical School/Dana Farber Cancer Institute); ERp72 and grp94 by Michael Green (St. Louis University); PDI by Marek Michalak (University of Alberta); BiP by Mary-Jane Gething (University of Texas–Southwestern Medical Center). Antibodies against FKBP13 were kindly provided by Steven Burakoff (Harvard Medical School), against BiP by David Bole (University of Michigan), against ERp72 and grp94 by Michael Green (St. Louis University), and against PDI by Charlotte Kaetzel (Case Western Reserve University).

Treatment conditions, Northern-blot analysis and actinomycin D chase

Confluent monolayers of Madin–Darby canine kidney (MDCK) cells growing in Dulbecco's minimal essential medium (DMEM; Gibco) containing 5 % fetal-calf serum were incubated in either (1) fresh DMEM containing 2 μg/ml or 10 μg/ml tunicamycin or (2) fresh minimal essential medium supplemented with dialysed 5 % fetal-calf serum [11], and containing either 5 μM A23187 or 5 μM ionomycin. Total RNA was isolated by phenol/chloroform extraction [12] at 4, 8, 12, 16 and 24 h after treatment with the drugs. RNA (5 μg) was electrophoresed on 1 % formamide/formaldehyde agarose gels, transferred to nitrocellulose and hybridized with random-primed ^{32}P-labelled cDNA [13]. In addition, actinomycin D chase experiments were performed. Briefly, control and ionomycin-treated (5 μM) MDCK cells were exposed to actinomycin D {20 μg/ml, which inhibited RNA synthesis, measured by [^{3}H]uridine uptake, by > 95 % (results

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Abbreviations used: FKBP, FK506-binding protein; ER, endoplasmic reticulum; BiP, immunoglobulin-binding protein; grp, glucose-regulated protein; ERp, ER protein; PDI, protein disulphide-isomerase; MDCK, Madin–Darby canine kidney; LDH, lactate dehydrogenase.

‡ To whom correspondence should be addressed, at: Renal Division, Brigham and Women's Hospital, Harvard Medical School, 75 Francis Street, Boston, MA 02115, U.S.A.

not shown)]. Total RNA was isolated and processed as described above at 1, 2 and 4 h after treatment with actinomycin D.

Immunoblot protein analysis

After growth in the presence of drugs, the medium was collected, cells were extracted [14], and protein samples were electrophoresed on a SDS/13%-polyacrylamide gel, subjected to Western blotting, probed with primary antisera, and developed as previously described [14,15]. In order to determine if any protein found in the conditioned media was the result of cell death, the media were assayed for lactate dehydrogenase (LDH) activity colorimetrically by using a LDH assay kit (Sigma).

Sequence analysis

The murine FKBP13 genomic clone (3 μg) isolated as previously described in pBluescript(+) [16] was sequenced by the dideoxy-nucleotide chain-termination method using the Sequenase enzyme (U.S. Biochemical Corp.) and analysed by using Geneworks (Intelligenetics).
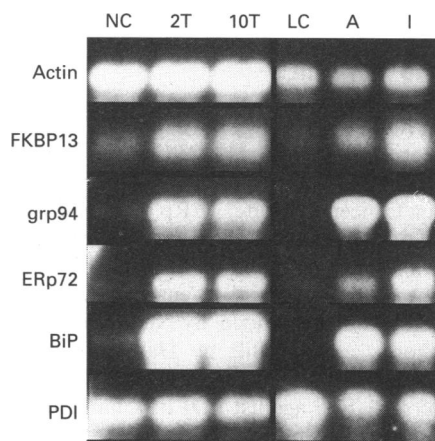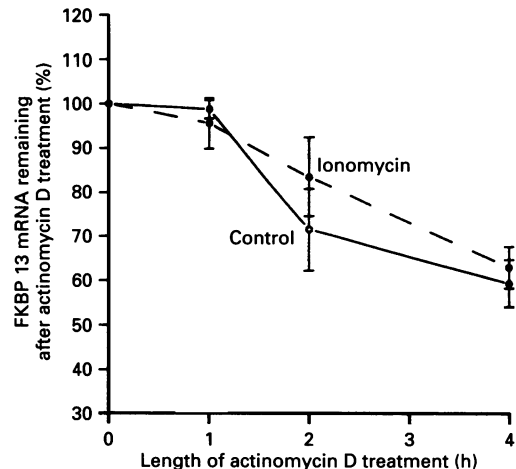
Figure 2 FKBP13 mRNA half-life

Graph showing percentage of FKBP13 mRNA remaining after 1, 2 and 4 h of exposure to actinomycin D in MDCK cells grown for 12 h in media with (O) or without (●) ionomycin (5 μM). Northern blots were probed with ^{32}P-labelled cDNA probes for glyceraldehyde-3-phosphate dehydrogenase (to quantify the gel load and normalize the mRNA levels) and FKBP13. Amounts of mRNA were quantified by densitometric analysis. Percentages were determined by comparing with zero time (100% = no actinomycin D treatment).

