Distinct roles of activating transcription factor 6 (ATF6) and double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK) in transcription during the mammalian unfolded protein response

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In response to accumulation of unfolded proteins in the endoplasmic reticulum (ER), a homeostatic response, termed the unfolded protein response (UPR), is activated in all eukaryotic cells. The UPR involves only transcriptional regulation in yeast, and approx. 6 % of all yeast genes, encoding not only proteins to augment the folding capacity in the ER, but also proteins working at various stages of secretion, are induced by ER stress [Travers, Patil, Wodicka, Lockhart, Weissman and Walter (2000) *Cell* (Cambridge, Mass.) 101, 249–258]. In the present study, we conducted microarray analysis of HeLa cells, although our analysis covered only a small fraction of the human genome. A great majority of human ER stress-inducible genes (approx. 1 % of 1800 genes examined) were classified into two groups. One group consisted of genes encoding ER-resident molecular chaperones and folding enzymes, and these genes were directly

regulated by the ER-membrane-bound transcription factor activating transcription factor (ATF) 6. The ER-membrane-bound protein kinase double-stranded RNA-activated protein kinase-like ER kinase (PERK)-mediated signalling pathway appeared to be responsible for induction of the remaining genes, which are not involved in secretion, but may be important after cellular recovery from ER stress. In higher eukaryotes, the PERK-mediated translational-attenuation system is known to operate in concert with the transcriptional-induction system. Thus we propose that mammalian cells have evolved a strategy to cope with ER stress different from that of yeast cells.

Key words: amino acid starvation, microarray, protein folding, translation.

INTRODUCTION

The endoplasmic reticulum (ER) is responsible for the quality control of proteins that pass through the secretory pathway [1,2]. Protein unfolding occurs in the ER not only when the productive folding process in the ER is perturbed by environmental stress conditions, but also when the amounts of newly synthesized secretory or transmembrane proteins exceed the folding capacity in the ER. In addition, proteins possessing genetic mutation(s) in their amino acid sequences may not be able to be folded properly. Unfolded proteins thus accumulated in the ER activate a homeostatic response, termed the unfolded protein response (UPR), in all eukaryotic cells [3–6].

The UPR mechanism in the budding yeast *Saccharomyces cerevisiae* involves transcriptional regulation alone and has been well characterized [3–6]. The most proximal component of the pathway is the transmembrane protein kinase/endoribonuclease Ire1p [7–9], which senses the presence of unfolded proteins in the ER and transduces a signal across the ER membrane [10–14], whereas the most distal component is the basic leucine zipper (bZIP)-type transcription factor Hac1p [15–17], which activates transcription of UPR-target genes via direct binding to the

cis-acting UPR element (UPRE) [18–20]. The primary targets of the UPR were originally thought to be proteins to cope with unfolded proteins directly, such as ER-resident molecular chaperones and folding enzymes [21–23]. However, microarray analysis of *S. cerevisiae* revealed that numerous genes (approx. 6 % of all yeast genes) encoding not only proteins to augment the folding capacity in the ER, but also proteins working at various stages of secretion, are induced by ER stress in a manner dependent on Ire1p and Hac1p [24]. This observation led Travers et al. [24] to propose that specific remodelling of the secretory pathway occurs during the UPR to minimize the amount and/or concentration of unfolded proteins in the ER in yeast cells, and this proposal was supported by genetic analysis conducted for *S. cerevisiae* [25].

Our understanding of the molecular mechanism of the transcriptional regulation in mammals has recently made great progress due to the discovery of the bZIP-type transcription factors activating transcription factor (ATF) 6 α and ATF6 β , which bind directly to the *cis*-acting ER stress-response element (ERSE) responsible for the mammalian UPR [26,27]. Both ATF6 α and ATF6 β are constitutively synthesized as type II transmembrane proteins in the ER and activated by regulated

Abbreviations used: AARE, amino acid-response element; Asn-S, asparagine synthetase; ATF, activating transcription factor; bZIP, basic leucine zipper; CHOP, CCAAT/enhancer-binding protein homologous protein; DMEM, Dulbecco's modified Eagle's medium; DOX, doxycyclin; DRAL, down-regulated in Rhabdomyosarcoma LIM protein; ECL® (Amersham Biosciences), enhanced chemiluminescence; eIF2 α , eukaryotic translation initiation factor 2 α ; ER, endoplasmic reticulum; EDEM, ER-degradation-enhancing α -mannosidase-like protein; ERp, ER protein; ERSE, ER stress-response element; FBS, fetal bovine serum; FHL2, four and a half LIM domains 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GCN2, general control non-derepressible-2; GRP, glucose-regulated protein; HMG-CoA-S, 3-hydroxy-3-methylglutaryl-CoA synthetase; pATF6 α (N) and pATF6 β (N), active nuclear forms of ATF6 α and ATF6 β respectively; pATF6 α (P) and pATF6 β (P), ER-membrane-bound precursor forms of ATF6 α and ATF6 β respectively; PERK, double-stranded RNA-activated protein kinase-like ER kinase; PAX6, paired box gene 6; SCAMP, secretory carrier membrane protein; SEC, secretion; SERCA2, sarcoplasmic/ER Ca²⁺-ATPase 2; Trp-tRNA-S, tryptophanyl-tRNA synthetase; UPR, unfolded protein response; UPRE, UPR element; VPS, vacuolar protein sorting; XBP, X-box-binding protein.

