BiP is feed-back regulated by control of protein translation efficiency

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Summary

The lumenal endoplasmic reticulum (ER) protein BiP, among its other functions, is believed to serve as an ER stress sensor, triggering the so-called 'unfolded protein response' or UPR. For this role, BiP levels are critical. Indeed, here we show that BiP expression is tightly controlled at a post-transcriptional level. Thus, an artificial increase in cellular BiP mRNA does not lead to increased synthesis of BiP in unstressed cells, and, consequently, protein levels remain constant. Under ER stress conditions, however, this homeostatic restriction is alleviated, and independent of transcript levels, the translation efficiency of BiP transcripts is enhanced, allowing the cells to produce more protein. We additionally show that this regulation is independent of elements in the 5′ and 3′ UTR of BiP mRNA, which rather points to a novel type of translational feed-back control. BiP is the first example of a lumenal protein whose expression is controlled at a translational level. The implications of these findings with respect to cellular stress are discussed.

Key words: Cellular stress, Endoplasmic reticulum, Polysome profile, Tunicamycin, Thapsigargin, UPR

Introduction

In eukaryotic cells, most secretory and membrane-bound proteins are translocated into the endoplasmic reticulum (ER) on the way to their final destination. Folding and assembly of newly translocated proteins is monitored by a specialized set of ER enzymes and molecular chaperones. Among these, the immunoglobulin heavy chain binding protein, BiP (Haas and Wabl, 1983), was the first ER component fulfilling the initial definition of a molecular chaperone (Ellis, 1987). BiP transiently binds to stretches of folding protein subunits to inhibit inter- and intramolecular interactions that could give rise to permanent misfolding or aggregation reactions (Gething and Sambrook, 1992; Hurtley et al., 1989). As an integral part of the ER quality control system, BiP retains misfolded proteins in the ER (Hammond and Helenius, 1995; Leitzgen and Haas, 1998). BiP also has a role in protein translocation through the ER membrane (Brodsky et al., 1995; Lyman and Schekman, 1997; Vogel et al., 1990; Young et al., 2001) and in occluding the translocation pore at the lumenal end when it is not used by translating ribosomes (Hamman et al., 1998).

In addition to these functions, BiP seems to fulfill a crucial role in the unfolded protein response (UPR). This response was first characterized as an upregulation of BiP at the transcriptional level, triggered by the accumulation of misfolded proteins in the ER (Kozutsumi et al., 1988; Kohno et al., 1993). Array hybridization experiments revealed 208 UPR target genes in the yeast *Saccharomyces cerevisiae* affecting multiple ER and secretory pathway functions (Travers et al., 2000). The mediator of the UPR pathway in *S. cerevisiae* is Ire1p (Cox et al., 1993; Mori et al., 1993), an ER-resident transmembrane kinase/nuclease. Activation of Ire1p, as induced for instance by tunicamycin treatment, which inhibits N-linked glycosylation, leads to the production of a transcription factor, Hac1p, that in turn drives the transcriptional response (reviewed in Chapman et al., 1998).

In mammals, the ER stress response is more diverse. Two homologues of IRE1 were identified (Tirasophon et al., 1998; Wang et al., 1998), which are most probably involved in transcriptional activation of UPR target genes. An additional transmembrane ER-resident kinase, PERK, not present in yeast, has a lumenal domain similar to that of IRE1 (Harding et al., 1999). This kinase is responsible for the phosphorylation of the translational initiation factor eIF2α (Harding et al., 1999), which interferes with the formation of an active 43S translation-initiation complex (Hinnebusch, 1994). The resulting inhibition of global protein synthesis (Brostrom and Brostrom, 1998; Prostko et al., 1992) is required for cells to survive ER stress (Harding et al., 2000b). Furthermore, ER stress also causes induction of growth arrest and programmed cell death in many cell types (Larsson et al., 1993; Nakashima et al., 1993), most probably because of downstream effects of the activated transcription factor CHOP/GADD153 (Wang et al., 1996; Zinszner et al., 1998).

Accumulating evidence supports the idea that BiP plays the key role in the UPR, not only as an ER-chaperone but also as an ER-stress sensor (Dorner et al., 1992; Leborgne-Castel et al., 1999; Little and Lee, 1995; Morris et al., 1997). Most strikingly, the lumenal domains of both IRE1 and PERK were shown to form a stable complex with BiP, and release of BiP binding correlated with both perturbation of protein folding in the ER and activation of the transmembrane kinases (Bertolotti et al., 2000).

If BiP indeed has stress sensor function, BiP levels are critical for UPR induction. For instance, too high levels of BiP

would delay or prevent UPR, whereas too low levels could cause a premature or prolonged UPR. Thus, a mechanism is needed that controls the amount of BiP. Furthermore, this mechanism should have a certain buffering capacity to prevent UPR induction by transient and/or small perturbances. Some reports point to post-transcriptional control of BiP expression (Lam et al., 1992; Leborgne-Castel et al., 1999; Ulatowski et al., 1993). In concert with classical transcriptional regulation, a translational mechanism controlling BiP expression could increase the efficiency of the ER stress response.

We have investigated the regulation of BiP expression in unstressed and stressed cells. Using the tetracycline-sensitive (tet-off) expression system (Gossen and Bujard, 1992), we introduced a construct encoding heterologous mouse BiP into human HeLa cells. This experimental system allowed us to investigate translation of BiP in cells in which BiP transcript levels were increased in the absence of stress. This system also allowed us to expose cells to ER stress and investigate translation of the same amount of mouse BiP transcript as in unstressed cells, because the heterologous BiP mRNA is not under UPR control. In addition, we performed polysome analyses to monitor the ribosome loading of BiP mRNA under normal and ER-stress conditions. Taken together, our data reveal the existence of a regulation mechanism that acts in a stress-dependent manner and specifically controls BiP expression at a translational level.

Materials and Methods

Cell culture

Bil11 and Bil58 are independent stable HeLa cell transfectants producing the tet-off transactivator protein and bearing a bi-cistronic construct under control of the tetracycline-sensitive expression system (Gossen and Bujard, 1992). The bi-cistronic construct carries the complete open reading frames of both mouse BiP (–5 to 2006, GenBank acc. no AJ002387) and firefly luciferase, separated by 60 nucleotides. Bilu33 is a stably transfected HeLa line expressing luciferase under control of the tetracycline-sensitive expression system but lacking the mouse BiP sequence. The cells were cultured as described previously (Gossen and Bujard, 1992). 1 μg ml^{-1} tetracycline was added to shut off transcription, for instance, during selection of stable transfectants.

Luciferase assay

Luciferase assays were performed as described previously (Gossen and Bujard, 1992). 79% of Bil11 and 96% of Bil58 cells tested positive for luciferase expression as confirmed by subcloning.

Cell lysis, western blotting and antibodies

Cell lysis and western blotting were performed as described before (Chillaron and Haas, 2000).