Figure 1 FKBP13 mRNA expression is co-ordinately increased with mRNA for putative ER molecular chaperones

Northern-blot analysis of total RNA from MDCK cells grown for 12 h in the absence (NC, normal-Ca^{2+} control) or presence of tunicamycin (2T, 2 μg/ml; 10T, 10 μg/ml) and in the absence (LC, low-Ca^{2+} control) or presence of Ca^{2+} ionophores (A, 5 μM A23187; I, 5 μM ionomycin). Blots were probed with ^{32}P-labelled cDNA probes for the gene products indicated at the left of the Figure.

RESULTS AND DISCUSSION

Treatment of animal cells with either tunicamycin, which inhibits N-linked glycosylation, or Ca^{2+} ionophores (A23187 or ionomycin), which deplete intracellular Ca^{2+}, is believed to result in accumulation of misfolded proteins in the ER and induction of transcription of the genes for BiP, grp94 and ERp72 [4–7,9,10]. To determine whether FKBP13 is also inducible under these conditions, MDCK cells were treated with either tunicamycin or Ca^{2+} ionophores (A23187 and ionomycin), and total cellular RNA was subjected to Northern analysis in order to determine the levels of mRNAs for FKBP13, BiP, grp94, ERp72, protein disulphide-isomerase (PDI) and actin (Figure 1). Treatment of MDCK cells with tunicamycin (2 or 10 μg/ml; equivalent results were obtained for either concentration) or Ca^{2+} ionophores produced significant and consistent increases in the mRNA levels for BiP, grp94 and ERp72, whereas the mRNA for PDI was induced minimally, if at all (Figure 1; Table 1). These results were consistent with previous studies which showed levels of induction for mRNAs encoding these proteins as follows: BiP >

Table 1 Induction of mRNA after treatment of MDCK cells with tunicamycin or Ca^{2+} ionophores

Induction was determined by dividing values for treated cells by those for untreated and normalizing with actin mRNA level. Only data for 10 μg/ml tunicmycin are shown; results for 2 μg/ml were comparable. Data for 4 and 24 h not shown. Results are means ± S.E.M. (n = 4 experiments). Abbreviations: TUNICA, tunicamycin; IONO, ionomycin.

Treatment time...	8 h			12 h			16 h		
	TUNICA	A23187	IONO	TUNICA	A23187	IONO	TUNICA	A23187	IONO
FKBP13	1.5 ± 0.3	2.6 ± 0.2	2.2 ± 0.3	2.0 ± 0.7	2.3 ± 0.8	2.6 ± 0.6	1.8 ± 0.3	3.0 ± 0.5	4.7 ± 1.6
grp94	6.6 ± 2.1	5.8 ± 0.5	7.2 ± 0.5	9.8 ± 0.1	8.6 ± 5.6	11 ± 7	9.9 ± 2.2	4.6 ± 2.1	8.2 ± 5
ERp72	2.5 ± 0.9	5.6 ± 3.7	8.4 ± 6.8	5.8 ± 3	3 ± 1.1	3.4 ± 2.8	8.5 ± 0.9	2.1 ± 0.1	4.1 ± 0.5
BiP	17.9 ± 8	13.3 ± 2.8	13.4 ± 2.9	12.2 ± 3.3	8.0 ± 2.2	6.6 ± 3.5	26.6 ± 8.3	9.4 ± 2.3	16.7 ± 8.7
PDI	0.9 ± 0.3	1.2 ± 0.1	1.2 ± 0.1	1.0 ± 0.3	0.5 ± 0.2	0.9 ± 0.7	1.4 ± 0.3	1.1 ± 0.4	1.8 ± 0.6

(a)