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intramembrane proteolysis in response to ER stress [28–34]. When the ER-membrane-bound precursor forms of ATF6 α [pATF6 α (P)] and ATF6 β [pATF6 β (P)] are cleaved in response to ER stress, their bZIP-containing N-terminal halves become soluble transcription factors, which are translocated into the nucleus and activate transcription of UPR-target genes via direct binding to the ERSE in collaboration with the general transcription factor nuclear factor-Y ('NF-Y'). Overexpression of the active nuclear forms of ATF6 α [pATF6 α (N)] and ATF6 β [pATF6 β (N)] is sufficient for transcriptional induction of the ER-resident molecular chaperone glucose-related protein (GRP) 78/immunoglobulin heavy-chain binding protein ('BiP'), a major target of the UPR, and of the transcription factors CCAAT/enhancer-binding protein homologous protein (CHOP) and X-box-binding protein (XBP) 1 [29,33]. Accumulating evidence strongly indicates that ATF6 α and ATF6 β are crucial transcriptional regulators of the mammalian UPR [29–33]. However, the ATF6-target genes have not been fully characterized.

In contrast with yeast cells, translational regulation occurs as a part of the UPR in higher eukaryotes [35]. This difference is ascribed to the presence of the transmembrane protein kinase pancreatic eukaryotic translation initiation factor 2 α (eIF2 α)-subunit kinase ('PEK')/double-stranded RNA-activated protein kinase-like ER kinase (PERK) in the ER of mammalian [36,37] as well as worm (*Caenorhabditis elegans*) [38] cells, but not of yeast cells. When activated in response to ER stress via oligomerization and autophosphorylation [12,13], PERK directly phosphorylates the α subunit of eIF2 α on Ser⁵¹, leading to general translational attenuation [37]. The primary effect of this signalling is to decrease the load of newly synthesized proteins into the ER when the productive folding process is not effective there. Cells lacking PERK or cells possessing a homozygous mutation at the eIF2 α phosphorylation site are much more sensitive to ER stress than wild-type cells [39–41], indicating the importance of this translational regulation for cellular survival under ER stress conditions. Interestingly and paradoxically, translational attenuation induces the translation of certain mRNAs, such as an mRNA encoding the bZIP transcription factor ATF4, resulting in the transcriptional induction of the transcription factor CHOP [42]. Thus the PERK pathway also plays a role in transcriptional regulation during the UPR.

In the present study, we conducted microarray analysis of HeLa cells to determine the nature of genes induced by ER stress in mammalian cells and to assess the contribution of the ATF6 and PERK pathways to the overall transcriptional regulation during the mammalian UPR.

MATERIALS AND METHODS

Plasmid construction

Recombinant DNA techniques were performed according to standard procedures [43]. A fragment of pCGN-ATF6(373) [28] containing the amino acid region 1–373 of ATF6 α tagged with the influenza virus haemagglutinin epitope at the N-terminus was inserted into pTRE2 (ClonTech, Palo Alto, CA, U.S.A.) to create pTRE-ATF6 α (1–373).

Cell culture and establishment of stable transformants

HeLa Tet-Off cells (ClonTech) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) Tet system-approved fetal bovine serum (FBS; ClonTech), 2 mM L-glutamine, 100 μ g/ml G418, 50 ng/ml doxycycline (Dox), 100 units/ml penicillin and 100 μ g/ml streptomycin sulphate. Cells were maintained at 37 °C in a humidified incubator

containing 5% CO₂ mixed with air. HeLa Tet-Off cells were transfected by electroporation with pTK-Hyg (ClonTech) and pTRE2. A stable transformant isolated by adding 300 μ g/ml hygromycin B to the culture medium was used after one round of cloning and termed HeLa-vector. HeLa Tet-Off cells were transfected similarly with pTK-Hyg and pTRE-ATF6 α (1–373). Dozens of stable transformants were isolated on the basis of their resistance to 300 μ g/ml hygromycin B and were subjected to one round of cloning. Expression of ATF6 α (1–373) was then checked by immunoblotting before and after removal of Dox from the culture medium. A stable transformant, in which ATF6 α (1–373) was detected only after removal of Dox and whose growth rate was similar regardless of the presence or absence of Dox, was selected and used after one more round of cloning as HeLa-pATF6 α (N).

Amino acid starvation

HeLa-vector and HeLa-pATF6 α (N) cells were grown in DMEM/Ham's F12 (Sigma, St. Louis, MO, U.S.A.) supplemented with 10% (v/v) Tet system-approved FBS, 2 mM L-glutamine, 100 μ g/ml G418, 300 μ g/ml hygromycin B, 50 ng/ml Dox, 100 units/ml penicillin and 100 μ g/ml streptomycin sulphate. Amino-acid-starvation experiments were conducted as described previously [44]. Cells at 70% confluency were washed three times with PBS and cultured for 8 h in DMEM/Ham's F12 lacking leucine (Sigma) supplemented with 10% (v/v) dialysed FBS (Sigma), 2 mM L-glutamine, 100 μ g/ml G418, 300 μ g/ml hygromycin B, 50 ng/ml Dox, 100 units/ml penicillin and 100 μ g/ml streptomycin sulphate.