Depending on the assay performed, various reagents were used to detect BiP: for specific immunoprecipitation of mouse BiP, a polyclonal antiserum was used that does not react with human BiP (a kind gift of L. Hendershot, Memphis, TN). To detect mouse but not human BiP in western blots, commercially available anti-Grp78 antiserum (PA1-014; 1:10.000; Affinity Bioreagents, Golden, CO) was used. When total BiP was analyzed, monoclonal anti BiP antibody (a kind gift of L. Hendershot, Memphis, TN) was used in immunoprecipitation and western blot experiments, because this antibody reacts with both mouse and human BiP.

Additional antibodies used in western blots were: anti-calreticulin (Affinity Bioreagents, Golden, CO; 1:2000), anti-calnexin, (a kind gift of E. Ivessa, Vienna, Austria), anti-Erp29 (a kind gift of S. Mkrtchian, Stockholm, Sweden), anti ERp72 (a kind gift of H.-D. Söling, Göttingen, Germany), anti-tubulin (kind gift of J. Wehland, Braunschweig, Germany) and HRP-conjugated secondary antibodies (BioRad, München, Germany). Signals were obtained using BM Chemiluminescence Blotting Substrate (Roche Diagnostics, Mannheim, Germany) and quantified by standard scanning densitometry using the NIH Image program version 1.6.

Synthesis of recombinant BiP-GST fusion protein

The amounts of mouse BiP and total BiP were quantified by use of defined amounts of a recombinant BiP-GST fusion protein detected by both antibody reagents. Recombinant BiP-GST was made with the pGEX fusion protein system (Amrad Cooperation Ltd., Melbourne, Australia) and contains glutathione-S-transferase of 26 kDa fused to the 23.8 kDa C-terminal portion of mouse BiP.

Pulse chase analysis and immunoprecipitation

Cells were washed twice in PBS and incubated in methionine-free RPMI 1640 medium/10% dialyzed FCS (1 hour, 37°C) prior to labeling (1.5 hour, 37°C, 100 μCi ml^{-1} [^{35}S]-methionine). Chase was initiated by replacing the label medium with DMEM/FCS containing an excess of methionine (4.5 mM). At various time points, cells were washed once with ice-cold PBS and lysed as described above. Incorporation of radioactivity into TCA-precipitated proteins was measured with a scintillation-counter (Beckman LS 6000TA, Beckman, München, Germany). Immunoprecipitations of BiP were performed from equal amounts of cell lysates using either a monoclonal anti-BiP antibody detecting equally well human and mouse BiP or a rabbit anti-BiP antiserum specifically reacting with mouse BiP (kind gifts of L. Hendershot). Labeled proteins were separated by SDS-PAGE under reducing conditions as described previously (Knittler and Haas, 1992) and visualized by autoradiography. Signals were quantified using a phosphoimager (BAS1000, Imaging Screen Plate BASIII, Fuji, Tokyo, Japan, using MACBAS version 1.0).

Determination of synthesis rates and induction of UPR

Cells were washed twice with PBS and cultured in methionine-free medium (1.5 hour, 37°C) prior to the addition of 100 μCi ml^{-1} [^{35}S]-methionine. UPR was induced by tunicamycin (1 μg/ml) or thapsigargin (200 nM), which was present during starvation and labeling. For actinomycin D (5 μg/ml), cells were treated 5 minutes before and during labeling. Cell lysis, performed at the time points indicated, measurement of radioactivity incorporated into proteins, immunoprecipitation and signal quantification are all described above.

Polysome analysis and isolation of total RNA

Cells at 50-60% confluency were treated for 90 minutes with tunicamycin (2 μg/ml) in DMSO or only the solvent. Cycloheximide (0.1 mg/ml) was added during the last 3 minutes of treatment. The plates were washed twice with ice-cold PBS containing cycloheximide. Cells from eight dishes were pooled, lysed in 500 μl polysome extraction buffer (15 mM Tris-HCl [pH 7.4], 15 mM $MgCl_2$, 0.3 M NaCl, 1% Triton X-100, 0.1 mg/ml cycloheximide, 1 mg/ml heparin, 1 mM phenylmethysulfonyl fluoride, 10μg/ml leupeptin, 5 μg/ml chymostatin, 3 μg/ml elastatinal and 1μg/ml pepstatin) and incubated on ice for 20 minutes. The lysates were cleared by centrifugation (10 minutes, 12,000 ***g***) and layered on top of a 10 ml 10-50% sucrose gradient prepared in extraction buffer without protease inhibitors. After a spin of 4 hours at 15,000 ***g*** in an SW41 rotor, the absorbance at 254 nm was read across the gradient.

The gradients were fractionated into 16 samples of 650 μl, proteins were removed by phenol/chloroform extraction, and RNA was precipitated with ethanol.

Poly A^+ RNA isolation and northern blot analysis

Poly A^+ RNA isolation was performed with Dynabeads mRNA DIRECT Kit (Dynal A.S., Oslo, Norway). The RNA was denatured in formamide and formaldehyde, separated on 1.2% denaturing agarose gels and blotted onto Hybond-N membrane (Amersham Pharmacia Biotech, Uppsala, Sweden) as described by the manufacturer. The two probes, covering position –5 to 1103 of mouse BiP cDNA (in this region it has more than 92% identity with human BiP cDNA) and the complete actin-coding sequence were radioactively labeled using a random primer DNA labeling kit (Biorad, München, Germany). Hybridizations were done according to standard procedures (Sambrook et al., 1989), and signal quantification was performed by phosphoimaging, as described above.

Results

We established stable human cell lines capable of conditionally overexpressing mouse BiP in human HeLa cells by using the tet-off activation system (Gossen and Bujard, 1992). For transfection, we used a bi-cistronic construct that contains the coding sequence of mouse BiP but lacks the mouse 5′ and 3′ UTR sequences. Firefly luciferase, encoded downstream of mouse BiP ORF, was used as a reporter to quantify indirectly the bi-cistronic mRNA. Most analyses were carried out with two different independent transfectants, Bil11 and Bil58. As the results obtained were basically the same, representative data are only shown for Bil11.

BiP expression is tightly controlled in unstressed cells

In the presence of tetracycline, transcription of the hetorologous sequence was disabled. Upon removal of tetracycline, expression was turned on and luciferase activity increased with similar kinetics to those described by Gossen and Bujard (Gossen and Bujard, 1992) (K.G. and I.G.H., unpublished). The kinetics of mouse BiP induction were investigated by a western blot analysis using anti-Grp78 antiserum, which recognizes only mouse BiP (Fig. 1A). Mouse BiP was detectable 24 hours after induction and only slowly increased thereafter. Steady state levels were reached only after more than 96 hours of tetracycline removal.

