IGFBP-1 regulation by ER stress is mediated by ATF4

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Running title: IGFBP-1 regulation by ER stress is mediated by ATF4

Endoplasmic reticulum stress induction of insulin-like growth factor binding protein-1 involves ATF4

Endoplasmic reticulum (ER) stress is sensed by cells in different physiopathological conditions in which there is an accumulation of unfolded proteins in the ER. A coordinated adaptive program called the unfolded protein response is triggered and includes translation inhibition, transcriptional activation of a set of genes encoding mostly intracellular proteins and ultimately apoptosis. Here we show that insulin-like growth factor binding protein-1 (IGFBP-1), a secreted protein which modulates IGF bioavailability and has other IGF-independent effects, is potently induced during ER stress in human hepatocytes. Various ER stress inducing agents were able to increase IGFBP-1 mRNA levels, as well as cellular and secreted IGFBP-1 protein up to 20 fold. A distal regulatory region of the human IGFBP-1 gene (-6682/-6384) containing an activating transcription factor 4 (ATF4) composite site was required for promoter activation upon ER stress. Mutation of the ATF4 composite site led to the loss of IGFBP-1 regulation. EMSA revealed an ER stress inducible complex which was displaced by an ATF4 antibody. Knockdown of ATF4 expression using two specific siRNAs impaired upregulation of IGFBP-1 mRNA, which highlights the relevance of ATF4 in endogenous IGFBP-1 gene induction. We conclude that, in addition to intracellular proteins involved in secretory and metabolic pathways, ER stress induces the synthesis of secreted proteins. Increased secretion of IGFBP-1 during hepatic ER stress may thus constitute a signal to modulate cell growth and metabolism and induce a systemic adaptive response.

The endoplasmic reticulum (ER) is the site of synthesis, folding and modification of secretory and cell surface proteins as well as the resident proteins of the secretory pathway. Perturbations that alter ER homeostasis lead to the accumulation of unfolded proteins which are detrimental to cell survival. To alleviate this stressful condition, the cell has evolved a coordinated adaptive program called the Unfolded Protein Response (UPR) (1-3). The most immediate response associated with ER stress is transient attenuation of protein synthesis to decrease the load on the ER (1,4,5). The second part of the response consists in specifically upregulating ER protein folding, ER-associated degradation (ERAD) efficiencies, and expression of a set of genes involved in the general adaptation to stress. Target genes include molecular chaperones such as BiP, GRP94, calreticuline (6), ERAD components such as EDEM (7) , genes involved in amino acid metabolism and resistance to oxidative stress (8)… But in addition to the recovery from ER stress, the UPR initiates proapoptotic pathways which could lead to programmed cell death if the stress is sustained (9).

The diversity of these responses is mediated by three ER transmembrane transducer proteins which sense the accumulation of unfolded protein in the ER lumen and activate signaling pathways. These transducers include the precursor form of the activating transcription factor 6 (ATF6) and two kinases : the double-stranded RNA-activated protein kinase-like ER kinase (PERK) and the kinase/endoribonuclease IRE1 (10). Upon ER stress, PERK phosphorylates the alpha subunit of eukaryotic initiation factor-2 (eIF2α) which reduces the level of eIF2-GTP available for translation initiation, leading to a general attenuation of translation (5). Paradoxically, eIF2α phosphorylation also increases translation of selective mRNAs such
as the mRNA coding for the bZIP activating transcription factor 4 (ATF4) which regulates the expression of some UPR target genes (8). Thus, by phosphorylating eIF2α, the PERK pathway regulates both translation and transcription during ER stress. The second transmembrane ER kinase, IRE1, is an endoribonuclease that splices XBP1 (X-box binding protein-1) mRNA in response to ER stress; this generates the potent bZIP transcription factor, XBP1, which regulates the transcription of a set of UPR target genes (11). Activation of the third transmembrane ER transducer, the precursor form of ATF6, in response to ER stress leads to its transport to the Golgi apparatus, where it is cleaved, releasing a cytoplasmic fragment, the bZIP transcription factor called ATF6 which activates transcription of UPR target genes (11).