Immunoblotting

Cells cultured in 60-mm dishes were washed with PBS, scraped with a rubber policeman and lysed in 100 μ l of Laemmli's SDS-sample buffer lacking the reducing reagent. The protein concentration was determined using a Micro bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL, U.S.A.). After addition of 2-mercaptoethanol (5% final concentration) and boiling for 5 min, 25 μ g (for detection of ATF6 α) or 50 μ g (for detection of ATF4) of protein was subjected to SDS/PAGE [7.5% (w/v) or 10% (w/v) gels respectively]. ATF6 α and ATF4 were detected with anti-(ATF6 α) [28] and anti-[cAMP-response element-binding protein ('CREB') 2] (C-20) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and the enhanced chemiluminescence (ECL®) Western blotting detection system (Amersham Biosciences, Uppsala, Sweden). Chemiluminescence was visualized using an LAS-1000plus LuminoImage analyser (Fuji Film, New York, NY, U.S.A.).

Northern blot hybridization

Total RNA was extracted from HeLa cells by the acid guanidinium/phenol/chloroform method using Isogen (Nippon Gene, Tokyo, Japan). Portions (10 μ g) of RNA were subjected to electrophoresis using 1% (w/v) agarose gels containing 2.2 M formaldehyde and analysed by using standard procedures [43].

Microarray analysis

Total RNA extracted from HeLa-vector or HeLa-pATF6 α (N) cells using Isogen was purified further using RNeasy Midi (Qiagen, Valencia, CA, U.S.A.). Microarray analysis was performed with the MICROMAX Direct cDNA Microarray System (PerkinElmer Life Science, Boston, MA, U.S.A.)

according to the manufacturer's instructions. Portions (100 µg) of total RNA prepared from control and 'treated' cells were labelled with cyanine 3-dUTP and cyanine 5-dUTP respectively, during reverse transcription. Equal amounts of non-human control RNAs supplied by the manufacturer were also labelled with cyanine 3-dUTP and cyanine 5-dUTP for data correction. The labelled cDNAs were mixed and hybridized with MICROMAX Human cDNA I on which 2400 human genes were spotted. Cyanine 3 and cyanine 5 fluorescence intensity was determined for each spot using an Affymetrix 428 Array Scanner (Santa Clara, CA, U.S.A.) and ImaGene software (BioDiscovery, Marina Del Ray, CA, U.S.A.). The fold induction caused by each treatment was defined as the ratio of the intensity of cyanine 5/cyanine 3 after normalization against the same ratio obtained with non-human control RNAs. Because our analysis was based on fold-induction values, genes showing extremely low fluorescence intensity were eliminated from each analysis to ensure accuracy. Namely, if cyanine 3 or cyanine 5 intensity for a gene was less than 0.1 % of the maximum value of cyanine 3 or cyanine 5 intensity respectively, obtained with 2400 genes in each experiment, the fold-induction value was not determined. Four independent experiments were carried out for tunicamycin treatment, ATF6 α (1–373) expression and amino acid starvation, and fold-induction values for 1868 out of 2400 genes were obtained at least three times and mostly four times in all cases.

RESULTS

Targets of the ATF6 pathway

To identify the spectrum of human genes inducible by ER stress, we isolated total RNA from HeLa cells that had been treated in the absence or presence of tunicamycin for 8 h, which elicits ER stress by inhibiting protein N-glycosylation [3,21], and the fold induction by tunicamycin treatment was determined for each of the 2400 human genes spotted on to a slide glass. Using the procedures described in the Materials and methods section, 22 genes were found to be induced by more than 2-fold among the 1868 genes successfully analysed (their fold-induction values are plotted on the abscissa of Figures 1A and 1B, with a scale of log₂). *ATF4* was induced less than 2-fold in our system, in contrast with the results published previously [41]. Two other known UPR-target genes present in the microarray, *KIAA0212* (also known as *EDEM*, where *EDEM* encodes ER-degradation-enhancing α -mannosidase-like protein) and *XBPI*, could not be analysed due to their low basal expression. Four of the 22 inducible genes, i.e. *asparagine synthetase (Asn-S)/temperature-sensitive mutant 11 ('ts11')*, *tryptophanyl-tRNA synthetase (Trp-tRNA-S)/IFP53* (where IFP53 represents the gene encoding interferon δ -inducible protein with a molecular mass of 53 kDa), *down-regulated in Rhabdomyosarcoma LIM (DRAL)/four and a half LIM domains 2 (FHL2)* and *paired box gene 6 (PAX6)/oculorhombin*, were duplicated in the microarray, due to two different names assigned to them (see Figure 1B). Thus the frequency of ER stress-responsive genes in humans was approx. 1 %, which is much lower than that (approx. 6 %) in yeast.