For detection of total BiP, we used a rat monoclonal anti-BiP antibody that recognizes mouse and human BiP equally well. The time-dependent increase in mouse BiP expression was not reflected by an increase in the amount of total BiP (Fig. 1B). Instead, the amount of total BiP stayed constant despite the additional expression of mouse BiP. To exclude the possibility that the amount of mouse BiP was too low to be detected as an increase in total BiP, we determined the amounts of BiP detected by the antibody reagents used. A BiP-GST fusion protein was generated that contains the epitopes recognized by both the monoclonal anti-BiP antibody and the anti-Grp78 antiserum. Using the BiP-GST fusion protein as a reference (Fig. 1C), we calculated the amount of mouse and of total BiP expressed in permanently activated HeLa cells. About 60-65% of total BiP consisted of mouse BiP, implying continuous displacement of human BiP by newly synthesized mouse BiP upon activation of mouse BiP expression (Fig. 1C).

These results reveal the existence of a cellular control mechanism maintaining BiP at a constant level in unstressed cells, underlining the role of BiP not only as a chaperone but also as stress sensor in the UPR. This mechanism is specific for BiP because the levels of other ER chaperones, like ERp29 (Mkrtchian et al., 1998), ERp72 (Mazzarella et al., 1990), Calreticulin (Smith and Koch, 1989) and Calnexin (Wada et al., 1994) were not affected by mouse BiP co-expression (K.G. and I.G.H., unpublished).

BiP expression is controlled at a translational level

To determine at which level BiP expression is controlled, BiP mRNA was first examined. Poly A^+-RNA was isolated from samples taken at various time points after removal of tetracycline and analyzed by northern blotting using a BiP cDNA probe that hybridizes with both human and mouse BiP mRNA (Fig. 2A). In the presence of tetracycline, only endogenous human BiP mRNA was detected. Bi-cistronic transcripts containing the mouse BiP sequence reached steady state levels within the first 24 hours of activation and remained constant thereafter. Quantification of the signals revealed that the amount of mouse BiP transcripts was about eight- to ten-fold that of human BiP transcripts. Remarkably, transcriptional activation of the mouse sequence did not affect the level of the endogenous BiP mRNA. These results show that control of BiP expression levels is post-transcriptional.

To investigate the level of regulation in more detail, we next investigated the rate of BiP protein synthesis in control cells (BiLu33) expressing only endogenous human BiP (and firefly luciferase) and in activated transfectants (Bil11 and Bil58) additionally expressing mouse BiP. Note that the amount of BiP mRNA in control cells (BiLu33) is basically identical to BiP mRNA levels in unactivated Bil cells expressing endogenous human BiP only (K.G. and I.G.H., unpublished). Synthesis rates were visualized as a time-dependent increase in the amount of total labeled BiP immunoprecipitated with the monoclonal anti-BiP antibody, and conditions were chosen such that the cell lines investigated could be directly compared (Fig. 2B). Strikingly, the rates of BiP synthesis were identical in all cells investigated, whether or not mouse BiP was co-expressed. This indicates that BiP synthesis is independent of the actual amount of BiP transcripts in unstressed cells. Consistently, the half-life of total BiP was identical (28-33 hours) in activated transfectants and control cells (Fig. 2C). Furthermore, the half-life of mouse BiP did not differ from that of the human homolog as confirmed by a supplementary assay for the half-life of mouse BiP in the activated transfectants (K.G. and I.G.H., unpublished). In no case was secretion of BiP observed.

Altogether, these findings argue strongly for a mechanism controlling BiP expression at a translational level. Variations in BiP mRNA levels do not affect either the synthesis rate or the half-life of BiP, reaffirming that BiP expression is feed-back regulated in unstressed cells.

UPR-mediated enhancement of BiP translation efficiency

Under conditions of ER stress, activation of PERK leads to inhibition of eIF2-dependent translation initiation, resulting in