The three signaling pathways of the UPR allow the regulation of gene expression by three transcription factors: ATF4, XBP1 and ATF6 which bind to different cis-acting elements. ER stress response element (ERSE, CCAAT-N9-CCACG) bind both XBP1 and ATF6 factors in the presence of the general transcription factor nuclear factor-Y (NF-Y) (12,13). UPRE sequences (consensus: TGACGTGG/A) specifically binds XBP1 without assistance of NF-Y (7,12). ATF4 composite sites (consensus: R/CTTR/TCRTCA, R=G or A) (14) include a set of sequences called amino acid response element (AARE) in the CHOP promoter (15,16), nutrient stress response element-1 (NSRE-1) in the asparagine synthetase gene (ASNS) (17), C/EBP-ATF composite site in the Herp promoter (18) and ATF site in the GADD34 gene (19). In the CHOP promoter, the ATF4 composite site binds ATF4 in combination with ATF2 (16); in the ASNS promoter, the NSRE1 site binds different transcriptional factors with a sequential order (20): during the initial phase following the stress (amino acid deprivation), NSRE1 mainly binds ATF4 and ASNS gene transcription is increased. Subsequently, binding of C/EBPβ and ATF3-full length to NSRE1 increased and ASNS transcription declined.

The three signaling pathways differ in their activation pattern: while Ire1/XBP1 and ATF6 pathways are specific to ER stress, the PERK pathway shares eIF2α’s phosphorylation and the induced « integrated stress response » (IRS) with unrelated stress : amino acid deprivation, viral infection and heme deficiency which activate specific kinases (8,21).

UPR target genes encode mostly intracellular proteins which carry biological functions helping the cell to cope with the accumulation of unfolded proteins or leading to cell death (8). It is unclear whether the UPR also includes the activation of signals involved in cell-cell communication. The possibility that secreted proteins expression could be regulated by ER stress is an important issue, since, in this case, the stressed cell would communicate the information to other cells and trigger a response at the tissue or systemic levels.

Insulin-like growth factors binding proteins (IGFBPs) are a family of secreted proteins which bind insulin-like growth factors (IGFs) with high affinities. The concentration of free IGFs is believed to represent the primary determinant of the tissue response to IGFs: free IGF bind to the IGF type I receptor and modulate developmental growth and metabolism. By regulating IGFs transport and half-life, IGFBPs modulate IGFs bioactivity. But IGFBPs also act on cell migration, growth and death by interacting with cell-surface, extracellular or intracellular partners (22).

IGFBP-1 displays tissue-specific expression; it is mostly secreted by hepatocytes, decidualized uterine endometrium, ovarian granulosa cells and kidney (23). IGFBP-1 acts both as an “endocrine” and an “autocrine/paracrine” factor. Although all the functions of IGFBP-1 are not understood, IGFBP-1 is kown to influence glucose homeostasis and to play a role in the female reproductive functions (23,24). Indeed, various transgenic mice overexpressing the IGFBP-1 gene consistently display impaired glucose tolerance and abnormalities in insulin action in addition to alterations of reproduction, intrauterine and postnatal growth restrictions (25-29). Gene knock-out studies in mice suggested another function of IGFBP-1 in the liver; IGFBP-1 may act as a pro-mitogenic and a protective protein of the injured liver probably through an IGF-independent mechanism (30,31)

IGFBP-1 differs from the other IGFBPs in its rapid regulation by metabolic status; IGFBP-1 serum level decreases following food intake and increases between meals; insulin down-regulation of IGFBP-1 gene is believed to be responsible for this daily fluctuation (32). In contrast, increased concentration of glucocorticoids and proinflammatory cytokines are believed to strongly regulate IGFBP-1
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In addition to these regulations, amino acid depletion and hypoxia were shown to upregulate IGFBP-1 gene expression (36,37). Furthermore, we have recently shown that the environmental contaminant dioxin induced IGFBP-1 gene expression (38). During the course of exploring the mechanisms of dioxin action, in particular the contribution of induced ER-localized cytochromes P450, we observed a potent induction of IGFBP-1 upon ER stress. We show here that IGFBP-1 is highly induced during ER stress in human hepatocytes; both mRNAs and secreted proteins are induced up to 20-fold by different chemicals inducing the UPR. This induction requires the transcription factor ATF4 which binds to a distal regulatory region of the human IGFBP-1 gene promoter.

MATERIALS AND METHODS

Chemicals. All chemical products including tunicamycin, brefeldin A and thapsigargin were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France). Oligonucleotides were obtained from Qiagen (Les Ulis, France) and siRNA from Eurogentec (Angers, France).

Cell culture. Human hepatocarcinoma HepG2 cells were cultured at 37°C in 50% Dulbecco’s minimal essential medium complemented with non essential amino acids and 50% Ham’s F-12 medium, supplemented with 10% fetal bovine serum, 200 U/ml penicillin, 50 µg/ml streptomycin (Invitrogen, Cergy-Pontoise, France) and 0.5 mg/ml amphotericin B (Bristol-Myers Squibb Co, Stamford, CT) in a humidified atmosphere in 5% CO2. Human hepatocytes were a kind gift from Dr. P. Maurel (Institut National de la Sante et de la Recherche Medicale 128, Montpellier, France) and were maintained as previously described (39).