To determine the spectrum of ATF6-target genes in humans, we established a stable HeLa cell line in which the expression of ATF6 α (1–373), representing pATF6 α (N), the nuclear, active form of ATF6 α , is controlled negatively by the presence of tetracycline in the culture medium using the so-called Tet-off system. This cell line is referred to as HeLa-pATF6 α (N), whereas a control cell line carrying the same vector alone is referred to as HeLa-vector. As shown in Figure 2(A), ATF6 α (1–373) was detected specifically in HeLa-pATF6 α (N) (Figure 2A, lanes 5–8),

but not in HeLa-vector (Figure 2A, lanes 1–4) from 1 day after removal of Dox, a tetracycline derivative. The level of ATF6 α (1–373) in these cells was similar to that of endogenous pATF6 α (N) detected after 1 h of treatment with tunicamycin (Figure 2B). Concomitantly, the level of endogenous GRP78 mRNA was elevated markedly in HeLa-pATF6 α (N) (Figure 2C, lanes 5–8), but not in HeLa-vector (Figure 2C, lanes 1–4), from 1 day after removal of Dox to an extent comparable with that obtained after tunicamycin treatment of HeLa-vector (Figure 2C, lane 10) or HeLa-pATF6 α (N) (Figure 2C, lane 12).

We then isolated total RNA from HeLa-pATF6 α (N) cultured in the absence or presence of Dox for 1 day and determined the fold induction caused by the expression of ATF6 α (1–373) for each of 1868 human genes (their fold-induction values are plotted on the ordinate of Figure 1A with a scale of log₂). As a result, seven genes (red circles in Figure 1A) were found to be induced similarly by both tunicamycin treatment and the expression of ATF6 α (1–373) (see Figure 3A, categorized into group A). Strikingly, all of these turned out to be ER-resident proteins. Some of the genes encode molecular chaperones (*GRP78*, *GRP94* and *calreticulin*) and others are folding enzymes that facilitate correct disulphide-bond formation, i.e. protein disulphide-isomerase and protein disulphide-isomerase-like proteins [*ER protein (ERp)* 72, *ERp61* and *P5*] [45]. In addition, the *sarcomasmic/ER Ca²⁺-ATPase 2 (SERCA2)* gene, coding for an ER-resident Ca²⁺ ATPase that is important for Ca²⁺ homeostasis in the ER, was found to be induced by tunicamycin treatment (2-fold), as reported previously [46]. However, the expression of ATF6 α (1–373) induced *SERCA2* to a lesser extent (1.3-fold). This observation is consistent with a recent report [47] showing that induction of *SERCA2* by Ca²⁺ depletion was mediated by both ATF6-dependent and -independent signalling pathways. Interestingly, the induction profile of the gene coding for the ER-resident 3-hydroxy-3-methylglutaryl-CoA synthase (*HMG-CoA-S*) was distinct from those of members of group A, and thus we categorized this gene into group A'. The *HMG-CoA-S* gene was not induced (but was rather suppressed) by tunicamycin treatment, but was induced 2-fold by the expression of ATF6 α (1–373). This pattern was confirmed by Northern blot hybridization (results not shown), although the basis for this differential regulation is currently unclear.

The results above clearly indicated that the primary targets of the ATF6 pathway are proteins that augment the protein folding capacity in the ER, and that the ATF6 pathway is responsible for the induction of proteins that cope with ER stress directly.

Synergistic induction of *CHOP* by the ATF6 and PERK pathways

In the microarray analysis, the gene with the highest induction by tunicamycin treatment was *CHOP* (16-fold); however, *CHOP* was induced only 2-fold by the expression of ATF6 α (1–373) (Figures 1A and 3A, and categorized into group C). This conclusion was firmly supported by Northern-blot hybridization analysis as shown in Figure 4(A) (compare lane 5 with lane 6). These results suggest that the ATF6 pathway plays a rather minor role in the induction of *CHOP*, in marked contrast with its major role in the induction of ER-resident chaperones and enzymes. Since it has been already shown that induction of *CHOP* by ER stress is lost in PERK-deficient mouse embryonic stem cells [42] and we have found that ATF4 protein was detected in lysates of HeLa cells only after tunicamycin treatment (Figure 4B), as observed in wild-type mouse embryonic stem cells [42], the PERK/eIF2 α phosphorylation/ATF4 pathway is the most promising candidate as an ATF6-independent pathway that is activated during the UPR in HeLa cells. Unfortunately,

Figure 1 Identification of human genes inducible by tunicamycin, ATF6 α (1-373) or amino acid starvation

(A) Microarray analysis was conducted using total RNA isolated from HeLa cells that had been treated in the absence or presence of tunicamycin (2 μ g/ml) for 8 h as well as total RNA isolated from HeLa-pATF6 α (N) cells that had been cultured for 1 day in the absence or presence of Dox. Fold induction by tunicamycin treatment and expression of ATF6 α (1-373) was plotted on the abscissa and ordinate respectively, with a scale of $\log_2$. (B) Microarray analysis was conducted using total RNA isolated from HeLa cells cultured for 8 h in the absence or presence of leucine in the medium. Fold induction following tunicamycin treatment and by amino acid starvation was plotted on the abscissa and ordinate respectively, with a scale of $\log_2$. Genes were categorized into group A and A' (red or pink circles), B (blue or light-blue circles) and C (yellow circles) (see Figure 3A). Genes marked by orange circles in (B) were not induced by tunicamycin treatment, but were induced by amino acid starvation. Abbreviations used: Asp-AT, aspartate aminotransferase; CRT, calreticulin; Gly-tRNA-S, glycyl-tRNA synthetase; GlnF6P-AT, glutamine:fructose-6-phosphate amidotransferase; neutral AA-T, neutral amino acid transporter; P5CR, pyrroline 5-carboxylate reductase; PDI, protein disulphide-isomerase.