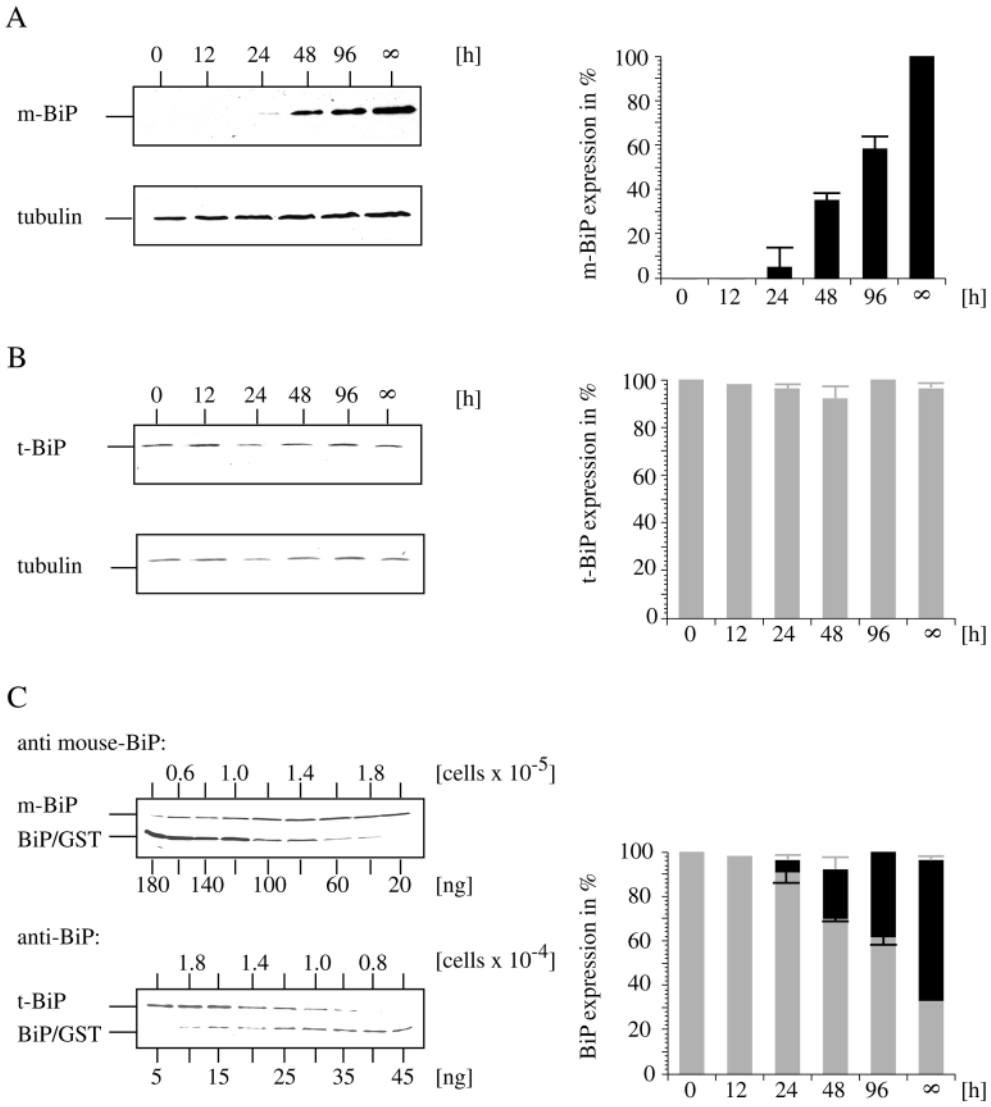

Fig. 1. BiP expression is tightly controlled in unstressed cells. (A) Left, detection of mouse BiP in lysates of Bil11. Bil11 cells were cultured in the presence of tetracycline, and lysates were prepared from equal amounts of cells taken at time points indicated after removal of tetracycline. Equivalents of 10^5 cells were loaded. The western blot was developed with anti-GRP78 antiserum, which detects only heterologous mouse BiP (m-BiP), and with anti-tubulin antibodies. Right, tubulin was used for normalization of the total cellular proteins loaded. Amounts of mouse BiP (black bars) were expressed as a percentage of steady state amounts of mouse BiP present in permanently activated cells. (B) Left, western blot analysis of total BiP (t-BiP) performed on one tenth of the lysates used in A. The blot was developed with monoclonal anti-BiP antibody detecting both human and mouse BiP equally and with anti-tubulin antibodies. Right, tubulin was used for normalization of the cellular proteins loaded. Amounts of total BiP (grey bars) were expressed as a percentage of steady state levels of total BiP present in permanently activated cells. (C) Left, quantification of mouse and total BiP in permanently activated cells. The amounts of lysate indicated were prepared from permanently activated cells and mixed with defined amounts of purified recombinant BiP-GST fusion protein (BiP/GST), as indicated. Mouse or total BiP was detected by using anti-GRP78 antiserum (upper panel) or monoclonal anti-BiP antibody (lower panel), respectively. Note that the BiP-GST fusion protein contains the epitopes for both anti-GRP78 antiserum and monoclonal anti-BiP antibody. Right, calculation of the ratio of mouse BiP according to the amount of total BiP after removal of tetracycline. Amounts of mouse BiP and total BiP giving signals in the linear range were assigned to a corresponding amount of BiP-GST fusion protein, allowing the determination of the ratio of mouse BiP to total BiP in permanently activated cells. The gradual replacement of human BiP (gray bars) by mouse BiP (black bars) after removal of tetracycline was monitored.

downregulation of global protein synthesis (Harding et al., 1999). In addition, IRE1 activation leads to increased cellular BiP mRNA levels, and more BiP protein is produced (Cox et al., 1993; Kohno et al., 1993; Kozutsumi et al., 1988; Li et al., 1994). However, the results shown above clearly demonstrate that a mere enhancement of BiP transcripts does not necessarily result in the production of more protein. If more BiP is to be produced, the translational restraint present in unstressed cells must be abrogated.

To characterize the novel regulation mechanism in more detail, we investigated the effects of treating cells with the UPR-inducing drug tunicamycin. As seen in Fig. 3A, the amount of endogenous BiP mRNA started to increase approximately 2 hours after drug addition. After 8 hours, approximately 10 times the amount of transcripts present in unstressed HeLa cells was reached. Shortly (60 or 90 minutes) after tunicamycin addition, we analyzed the rates of synthesis of total BiP, mouse BiP and total cellular proteins in unstressed and stressed cells. Tunicamycin has been reported to only moderately inhibit protein synthesis in HeLa cells (Brostrom and Brostrom, 1998). Consistently, we found that tunicamycin treatment for 90 minutes lowered the subsequent incorporation of radioactive methionine into TCA-precipitated material to approximately 80% of control values (Fig. 3B). This downregulation of global protein synthesis is a very rapid process in UPR and is already detectable 5 minutes after tunicamycin addition (K.G. and I.G.H., unpublished). Inhibition of protein synthesis was also reflected by a decrease in polysome size and an increase in free ribosomal subunits and monosomes (see Fig. 5).

In sharp contrast, we found BiP synthesis to be increased (by a factor of two to four after 90 minutes of tunicamycin