Northern blots. Total RNA were isolated using the RNeasy kit from QIAGEN. Northern Blots were performed using 10 µg of total RNA per lane. The probes used to detect IGFBP-1, BiP and GRP94 mRNAs were previously described (38). Herp probe was isolated by reverse transcription of HepG2 RNAs and specific amplification by PCR using the following oligonucleotides : Herp forward: 5’-CTTCCAAAGGAGGAAAAACG-3’ and reverse 5’-TCCATCCAAACATCTG-3’. Real time quantitative RT-PCR measurements were performed on an ABI Prism 7900 Sequence Detector system (Applied Biosystems). PCR cycles proceeded as follows: Taq activation (15 min), denaturation (15 s, 95°C), annealing (30 s, 60°C) and extension (30s, 72°C). The relative mRNA levels were estimated by the standard method using ribosomal protein L13a as the reference gene.

Cellular and medium protein extracts. Ten million HepG2 cells were treated with 2µg/ml tunicamycin, 0.25 µM thapsigargin, 0.25 µg/ml brefeldin A or the appropriate vehicle (ethanol or dimethyl sulfoxide (DMSO) ) in 6 ml of serum-free medium over 24 h. Culture supernatant containing secreted IGFBP-1 were saved, and protein extracts from cells were prepared. Cells were washed twice with Hanks’ balanced salt solution and centrifuged at 1500 rpm for 5 min. The pellet was resuspended in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA containing an antiprotease inhibitor cocktail tablet (Roche Diagnostics, Meylan, France) and lysed by sonication 30 s with a Vibracell (Fisher Bioblok Scientific, Illkirch, France). Centrifugation for 20 min at 15,000 rpm was performed for 45 min at 65°C with 2X standard saline citrate and 0.1% SDS and for 35 min with 0.5X standard saline citrate and 0.1% SDS. Quantifications were performed with a PhosphorImager and the ImageQuant software (Amersham Biosciences).

Real time quantitative RT-PCR. Reverse transcription was performed on each RNA sample (2 µg) using the cDNA High-Capacity Archive kit from Applied Biosystems (Courtaboeuf, France) in a final reaction volume of 50 µl. Sequences of oligonucleotides were previously described in (38) except for CHOP (16). Additional oligonucleotides are: GRP94 forward 5’TTGGTGTCGGTTCTATCC and GRP94 reverse GCTGGGTATCGTTGTGGT ; Herp forward 5’-CTCCAGGCCCTATTCAACAC-3’ and reverse 5’-TCCAATCCAACCAATCTCG-3’ and EDEM forward 5’-GAAATGAAAGGGGACAGAAG-3’ and reverse 5’-GCCAGCAAAGTGAAAGAC-3’.

Membranes were washed for 45 min at 65°C with 2X standard saline citrate and 0.1% SDS and for 35 min with 0.5X standard saline citrate and 0.1% SDS. Quantifications were performed with a PhosphorImager and the ImageQuant software (Amersham Biosciences).
then performed and the pellets were resuspended in 200 µl of 100 mM NaPO₄, 10 mM MgCl₂ and 20% glycerol pH 7.4. Protein concentration of cell lysates and medium extracts were measured using the BCA protein assay reagent (Pierce, Rockford, IL) and bovine serum albumin as a standard.

**Western blots.** Each sample (40 µg cellular protein extracts or 20 µg of culture cell medium) in 10% glycerol, 75 µM Tris pH 6.8, 1% SDS and 0.005% pyronine were run in a 9% polyacrylamide gel. Blots were incubated 3 h in phosphate buffer saline/0.05% Tween-20 with 1% polyvinyl pyrrolidone (Sigma-Aldrich) and then overnight at 4°C with either a mouse monoclonal anti-human IGFBP-1 (Mediagnost, Reutlingen, Germany) or a goat polyclonal BiP antibody (tebu-bio, Le Perray en Yvelines, France).