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TTTCATCTTCTACAAACTCTAATTTTACACTCCCCTCTACTCTAGCCAGT    -509

TTGGTTCCCGACCCCCAAGAGCTCAAGTTGGGAGGTGCCCCTGGGAGAGC    -459

TACGGGGCCGGACTGGGGGGTGCCCGAGACCGGGCTCCTCAGAGCCACCT    -409
    * *****

AGTGGAGATGTGTGGGTGCCTCGGGGCCGAACACCTGCAGCACCAATTTG    -359
                      ******

GGTGTGCTGACAGGGGGCAGCTGGGTGTGGCGGGAGTGGGGTCGTCCAGC    -309
                ____    ******

GGGCAAATCCGGTGCAATCAGATAGGTTTGGACTTTGCGTAAAGGGCTAG    -259

CGGGAACTGAAGAACGGAGAGGGGACTGGGAGGGGAGAGAAGGTCAGGTA    -209
                          *******

GTGAGCAAAGGGAGGGGGAAAAGGAGGGGCACTGGCTGCTGTGGCCTCCC    -159
    ******

CTGACCCCCTCCCCTCGCTGCTGGGGTCCTCGGTCAAGCCCCCTTCTCAC    -109

TGGACTGGTAAGTGTGGCTACAGAGCCCAGGGGAGGGGGTGGTGACCCAG    -59
                          *******

GACTGTGGGATTCCCCAGAAACAGGCAAGATATAATGGGGAGTTGGCGTC    -9

CTCCTGATACAGACTGAAGACGTCCCATGACTCCACCCCCCAGTTTTTTG    41

GTTGGGGGTGGGGTGGCCTTTGCCTACAGACATG    75
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(b)

hgrp94	CCAATCGGAAGGA-----GCCACGCTTCG-GGCATCGGTCACCG	-158
	: :::::: :: :::: :::: : ::: : :: :: :	
hBiP	CGAATCGGC-GGC----GGCCA-GCTTGGTGGCCTGGGCCAATG	-89
	: ::: ::: : : ::: : :: ::: : ::	
mFKBP13	CCAATTTG--GGTGTGCTGACA-G---GG-GGCAGCTGGGTGTG	-366
	::::: : :: : : : ::::: : :	
hgrp94	CCAATCGGAAGGA-----GCCACGCTTCG-GGCATCGGTCACCG	-158
Consensus	CcAATCgG--GG------GcCA-GcttgG-GGCatcgG-ca-tg	

Figure 3   Analysis of the 5′ flanking region of FKBP13

Murine FKBP13 genomic clone was sequenced by the dideoxynucleotide chain-termination method using Sequenase enzyme (U.S. Biochemical Corp). The numbers to the right of the Figure refer to the nucleotide positions relative to the putative transcription initiation site. (a) *ATG*, the methionine initiation codon is in **bold** and *italics*. The TATA box and CCAAT sequence are underlined and CCAAT-like sequences are in **bold**. Three putative SP-1-like binding sites are underscored with asterisks, and for GGGNNGGG sequences are overlined with asterisks. The palindrome CAGCTG is overlined. (b) Sequence from the 5′ flanking region of FKBP13 centred around the perfect CCAAT sequence and alignment with sequences from the putative promoter regions for human grp94 and human BiP (from [20]) that have previously been shown to share similarity and which are believed to be responsible for mRNA induction in response to unfolded proteins in the ER. Colons indicate nucleotide matches and dashes represent gaps. Upper-case letters in the consensus sequence represent a 3-out-of-3 match, and lower-case letters represent a 2-out-of-3 match.

Table 2   Comparison of FKBP13 5′ flanking region with putative promoter regions of BiP and grp94

Sequence	Position(s) in FKBP13	Present in BiP	Present in grp94
TATAA (TATA Box)	-28	+	+
CCAAT (CCAAT box)	-366	+	+
GGGNNGGG	-47, -79, -198, -231	+	+
SP1-like (GGGCGG)	-330, -386, -454	+	+
CAGCTG (palindrome)	-341	+	-

grp94 > ERp72 ≫ PDI [5]. FKBP13 mRNA levels were found to increase with both tunicamycin (2-fold) and Ca^{2+} ionophores (up to 5-fold) (Figure 1; Table 1). Moreover, the increase in FKBP13 mRNa after 16 h of treatment with Ca^{2+} ionophores was comparable with that observed for ERp72 mRNA (Table 1). Nevertheless, FKBP13 protein levels were not increased (Figure 4a) {only a slight increase in protein levels (< 1.5-fold) was observed
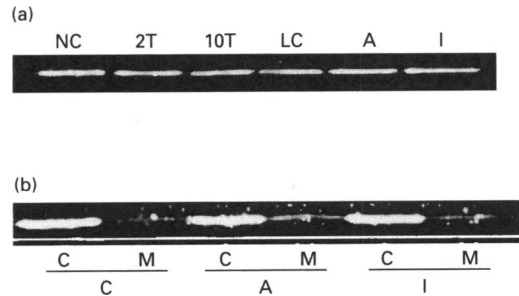

Figure 4   (a) FKBP13 protein levels are unchanged, and (b) FKBP13 protein is not significantly secreted, after treatment with tunicamycin and Ca^{2+} ionophores