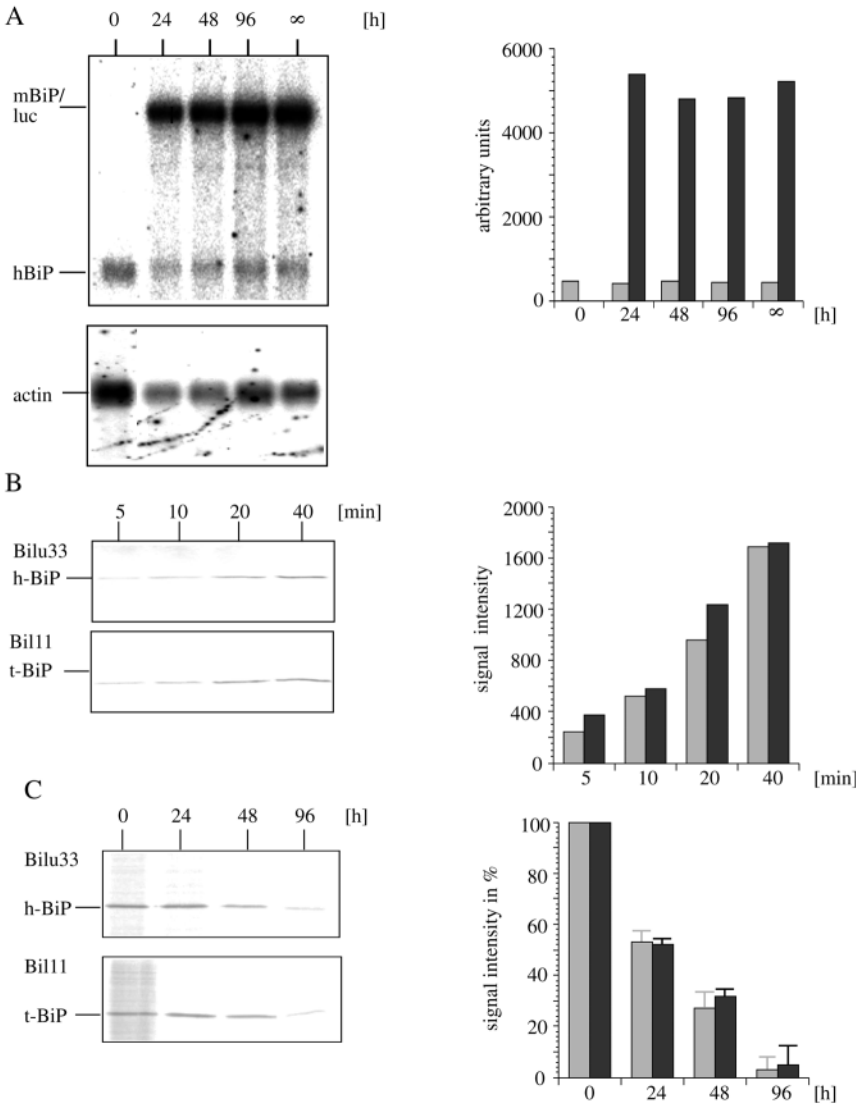

Fig. 2. BiP is controlled at a translational level. (A) Left, northern blot analysis of poly A^+ RNA isolated from Bil11 cells activated for various time periods, as indicated. The probe detecting both mouse (m-BiP) and human BiP (h-BiP) corresponds to a 1.1 kb fragment of the mouse BiP coding sequence (which has more than 92% identity to the human sequence). Actin mRNA was probed with the complete actin coding sequence. Right, quantification was performed by phosphoimaging and normalized for actin signals. Relative amounts of endogenous human BiP mRNA (grey bars) and heterologous mouse BiP mRNA (black bars) are presented. (B) Left, determination of the rate of BiP synthesis in Bilu33 cells expressing only endogenous human BiP (h-BiP) and activated Bil11 cells co-expressing human and mouse BiP (t-BiP). Cells were cultured in the absence of tetracycline, and lysates were prepared at time points indicated after ^{35}S-methionine addition. BiP was immunoprecipitated by monoclonal anti-BiP antibody. To directly compare BiP synthesis rates in the different cells, the respective amounts of cell lysates were normalized according to the amount of TCA-precipitated radioactivity determined in the first samples. Right, gels were exposed for the same time period and relative signal intensities of BiP in Bilu33 (grey bars) and Bil11 cells (black bars) were determined by phosphoimaging. (C) Left, kinetics of BiP degradation in Bilu33 expressing only endogenous human BiP (h-BiP) and activated Bil11 co-expressing human and mouse BiP (t-BiP). Cells were pulse labeled for 1.5 hours with ^{35}S-methionine, and identical volumes of culture were taken to prepare lysates at the time points indicated after initiation of the chase. Total BiP was immunoprecipitated by monoclonal anti-BiP antibody and signals quantified by phosphoimaging. Right, the amount of labeled BiP (grey bars: human BiP in Bilu33; black bars: total BiP in Bil11) is expressed as a percentage of labeled BiP isolated directly after the pulse.

treatment compared with the control values, Fig. 3C). These findings were confirmed by an analysis performed under the same UPR conditions as described above using a different HeLa cell transfectant producing a murine immunoglobulin light chain, κ_{NS1}. Synthesis of the κ-light chain as well as that of total proteins decreased again to approximately 80% of control values, whereas the rate of BiP protein synthesis increased by a factor of two to four (K.G. and I.G.H., unpublished).

The reverse effect of UPR on BiP translation and general protein synthesis is seen when mouse BiP and firefly luciferase are compared. Although both proteins are translated from the same transcript, mouse BiP translation increased (note that the bi-cistronic construct is not under control of a UPR sensitive promotor; Fig. 3D), whereas luciferase activity dropped to 80% (K.G. and I.G.H., unpublished). Interestingly, recovery of luciferase activity is observed 4 hours after ongoing tunicamycin treatment (K.G. and I.G.H., unpublished). Recovery of translation is also seen in measurements of total protein synthesis (K.G. and I.G.H., unpublished) and was described previously for NIH 3T3 cells (Brostrom and Brostrom, 1998).

Translational regulation is often mediated by elements located in the 5′ and/or 3′ UTR of the respective transcript (Hershey, 1991; Preiss and Hentze, 1999). Although mouse BiP lacks both of these sequences, this protein is subject to feed-back control, indicating a control element located in the coding sequence itself.

Our data show that the translational restraint acting in unstressed cells is alleviated under UPR conditions. Translational upregulation is already seen before transcript levels significantly rise. Furthermore, analysis of the mouse protein demonstrates that the translational upregulation is indeed uncoupled from the transcriptional response, because although the mouse BiP transcript is not under UPR control, mouse BiP translation was increased whereas synthesis of other proteins was downregulated. From these data, we conclude that translational and transcriptional upregulation of BiP are autonomous steps during UPR. In line with our data, enhanced BiP synthesis in the absence of transcriptional upregulation was also observed with glucocorticoid-treatment of S49 mouse lymphoma cells (Lam et al., 1992).

Ron's group recently reported that the thapsigargin-induced increase in BiP synthesis is not seen in the presence of the