**Cloning of IGFBP-1 and BiP genes fragments and plasmid construction.** The first 1205-bp fragment of the human IGFBP-1 promoter was already cloned and sequenced (40). Comparison of this sequence with the GenBank database (BLAST) allowed us to identify BAC number RP11-132L11 (GenBank accession number AC091524) which contains 7400 bp upstream of the transcription initiation site of the human IGFBP-1 gene. Using this sequence, we designed oligonucleotides that allowed the amplification of various fragments of the promoter. Nucleotide numbering represents the distance 5' (negative) or 3' (positive) to the mRNA capsite (nucleotide 1).

| Oligonucleotide   | Sequence                        |
|------------------|--------------------------------|
| OL+1135:5'-...-3' | TCACTGTTTCTTAGGGGGGCAAC-3'    |
| OL+493:5'-...-3' | CTTGCGTGCAGGAGTCTGA-3'        |
| OL+493:5'-...-3' | CTTGCGTGCAGGAGTCTGA-3'        |
| OL-1618:5'-...-3'| AAGGTAGCCCCAGGATTTAT-3'       |
| OL-2523:5'-...-3'| GGAACCTAGCAACAGGGTTTTC-3'     |
| OL-2644:5'-...-3'| TTGCAAGCTGAGGAAGTCAG-3'       |
| OL-3778:5'-...-3'| TGGTCTTGGAGGGTAAGTGG-3'       |
| OL-3966:5'-...-3'| CCTGCCTGTTATTCCAGCTC-3'       |
| OL-4030:5'-...-3'| CACCCACCACAGGGGTATAA-3'       |
| OL-4429:5'-...-3'| TTCCCTCTTCTTCTCATCTCT-3'      |
| OL-6682:5'-...-3'| CAGGGACACCTCAGAAAGGA-3'       |
| OL-6819:5'-...-3'| GCTTTGGCCTGGACACAT-3'         |
| OL-7129:5'-...-3'| GTGCCTGTTTTGGCAGGATGT-3'      |

PCR fragments were generated using the HotStar TaqDNA polymerase (QIAGEN) and HepG2 genomic DNA as a matrix and subcloned into pGL3 Basic vector (Promega, Charbonnieres, France). Fragments +493/+1135 ; -3966/-2523 ; -4429/-3778 ; -6819/-4030 ; 7129/-6288 ; -6682/-6384 were sequenced. The sequence correspond to the one present in the GeneBank database (accession numbers AC091524 and AY434089).

A fragment of the Human BiP promoter containing the proximal ERSE motifs was also generated by PCR and subcloned into PGL3 basic vector : p-339/+41-BiP-FL.

**Site-directed mutagenesis.** The human IGFBP-1 promoter was mutated between nucleotides -6629 and nt -6622 (UPRE 2 motif) or between nt -6481 and nt-6473 (ATF4 composite site) (see sequences on figure 5B) by a two step PCR to give rise respectively to p-6682/-6384-mutUPRE2-TATA-FL and p-6682/-6384-mutATF4-TATA-FL.

**Transient transfection experiments.** Expression vector of human ATF4 (pMycATF4) and its control vector (pCMV5myc vector) were generous gifts from Dr A. S. Lee (41). Expression vector of the human α1-antitrypsin mutant Hong-Kong, pA1ATΔTC was a kind gift from Dr N. Hosokawa (42).

HepG2 cells (4.10⁵ cells/well of six-well plate) were transfected in triplicate by the calcium phosphate coprecipitation technique (2 µg of plasmid-FL/well) except that glycerol shock was omitted. Thirty hours later, cells were treated with DMSO or tunicamycin 2 µg/ml over 16 h and cells were lysed in 200 µl of 1X passive lysis buffer (Promega). Firefly luciferase was assayed with the Promega kit. When cotransfection experiments were performed, either 500 ng of pMycATF4 or pCMV5myc or 2 µg of pA1ATΔTC vectors were included in the precipitate and firefly luciferase was assayed 40 h after transfection.

**Electrophoretic Mobility Shift Assay.** Eight millions HepG2 cells were treated with 2µg/ml tunicamycin or DMSO for 5 h. Nuclear extracts were prepared as previously described (38).

Synthetic double-stranded DNA probes (4 pg) were labeled with [α-32P]dCTP (Amersham Biosciences) and the large Klenow fragment of DNA Polymerase I (Ozyme, Saint Quentin en Yvelines, France). HepG2 nuclear
extracts (10 µg) were preincubated on ice for 15 min in the presence of 3 µg poly(dI-dC) (Amersham Biosciences) in a reaction mixture containing 25 mM Hepes pH 7.9, 60 mM KCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 0.75 mM DTT, 1 mM PMSF and 5% glycerol in the presence or absence of a 75-fold molar excess of the unlabeled competitors. Fifty femtomoles (100,000 cpm) of the labeled probe was then added and incubated for further 15 minutes. To test the effect of specific antibodies, 1 µg ATF4 antibody (also named CREB-2, tebu-bio) or IgG control was added to the incubation mixture on ice 2 h prior to the addition of the labeled probe. DNA-protein complexes were separated for 2 hours at 4°C on a prerun (30 min) 6% (w/v) polyacrylamide gel containing 2.5% glycerol, with 1X TGE  (25 mM Tris base, 190 mM glycine, 1 mM EDTA pH 8.5) as a running buffer.