Figure 2 Regulated expression of the nuclear active form of ATF6 α

(A) HeLa-vector and HeLa-pATF6 α (N) cells were cultured in the absence of Dox in the medium for the indicated period. Cell lysates were prepared and analysed by immunoblotting using an anti-ATF6 α antibody. The migration positions of endogenous pATF6 α (P) as well as exogenously introduced ATF6 α (1-373) are indicated. The asterisk indicates a non-specific band. Molecular-mass markers (in kDa) are indicated on the right. (B) HeLa-pATF6 α (N) cells were cultured for 1 day in the absence (-) or presence (+) of Dox in the medium, or were treated with tunicamycin (TM, 2 μ g/ml) for the indicated period. Cell lysates were prepared and analysed as in (A). Exogenous ATF6 α (1-373) migrated more slowly than endogenous pATF6 α (N) due to the influenza virus haemagglutinin epitope tag introduced at the N-terminus. pATF6 α (P*) represents the non-glycosylated form of pATF6 α (P). The asterisk indicates a non-specific band. Molecular-mass markers (in kDa) are indicated on the right. (C) HeLa-vector and HeLa-pATF6 α (N) cells were cultured in the absence of Dox in the medium for the indicated period or treated for 8 h without (-) or with (+) tunicamycin (TM, 2 μ g/ml). Total RNA was extracted and analysed by Northern-blot hybridization analysis using a cDNA probe specific for GRP78 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

however, involvement of the PERK/ATF4 pathway in the induction of *CHOP* could not be investigated directly in our system for the following reasons. First, overexpression of ATF4 alone was not sufficient for induction of *CHOP*, in contrast with ATF6 [29], but consistent with a previous report using mouse embryonic stem cells [42]. Second, overexpression of PERK is known to be extremely toxic to cells, as it causes cell-cycle arrest [48]. To circumvent this technical problem, we took advantage of

Figure 3 Grouping of human ER stress-inducible genes

(A) Fold induction of ER stress-inducible genes by tunicamycin treatment (open bars), expression of ATF6 α (1–373) (closed bars) and amino acid starvation (grey bars), determined as shown in Figure 1, was expressed as the means $\pm$ S.D. from four independent experiments for 21 genes. On the basis of these results, human ER stress-inducible genes were categorized into three groups (A and A', B and C). (B) Fold induction by tunicamycin treatment (open bars), expression of ATF6 α (1–373) (closed bars) and amino acid starvation (grey bars) of genes encoding typical cytoplasmic chaperones and folding enzymes as well as mitochondrial chaperonins (group D), and genes coding for proteins involved in translocation into the ER and intracellular transport (group E). Values are means $\pm$ S.D. from four independent experiments. Abbreviations used: Asp-AT, aspartate aminotransferase; CCT, chaperonin-containing T-complex polypeptide 1; FKBP, FK506-binding protein; Gly-tRNA-S, glycyl-tRNA synthetase; GlnF6P-AT, glutamine:fructose-6-phosphate amidotransferase; HSP, heat-shock protein; neutral AA-T, neutral amino acid transporter; P5C reductase, pyrroline-5-carboxylate reductase; PDI, protein disulphide-isomerase.

the fact that phosphorylation of eIF2 α , and subsequent translational induction of ATF4 can be evoked by amino acid starvation via activation of another eIF2 α kinase, general control non-derepressible-2 (GCN2) [42]. Thus the consequences of PERK activation by ER stress are identical with those of GCN2 activation by amino acid starvation (see Figure 7). Indeed, as shown in Figure 4(C), induction of ATF4 in lysates of both HeLa-vector (lanes 1–3) and HeLa-pATF6 α (N) (lanes 4–6), as well as concomitant induction of CHOP mRNA, was observed after leucine deprivation in the culture medium as expected.

We then isolated total RNA from HeLa cells cultured normally, as well as from those cultured in the absence of leucine for 8 h, and the fold induction by amino acid starvation was determined for each of the 1868 human genes (their fold-induction values are plotted on the ordinate of Figure 1B with a scale of log₂). CHOP was found to be induced 9-fold by amino acid starvation, indicating that the contribution of the

PERK/ATF4 pathway to the induction of CHOP was dominant to that of the ATF6 pathway (Figure 3A, group C). The results also indicated that the 16-fold induction of CHOP by tunicamycin treatment was much greater than the summation of the 2-fold induction by the expression of ATF6 α (1–373) plus the 9-fold induction by amino acid starvation. On the other hand, the 2-fold induction of OS-9, a gene frequently amplified in osteosarcoma [49], by tunicamycin treatment appeared to be explained by the additive effects of the ATF6 and PERK pathways (Figure 3A, group C).