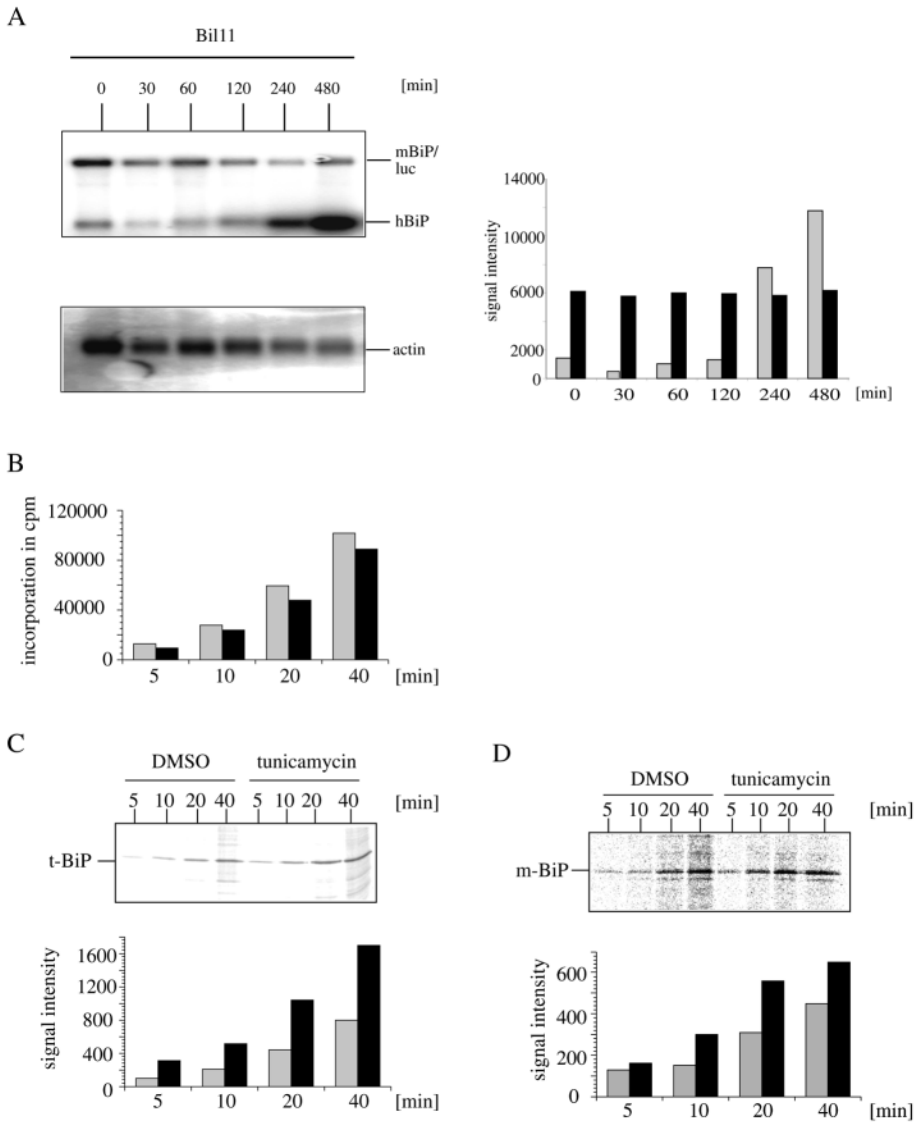

Fig. 3. Kinetics of upregulation of BiP expression during UPR. (A) Activated Bil11 cells expressing endogenous human and additional mouse BiP were treated with tunicamycin for 8 hours, and RNA was prepared at the different time points indicated. A northern blot of poly A^+ RNA was hybridized with the BiP-specfic probe (also used in Fig. 2A), detecting mouse BiP/luciferase mRNA (mBiP/luc) and endogenous human BiP mRNA. Quantification was performed by phosphoimaging and normalized for actin signals. Black bars represent mouse BiP/luciferase mRNA, and grey bars represent human BiP mRNA. (B) Determination of global protein synthesis. DMSO or tunicamycin (in DMSO) was added to activated Bil11 cells for 1.5 hours prior to addition of ^{35}S-methionine. Lysates were prepared from equivalent amounts of cells harvested at the time points indicated after ^{35}S-methionine addition. For determination of the rate of total protein synthesis, incorporation of ^{35}S-methionine into TCA-precipitated material from lysates of tunicamycin- (black bars) or mock-treated (gray bars) cells was measured. (C) Upper panel, for determination of the rate of BiP synthesis, monoclonal anti-BiP antibody was used for immunoprecipitation of total (human and mouse) BiP (t-BiP). Lower panel, relative signal intensities of total BiP isolated from lysates of tunicamycin- (black bars) or mock-treated (gray bars) cells. (D) Upper panel, for determination of the rate of mouse BiP (m-BiP) synthesis, anti-BiP antiserum was used for immunoprecipitation of mouse BiP. Lower panel, relative signal intensities of mouse BiP isolated from lysates of tunicamycin- (black bars) or mock-treated (gray bars) cells.

transcription inhibitor actinomycin D (Harding et al., 2000a). From this finding, it could be concluded that a transcriptional increase in BiP mRNA is a prerequisite for upregulation of BiP expression. Because our data indicate that translational upregulation of BiP is not dependent on increased mRNA levels, we postulated that actinomycin D might have a direct or indirect effect on BiP translation itself. To clarify this issue, we analyzed the effect of actinomycin D on BiP synthesis in thapsigargin-treated HeLa cells. Thapsigargin induces a more rapid and stronger UPR than tunicamycin. BiP transcript levels in BiLu33 cells were already elevated 30 minutes after thapsigargin addition (K.G. and I.G.H., unpublished), and the concomitant inhibition of protein synthesis was approximately 50% in activated Bil11 cells (Fig. 4A) as well as in BiLu 33 cells (K.G. and I.G.H., unpublished). We also analyzed the rate of BiP translation in thapsigargin-treated cells in the absence (Fig. 4A-C) or presence of Actinomycin D (Fig. 4D-F), which was added only 5 minutes prior to cell labeling. Under these conditions, actinomycin D should not drastically affect BiP synthesis, because BiP transcripts are already elevated. However, actinomycin D strikingly blocked the thapsigargin-induced increase in BiP synthesis, both total (compare 4B and E) and mouse (compare 4C and F) BiP. Thus, actinomycin D affects BiP translation, either directly or indirectly, possibly by preventing transcription of a short-lived protein required for translation of BiP.

To summarize, upregulation of BiP expression during UPR can be described as a two step process: (i) alleviation of the translational restraint present in unstressed cells resulting in increased translation efficiency of BiP mRNA and (ii) increased transcription of BiP from the well known classic UPR response.

Increased translation efficiency of BiP mRNA is probably caused by increased ribosome transit

To gain more insight into the mechanism underlying control of BiP mRNA translation, we next investigated polysome patterns in unstressed and stressed cells by sucrose gradient centrifugation of solubilized HeLa (Fig. 5A). Because of its moderate stress-inducing effect, tunicamycin allowed us to compare cells that – stressed or unstressed – contained the