(a) Western-blot analysis of the steady-state cellular levels of FKBP13 protein in MDCK cells incubated for 16 h in the absence (NC, normal-Ca^{2+} control) or presence of tunicamycin (2T, 2 μg/ml; 10T, 10 μg/ml) and in the absence (LC, low-Ca^{2+} control) or presence of Ca^{2+} ionophores (A, 5 μM A23187; I, 5 μM ionomycin). Blots were probed with antibody against human FKBP13. Steady-state levels of BiP and grp94 were increased < 1.5-fold. (b) Western-blot analysis of levels of FKBP13 protein in MDCK cells (C) and growth medium (M) after 16 h of incubation in the absence (C, control) or presence of Ca^{2+} ionophores (A, 5 μM A23187; I, 5 μM ionomycin). In all cases, <10% of the protein was detected in the medium. Colorimetric determination of LDH activity of the culture media revealed no difference in activity between controls and MDCK cells grown for 16 h in the presence of 5 μM ionomycin.

for BiP and grp94, consistent with what has been reported previously [17]}. In order to determine if the accumulation of FKBP13 mRNA was due to an increase in message stability, actinomycin D chase experiments were performed. A concentration of 20 μg/ml actinomycin D resulted in 95 % inhibition of mRNA synthesis in MDCK cells, as determined by [^{3}H]uridine uptake (results not shown). At this concentration of actinomycin D, treatment of MDCK cells with ionomycin [5 μM, a treatment which resulted in greatest increase in the mRNA for FKBP13 (Figure 1; Table 1)] was found to have no effect on the half-life of the mRNA for FKBP13 (Figure 2). This result suggests that the increase in FKBP13 mRNA is not due to an increase in the stability of the mRNA and probably reflects transcriptional regulation.

These results suggest that the gene for FKBP13 is transcriptionally activated under conditions which activate transcription of BiP, grp94 and ERp72, and raise the possibility that these genes respond to some common stimulus brought about by physiological stress. The 5′ flanking regions of the genes for BiP and grp94 share strong similarity, including several putative transcriptional regulatory sites [18-20] and the region believed to be responsible for the induction of BiP by unfolded proteins in the ER [9,10,21]. Since FKBP13 is co-ordinately regulated with the gene products for these chaperones, we analysed the 5′ flanking region of its gene for similar sequences. A 3 kbp genomic clone encoding the murine FKBP13 gene [16] was sequenced 509 bp 5′ to the approximate transcription start site (Figure 3a) and compared with the 5′ flanking region of the genes for human BiP and human grp94. We found that the murine FKBP13 sequence shared significant similarity to corresponding human sequences in BiP and grp94 (Figures 3a and 3b; Table 2), including a number of putative transcriptional regulatory sites (Table 2) [9,19]. Among these are interrupted poly(G) sequences, which have been suggested to function as recognition sites for transcriptional control [20]. For example, the sequence GGGNNGGG (where N is any base), which occurs multiple times in the promoter regions of FKBP13 (Figure 3a; Table 2), BiP and grp94 [9,20], has been described in chicken globin genes

as a nuclear transcription factor-binding site [20,22,23]. Furthermore, palindromes, such as CAGCTG, which is present in both FKBP13 (Figure 3a; Table 2) and BiP [20], are thought to be binding sites of transcriptional regulatory proteins. Thus the FKBP13 gene shares a number of similar putative transcriptional regulatory domains with the genes for BiP and grp94. In addition, a 37 bp sequence in the 5'-untranslated region of murine FKBP13 was found to have ~ 50% identity with the putative unfolded protein response element (UPR) of human BiP (Figure 3b). Although it remains to be determined if this sequence is actually the UPR for FKBP13 in mammalian cells, taken together with the other sequence data, these findings do suggest a possible mechanism for the co-ordinate transcriptional regulation of FKBP13 with other ER molecular chaperones in response to unfolded proteins.

There has been some debate surrounding the effects of tunicamycin and Ca^{2+} ionophores on the steady-state level and cellular localization of ER-resident proteins. Ionophore A23187 has been reported to decrease the protein levels of BiP and PDI in NIH 3T3 cells, apparently due to secretion of these ER luminal proteins as a result of ER Ca^{2+} depletion [24]. We therefore analysed FKBP13 protein levels after ionophore and tunicamycin treatment. In MDCK cells, Western-blot analysis indicated that there was minimal (< 10%) secretion of FKBP13 into the medium (Figure 4b). There was no increase in LDH activity in the media after ionomycin treatment, indicating that the apparent secretion of FKBP13, albeit very small, was not the consequence of cell death. Furthermore, none of the other proteins that we examined (BiP, grp94, ERp72 and PDI) appeared to be appreciably induced (protein level) or significantly secreted (results not shown).

Thus our data indicate that FKBP13 shares an important distinguishing characteristic of ER molecular chaperones (i.e., induction in response to the accumulation of misfolded proteins in the ER) and support the notion that FKBP13 may play a role in protein folding in the ER. Moreover, these results are compatible with the intriguing possibility that FKBP13 and rapamycin could exert some of their effects by interfering with protein folding in the ER [1].

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