To determine whether there is any co-operativity between the ATF6 and PERK pathways in induction of CHOP, we carried out further Northern-blot hybridization analyses (Figure 5). As compared with the control (lane 7), CHOP mRNA was induced 2-fold by the expression of ATF6 α (1–373) for 1 day (lane 8), whereas it was induced 5-fold by leucine deprivation for 8 h (lane 11). Importantly, when leucine was depleted for 8 h after

Figure 4 Comparison of induction of CHOP and Asn-S mRNAs by tunicamycin, ATF6 α (1–373) and amino acid starvation

(A) HeLa-vector and HeLa-pATF6 α (N) cells were cultured for 1 day in the absence (—) or presence (+) of Dox in the medium, or were treated for 8 h with (+) tunicamycin (TM, 2 μ g/ml). Total RNA was extracted and analysed by Northern-blot hybridization analysis using a cDNA probe specific for CHOP, Asn-S or GAPDH. (B) HeLa cells were treated with tunicamycin (TM, 2 μ g/ml) for the indicated period. Cell lysates were prepared and analysed by immunoblotting using an anti-(ATF4) antibody. (C) HeLa-vector and HeLa-pATF6 α (N) cells were cultured in the absence of leucine in the medium for the indicated period. Total RNA was extracted and analysed by Northern-blot hybridization using a cDNA probe specific for CHOP, Asn-S or GAPDH. Cell lysates were also prepared and analysed by immunoblotting using an anti-(ATF4) antibody (bottom panel).

1 day of expression of ATF6 α (1–373), CHOP mRNA was induced more than 20-fold (lane 12), an extent comparable with that obtained after tunicamycin treatment of HeLa-pATF6 α (N) cultured in the absence (18-fold; lane 10) or presence (16-fold; lane 9) of Dox. In contrast, the 5-fold induction of CHOP mRNA by leucine deprivation (lane 5) was not affected by 1-day removal of Dox in HeLa-vector (lane 6) as expected. We conclude that the ATF6 and PERK pathways work synergistically to induce CHOP.

Targets of the PERK pathway

Microarray analysis showed that most of the genes induced by tunicamycin treatment, but not by the expression of ATF6 α (1–373) (blue circles in Figure 1A), were induced more

Figure 5 Synergistic induction of CHOP mRNA but not Asn-S mRNA by ATF6 α (1–373) and amino acid starvation

HeLa-vector and HeLa-pATF6 α (N) cells were cultured for 1 day in the absence (—) or presence (+) of Dox in the medium, and then cultured for 8 h in the without (control) or with either medium lacking leucine (AAS) or tunicamycin (TM, 2 μ g/ml). Total RNA was extracted and analysed by Northern-blot hybridization using a cDNA probe specific for CHOP, Asn-S or GAPDH.

CHOP	AARE1(Human)	-310	ATTGCATCA	-302
CHOP	AARE2(Human)	-778	GTTTCACCA	-770
Asn-S	AARE1(Human)	-60	GTTTCATCA	-68
Asn-S	AARE2(Human)	-886	ATTACATCA	-894
Trp-tRNA-S	AARE1(Human)	-258	ATTGCATCA	-266
Trp-tRNA-S	AARE2(Human)	-530	GTTTCCTCA	-538
Trp-tRNA-S	AARE3(Human)	-874	GTTTCCTCA	-882
Neutral AA-T	AARE1(Human)	+39	TTTGCATCA	+47
Neutral AA-T	AARE2(Human)	-1090	ATTTCATCT	-1098
DRAL/FHL2	AARE (Human)	-781	TTTCCATCA	-773
PAX6	AARE (Human)	-97	ATTGCACCA	-89
Asp-AT	AARE (Human)	-234	GTTGCATCA	-226
P5C reductase	AARE1(Human)	-282	GTTGCATCA	-290
P5C reductase	AARE2(Human)	-813	GTTTCACCA	-805
α -L-iduronidase	AARE (Human)	-1228	ATTTCACCA	-1220
consensus	AARE		RTTKCATCA	

Figure 6 Presence of AARE in the promoter regions of group B genes

Analysis of the AAREs present in the *CHOP* and *Asn-S* promoters led to the proposal of the consensus RTTKCATCA [52]. Potential AARE sequences identified in the promoter regions of group B genes are aligned with *CHOP* and *Asn-S* AAREs. Shaded nucleotides are identical with those of the consensus. Numbers denote nucleotide positions, with respective transcription start site set at 1. Abbreviations used: Asp-AT, aspartate aminotransferase; neutral AA-T, neutral amino acid transporter; P5C reductase, pyrroline-5-carboxylate reductase.