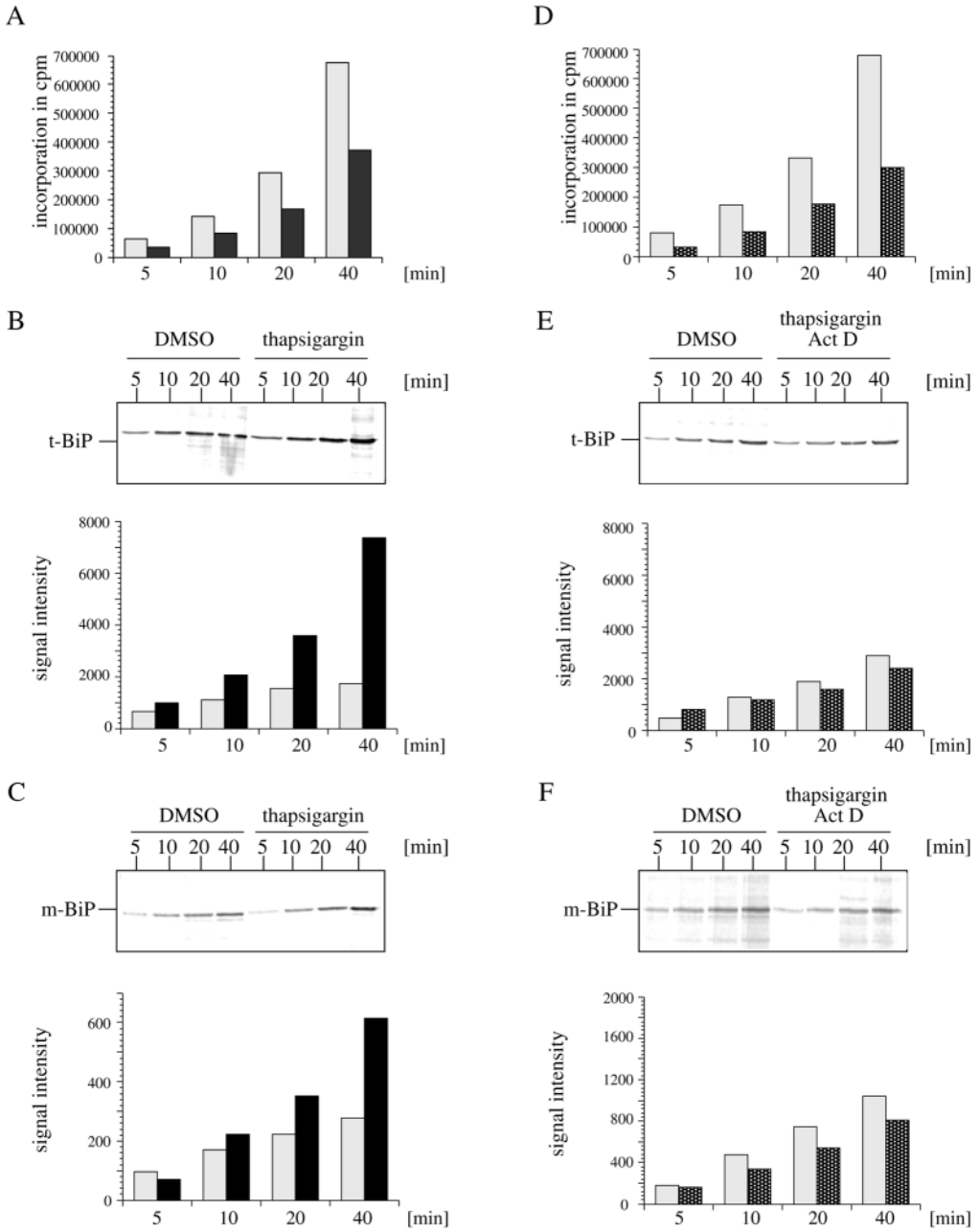

Fig. 4. UPR induction by thapsigargin and effect of actinomycin D on BiP synthesis in activated Bil11 cells. (A) Determination of ^{35}S-methionine incorporation into TCA-precipitated material from lysates of thapsigargin- (black bars) or mock- (DMSO-)treated cells (gray bars) prepared at the time points indicated after ^{35}S-methionine addition. (B) Upper panel, determination of BiP (t-BiP) synthesis rate was performed as described in Fig. 3C. Lower panel, relative signal intensities of total BiP in thapsigargin- (black bars) or mock- (DMSO-)treated (grey bars) cells. (C) Upper panel, determination of mouse BiP synthesis rate was performed as described in Fig. 3D. Lower panel, relative signal intensities of mouse BiP isolated from thapsigargin- (black bars) or mock-treated (gray bars) cells. (D) Determination of ^{35}S-methionine incorporation into TCA-precipitated material from lysates of activated Bil11 cells treated with thapsigargin during starvation (90 minutes) and pulse, and additionally with actinomycin D 5 minutes prior to and during pulse (black-checkered bars). Mock- (DMSO-)treated cells are also shown (gray bars). (E) Upper panel, determination of BiP (t-BiP) synthesis rate was performed as described in Fig. 3C. Lower panel, relative signal intensities of total BiP in thapsigargin and actinomycin D- (black-checkered bars) or mock (DMSO)-treated (grey bars) cells. (F) Upper panel, determination of mouse BiP synthesis rate was performed as described in Fig. 3D. Lower panel, relative signal intensities of total BiP in thapsigargin and actinomycin D- (black-checkered bars) or mock- (DMSO-)treated (grey bars) cells.

same amount of BiP transcripts. UPR caused a drastic increase of monosomes and free ribosomal subunits as was previously described (Harding et al., 2000b). To investigate how UPR affected ribosome association with specific transcripts, total RNA isolated from the fractions was analyzed by northern blotting using BiP and actin-specific probes (Fig. 5B). Signals were detected only in the polysome fractions, indicating that all transcripts for the genes analyzed were associated with

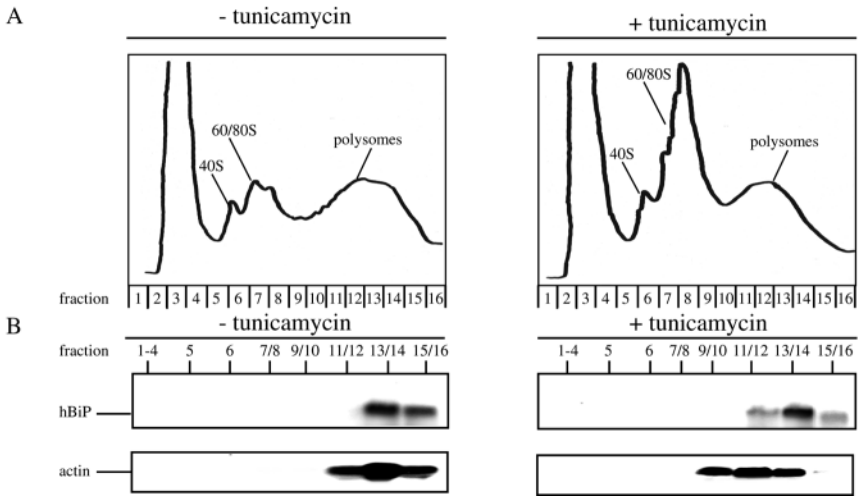

Fig. 5. Analysis of ribosome distribution in unstressed and stressed HeLa cells. (A) Upper panel, polysome profiles of mock- (left) or tunicamycin-treated (90 minutes; right) HeLa cells. Absorbance at 254 nm (y axis, indicating RNA concentration) is plotted against migration in the 10-50% sucrose gradients (x axis). The positions of polysomes and ribosomal subunits are indicated. The monosomal 80S peak is not resolved from the 60S subunit peak in these gradients. (B) Fractions corresponding to the profile shown in A were pooled as indicated and analyzed by northern blot using a BiP-specific probe (upper panel) that detected endogenous human BiP mRNA (hBiP) or an actin-specific probe (lower panel). Note that after 90 minutes of tunicamycin treatment, increase in BiP mRNA was still below a factor of 1.5.

ribosomes. In HeLa cells, actin mRNA (main peak in fraction 13/14) shifted to lighter fractions after tunicamycin treatment (main peak in fraction 11/12) indicating that actin transcripts were associated with fewer ribosomes during UPR (Fig. 5B). A similar but very weak shift was observed with human BiP mRNA, the majority of which remained in the same fraction as without UPR (fraction 13/14; Fig. 5B), showing a minor influence of inhibition of translational initiation on human BiP mRNA.

These results clearly show that the overall polysome organization of the BiP-encoding transcript is not drastically affected by the UPR. Thus, the increased translation efficiency of BiP mRNA observed under UPR conditions is most probably caused by a higher ribosome transit rate on BiP transcripts.

Discussion

A crucial requirement for an effective biological sensor is that small perturbations within strictly defined limits produce no response, but when a particular threshold value is reached, the response is rapidly induced. The work presented here describes, for the first time, feed-back regulation of the ER-lumenal chaperone BiP. By a novel mechanism, specific control of BiP mRNA translation efficiency, BiP protein expression levels are kept constant in unstressed cells, and rapidly increase in cells exposed to ER stress. According to our results, BiP meets all the properties a biological sensor should have.

Use of the tet-off activation system (Gossen and Bujard, 1992) allowed us to establish stable human cell lines capable of conditionally overexpressing mouse BiP in the absence of cellular stress. Antibodies directed to the C-terminus of rodent BiP specifically detected the protein derived from the construct introduced, and a monoclonal antibody recognizing both mouse and human proteins was used to quantify total cellular BiP. Firefly luciferase, encoded downstream of mouse BiP, was used as a reporter to indirectly quantify the bi-cistronic transcript. We show that the heterologous mRNA reached maximal levels (approximately 10 times the amount of endogenous BiP transcripts) 24 hours after transcriptional activation without affecting endogenous BiP mRNA levels. Mouse BiP protein increased very slowly, and steady state levels were reached only after more than 4 days of transcriptional activation. Strikingly, total cellular BiP levels did not increase. HeLa cells activated to produce mouse BiP transcripts maintain protein expression at a constant level by slowly displacing human BiP by the mouse protein. The slow increase in mouse BiP presumably reflects the low turnover rate of the long-lived protein. Since endogenous human BiP is degraded very slowly, mouse BiP can replace it only very slowly too. Under steady state conditions, mouse BiP protein made up approximately 60% of total cellular BiP, implying that human BiP transcripts translate into less protein when mouse BiP is co-expressed. In unstressed cells, translation of mouse BiP is evidently restricted as well, since the same amount of transcript is translated more efficiently under stress conditions. It is true that the final proportions of mouse to human BiP (65% and 35%, respectively, i.e. ~2:1) did not reflect the proportions of the respective transcripts present in permanently activated cells (90% and 10%, respectively). However, this could be related to the lack in the mouse BiP construct of the 5′ untranslated portion responsible for efficient 5′ cap-dependent or -independent translation initiation (Kozak, 1986; Sarnow, 1989). Taken together, our data show that BiP expression is feed-back regulated by restricted translation of BiP mRNA in unstressed cells. Reduced translation efficiency was not observed for other ER proteins, indicating that the mechanism described here is BiP specific.

In other systems, overexpression of BiP has been reported (Dorner et al., 1992). In unstressed cells, this may be possible when transcript levels are high enough to override the translational control mechanism presented here. Careful studies in transgenic tobacco plants carrying additional wild-type BiP copies showed that a 100- to 150-fold increase in BiP mRNA (in the system presented here, BiP mRNA is increased approximately 10 fold) only led to a modest five-fold increase in BiP protein (Leborgne-Castel et al., 1999), pointing to a mechanism regulating BiP expression in this system as well. When cellular BiP levels cannot be maintained by translational control, degradation may additionally be induced, as indicated by lower molecular mass bands probably representing BiP degradation products. Interestingly, loss of BiP also seems to be counterbalanced. Yeast mutants in p24 gene family members, implicated in vesicular transport, secrete KDEL-

bearing ER proteins such as BiP (Marzioch et al., 1999). However, BiP export does not lead to a reduction in intracellular BiP levels. It is tempting to speculate that the translation efficiency of BiP is enhanced in these mutants.

UPR is known to upregulate a number of target genes, including BiP. However, our findings definitively indicate that an increased amount of BiP transcripts is not sufficient to raise the protein levels, because, in unstressed cells, elevated transcript levels led neither to increased translation of BiP nor to increased protein levels. Consequently, the constraint limiting BiP expression in unstressed cells must be revoked when more BiP is needed. Indeed, under conditions of ER stress, we found BiP synthesis to be more efficient. Thus, an increase in BiP expression during UPR is possible by revoking the mechanism controlling BiP expression in unstressed cells rather than by the mere enhancement of BiP transcripts. Moreover, it seems that translational and transcriptional upregulation of BiP are independent events. Increased BiP translation rates upon UPR induction with both tunicamycin and thapsigargin could be detected before BiP mRNA levels rose. Even more strikingly, mouse BiP translation was enhanced under UPR conditions even though mouse BiP transcription is not under UPR control. Thus, in addition to the classical ER stress response, namely transcriptional upregulation of UPR genes, and downregulation of general protein synthesis, UPR also leads to a very rapid increase in the translation efficiency of BiP mRNA. The fast translational response allows the cell to adapt to small perturbations without inducing the transcriptional response. Only if a certain threshold value is reached is the final transcriptional upregulation of UPR genes turned on.

The increase of BiP translation efficiency under UPR conditions deserves further discussion. As already mentioned, UPR also leads to PERK-mediated phosphorylation of the translation initiation factor eIF2α (Harding et al., 1999). Since inactivation of a translation initiation factor seems to be a very general mechanism to downregulate protein translation, it is difficult to explain how BiP can evade this inhibition and even show increased translation efficiency. It has been shown that an IRES element situated upstream of the initiation AUG in BiP mRNA is involved in some alternative pathway of translation initiation (Sarnow, 1989). Further examples for the involvement of 5′ and 3′ untranslated regions in translational regulation have been described (Hershey, 1991; Preiss and Hentze, 1999). Our experimental system allowed us to investigate the role of the 5′ or 3′ untranslated regions of BiP mRNA, as both regions are missing in the mouse sequence employed. If elements in these regions were essential for translational control, BiP would be expected to be downregulated upon UPR induction as is any other cellular protein, for example, actin or luciferase. However, this was clearly not the case. These findings suggest that translational upregulation of BiP is not dependent on a cis-acting IRES element or on other regions present in the non-coding portion.

BiP translation efficiency does not seem to be affected by inactivation of the trans-acting initiation factor eIF2α, although this elicits disassembly of polysomes and a reduced rate of global protein synthesis (Brostrom and Brostrom, 1998; Prostko et al., 1992). Despite the slightly decreased polysome size of BiP transcripts under UPR conditions, BiP translation is clearly enhanced. These findings are consistent with a model in which BiP expression is controlled at the level of translation elongation. If the BiP translation elongation rate was low in unstressed cells, enhancement of the elongation rate in stressed cells could allow faster migration of ribosomes and the same or even a decreased amount of ribosomes would be able to synthesize more BiP protein from the same amount of transcript (protein synthesis rate=([number of active ribosomes]×[number of amino acid residues])/[ribosome transit time]; (Hershey, 1991)). In this context, it is interesting to note that regulation of chicken reticulocyte hsp70 mRNA translation is at the level of elongation (Theodorakis et al., 1988).

There seem to be various translational mechanisms acting during UPR. Another protein described as escaping stress-induced inhibition of translation initiation is the transcription factor ATF4 (Harding et al., 2000). In the absence of stress, ATF4 synthesis is inefficient because translation is initiated at an out-of-frame ATG situated upstream of the real start codon. Under UPR conditions, however, more ATF4 is produced because the ribosomes initiate translation at the correct start codon, shifting ATF4 transcripts into higher polysome fractions (Harding et al., 2000a).

The translation elongation rate of BiP mRNA could be controlled either by some structural motifs present in the coding region of BiP mRNA or by signals in the protein itself. Control at the level of translocation, for instance, is a possibility to slow down translation elongation. Interestingly, an event that limits protein translocation into the ER seems to limit apolipoprotein B synthesis in liver cells when the lipid supply for very low density lipoprotein formation is insufficient (Bonnardel and Davis, 1995). Whatever the precise mechanism is, with regard to the many functions of BiP, it is not surprising that even subtle changes in BiP concentrations have deleterious effects on the cells. We propose that this mechanism is to ensure on/off switch functions of BiP by allowing the cells to fine-tune BiP levels in order to prevent untimely UPR.

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