than 2-fold by amino acid starvation (Figure 1B), and that nine of the ten genes were induced similarly by tunicamycin treatment and amino acid starvation (Figure 3A, categorized into group B). A typical example for this class of genes was the *Asn-S* gene. *Asn-S* has been shown [50,51] to be inducible not only by amino acid starvation, but also by ER stress via a common promoter element distinct from ERSE, a binding site for ATF6. Therefore we characterized the induction profile of *Asn-S* by Northern-blot hybridization. Asn-S mRNA was induced by both tunicamycin treatment (Figure 4A, lanes 3 and 6) and amino acid starvation (Figure 4C, lanes 3 and 6), but not by the expression of ATF6 α (1–373) (Figure 4A, lane 5). Accordingly, the induction

Figure 7 Transcriptional regulation by the ATF6 and PERK pathways during the mammalian UPR

ATF6 is activated by ER stress-induced proteolysis [28–34]. The molecular basis for the PERK-dependent transcriptional induction mechanism has been revealed previously by Ron and co-workers [42]. The results reported in the present study indicate that the ATF6 and PERK pathways have distinct roles in transcription during the mammalian UPR as depicted.

of *Asn-S* mRNA by amino acid starvation was not affected by the expression of ATF6 α (1–373), in contrast with the *CHOP* mRNA (Figure 5, compare lane 12 with lane 11). These findings are consistent with those from previous reports [50,51] as well as the data shown in Figure 3(A), further supporting our notion that the results obtained with the microarray analysis in the present study were highly reliable.

The nucleotide sequences of the promoter regions are available for eight of the ten genes categorized into group B in Figure 3(A). Using a computer-based search, we found that all of them contained sequence(s) that perfectly match, or closely resemble, the *cis*-acting amino acid-response element (AARE), as in the cases for *CHOP* and *Asn-S* [52] (Figure 6). *CHOP* AARE has been shown to be necessary and sufficient for the induction of *CHOP* by amino acid starvation [53] and for binding directly to ATF4 [54]. *Asn-S* AARE was also shown to be required for induction of *Asn-S* by both amino acid starvation and ER stress [51].

The above results strongly suggest that the PERK pathway is responsible for the majority of ATF6-independent transcriptional induction during the UPR in HeLa cells. Furthermore, its effect is mediated by the interaction of ATF4 with the AARE, although ATF4 appears to require an ER stress-induced modification or a specific partner protein, because overexpression of ATF4 alone is not sufficient for AARE-mediated transcriptional activation, as summarized in Figure 7.

DISCUSSION

The microarray analysis conducted in the present study for human cells revealed features of the mammalian UPR quite different from those of the yeast UPR. In yeast, a single signal transduction system, i.e. the Ire1p/Hac1p pathway, is responsible

for the transcriptional induction of all of ER stress-inducible genes [24]. In contrast, at least two signal transduction systems operate in mammalian cells, namely the ATF6 and PERK pathways, which appear to account for induction of most of the human ER stress-inducible genes (Figures 1 and 3). Importantly, targets of the ATF6 pathway (group A genes in Figure 3A) do not overlap with targets of the PERK pathway (group B genes), except for *CHOP* and *OS-9* (group C genes), which were induced synergistically and apparently additively respectively, by the two pathways, indicating that the ATF6 and PERK pathways play distinct roles in the mammalian UPR. As group A genes encoded ER-resident molecular chaperones and folding enzymes, the primary consequence of activation of ATF6 is augmentation of the folding capacity in the ER to cope with unfolded proteins accumulated under ER stress conditions directly. On the other hand, many group B genes code for proteins related to protein synthesis or amino acid biosynthesis/metabolism, such as *Asn-S*, *Trp-tRNA-S*, neutral amino acid transporter, aspartate aminotransferase, pyrroline-5-carboxylate reductase, glycyl-tRNA synthetase and glutamine:fructose-6-phosphate amidotransferase. Translation is attenuated during the UPR via PERK-mediated phosphorylation of eIF2 α in mammalian cells. Thus our observations may imply that mammalian cells are preparing for the next burst of new protein synthesis by activating the PERK-mediated transcriptional induction system, while simultaneously restricting protein synthesis by activating the PERK-mediated translational regulation system to facilitate recovery from ER stress. Roles of PERK-mediated transcriptional induction of the transcription factor PAX6/oculorhombin and transcriptional coactivator DRAL/FHL2 in the mammalian UPR remain to be investigated.

We noted that the somewhat weak induction of ATF4 that we observed appeared to depend solely on the PERK pathway

(results not shown), consistent with previous results [41]. Thus, among known the UPR-target genes present in the microarray, only two genes, *KIAA0212* (*EDEM*) and *XBPI*, could not be categorized in this analysis due to their low basal expression. The mechanism of transcriptional induction of the *EDEM* genes is currently under investigation, whereas it was shown previously that activation of the ATF6 pathway caused induction of *XBPI* mRNA [29]. However, data from a previous study [55] should be borne in mind in which cells deficient in PERK failed to induce the *XBPI* gene in response to tunicamycin treatment. Similarly, transcription of the *GRP78* and *Erp72* genes induced by tunicamycin treatment was greatly mitigated in cells possessing a homozygous mutation at the eIF2 α phosphorylation site and thus unable to activate the PERK pathway in response to ER stress [41]. Our present results showed that phosphorylation of eIF2 α and subsequent induction of ATF4 was not sufficient for induction of ER-resident molecular chaperones and folding enzymes (Figures 1 and 3). These results indicate the presence of cross-talk or interdependency between the ATF6 and PERK pathways to achieve maximal induction of the UPR-target genes.

The frequency of ER stress-inducible genes in HeLa cells was approx. 1%, which is much lower than the 6% in yeast cells, although our analysis covered only a small fraction of the human genome. In addition, genes encoding proteins involved in translocation into the ER [*secretion* (*SEC*) 61 β and *SEC62*] as well as intracellular transport [*SEC14L*, *SEC23B*, *vacuolar protein sorting* (*VPS*) 45A, *secretory carrier membrane protein* (*SCAMP*) 1 and *SCAMP3*] were not found to be inducible by ER stress in human cells (Figure 3B, group E). This is in contrast with yeast cells, in which *SEC61*, *SEC62*, *YKL091C* and *YLR380W/CSR1* (both are similar to *SEC14*), but not *SEC14* itself, *SEC23* and *VPS45*, are inducible by ER stress [24]. Yeast cells do not have PERK and thus appear to be unable to attenuate translation in response to ER stress. This may be why so many genes are up-regulated in yeast upon ER stress. Therefore specific remodelling of the secretory pathway must occur to decrease the amount and/or concentration of unfolded proteins accumulated in the ER. In contrast, mammalian cells are probably able to deal with the accumulation of unfolded proteins in the ER without the participation of organelles other than the ER. The ATF6 pathway-mediated augmentation of the folding capacity in the ER and PERK-dependent decrease of the load of proteins in the ER occur simultaneously in response to ER stress. The advent of PERK may have changed the cellular strategy for coping with ER stress.

It should be noted that the 1868 genes analysed in the present study include many cytosolic molecular chaperones [heat-shock proteins and chaperonin containing T-complex polypeptides ('CCTs')] and folding enzymes [FK506-binding proteins and cyclophilins] as well as mitochondrial chaperonins, but none of them were induced significantly (rather suppressed) by tunicamycin treatment, ATF6 α (1–373) expression or amino acid starvation (Figure 3B, group D), implying that there is compartment-specific management of the unfolded proteins that are accumulated in mammalian cells, as shown previously for yeast cells [56]. The reason for down-regulation of transcription of many genes observed in our analysis (Figure 3B) is currently unclear. It may simply be because the relative abundance of their transcripts falls due to induction of particular responsive genes. Alternatively, transcriptional suppression may actively occur in response to each treatment via yet unknown mechanisms.

Recently, a third signalling pathway was found to play an important role in transcriptional regulation during the UPR in metazoan cells, namely the IRE1/*XBPI* pathway; *XBPI* mRNA encoding the bZIP transcription factor *XBPI* is spliced by IRE1

to remove a small intron in response to ER stress in both worm and mammalian cells [38,55,57]. This unconventional (spliceosome-independent) mRNA splicing causes C-terminal replacement of *XBPI* ('frame switch' of *XBPI* mRNA-encoded open reading frames), resulting in production of a highly active transcription factor [57]. Targets of this 'frame switch' splicing pathway should overlap with targets of the ATF6 pathway, partly because both *XBPI* and ATF6 bind to the *cis*-acting ERSE, and therefore expression of the spliced form of *XBPI* should up-regulate transcription of genes encoding ER-resident molecular chaperones and folding enzymes, as in the case of expression of pATF6 α (N) and pATF6 β (N). Nonetheless, the presence of *XBPI*-specific target genes is predicted, since *XBPI*, but not ATF6, can bind efficiently to the *cis*-acting mammalian UPRE [57], which was identified previously artificially by *in vitro* binding site selection experiments and called the ATF6 site [30]. In this sense, the IRE1 pathway may be involved in the induction of *SERCA2*, because the induction of *SERCA2* by tunicamycin treatment did not appear to be fully explained by the ATF6 pathway (Figure 3A), and we found that sequences that closely resemble the mammalian UPRE (TGACGTGG/A) are present in the promoter region of *SERCA2* (TGACGAGG from –1018 to –1024 and TGACGTGC from –1617 to –1611). However, almost all human ER stress-inducible genes were categorized into three groups (A, B and C) in our analysis, suggesting that the IRE1/*XBPI* pathway-specific target genes, other than *SERCA2*, were not included in the approx. 1800 genes that we analysed in the present study. A much more extensive search will be required to identify the role of the IRE1/*XBPI* pathway in the mammalian UPR.

In conclusion, we have succeeded in identifying and characterizing target genes of the ATF6 pathway and showed that they encode proteins necessary to cope primarily with ER stress. In addition, we provided evidence suggesting that the PERK pathway is responsible for ER stress-induced transcriptional induction of not only *CHOP*, but also many other genes that carry AARE sequences in their promoter regions and which may play an important role in the recovery step from ER stress. Thus the ATF6 and PERK pathways possess clearly distinct roles in the mammalian UPR. We propose that mammalian cells have gained versatility by evolving three different *cis*-acting elements (ERSE, UPRE and AARE) and three different signalling pathways (ATF6, IRE1/*XBPI* and PERK/ATF4) for the transcriptional induction systems that are activated during the UPR.

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