The double stranded DNA sequences used in this study contained the putative wild type ATF4 composite site of human IGFBP-1 gene, -6484/-6464 AGATTTACATCATCCCCTG (ATF4-IGFBP-1), the mutated ATF4 composite site of human IGFBP-1 gene AGATAGCGCAACTCCCCTGTG (mutATF4-IGFBP-1), ATF4 composite site of human CHOP gene (AARE-CHOP) (16) and a consensus sequence which binds the transcription factor SP1 (GGATTCGATCGGGGCGGGGCGAGC) (SP1).

SiRNA knock-down studies. Sequences of anti-ATF4 siRNA (called hereafter siRNA A) and the mutated siRNA (siRNA mutA) were previously published (16) and erratum in J. Biol. Chem. 2004 Apr9; 279(15):15701. A second siRNA directed against ATF4 (called siRNA B) was selected according to similar criteria as siRNA A. siRNA B1 : 5'-GCACUUCACACUACUAGGdtdTdT-3'; siRNA B2: 5'-CCCCUGAGGUUGAAGUGCdTdT-3'.

One day before transfection with siRNA, HepG2 cells were plated on 6 well plates (500 000 cells /well). Then, 2 µg of siRNA were introduced into the cells using the calcium phosphate method as described above. Thirty hours later, cells were treated for 16 h with DMSO or 2 µg/ml tunicamycin and mRNA levels were measured by real time quantitative RT-PCR.

RESULTS

ER stress induces IGFBP-1 mRNA in primary cultures of human hepatocytes. The mRNAs of primary cultures of human hepatocytes treated or not for 24 h with 2 µg/ml tunicamycin or DMSO were analyzed by Northern blot using probes for IGFBP-1, for markers of ER stress: the chaperones BiP, GRP94 and Herp (involved in ERAD) as well as for 18S ribosomal RNA. Figure 1A shows that tunicamycin potently increased the mRNA levels of IGFBP-1 as well as those of the three ER stress markers. A 14-fold induction of IGFBP-1 was observed in Northern blot experiments whereas classical ER stress-sensitive mRNAs were induced 4.5- to 8-fold. Data from Northern blot analysis were confirmed by real time quantitative RT-PCR. Tunicamycin was shown to increase IGFBP-1 mRNA 25-fold; the other ER stress inducible mRNAs were induced 7- to 20-fold (figure 1B).

We then asked whether other components of the IGF/IGFBP system were regulated by tunicamycin. The mRNA levels of other IGFBPs expressed in human hepatocytes, IGFBP-2 and IGFBP-4, were not modified while the acid-labile subunit expression (a protein which interacts with IGFBP-3 and IGFBP-5 to form stable ternary complex with IGFs in serum) was induced about 2-fold (figure 1B). IGF-1 mRNAs were decreased by approximately 70% whereas IGF-II and IGF receptor type I (IGF-IR) mRNAs were not regulated. Thus, the potent induction of IGFBP-1 mRNAs by tunicamycin in hepatocytes is specific among the IGF/IGFBP system and seems to be associated with a decrease of IGF-I mRNAs.

Diverse ER stress inducers increase IGFBP-1 mRNA and protein levels in HepG2 cells. The effect of three different ER stress-inducers was tested on IGFBP-1 mRNA levels in the human hepatocarcinoma HepG2 cells. After a 24 hours treatment by tunicamycin (2 µg/ml), thapsigargin (0.25 µM) and brefeldin A (0.25 µg/ml) or the appropriate vehicle (DMSO for tunicamycin, thapsigargin, ethanol for brefeldin A), mRNAs were prepared and Northern blot hybridizations were performed. As shown in figure 2A, all treatments elicited a several-fold induction of IGFBP-1. Three ER-stress markers, BiP, Herp and GRP94 were used to evaluate the efficiency of the different treatments to produce an ER stress. Semi-quantifications of three different Northern blot experiments confirmed the induction of IGFBP-1 by all treatments with similar efficiencies (about 20-fold, data not shown).
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All treatments elicited an induction of the ER stress-markers expression; the magnitude of the inductions varied from 3 to 19-fold. In conclusion, these data show that IGFBP-1 mRNA level is very potently induced by ER stress in both primary cultures of human hepatocytes and the HepG2 hepatoma cells. Time course studies revealed that the induction of IGFBP-1 by tunicamycin (2 µg/ml) could be detected 10 hours following addition of the drug and was maximal at 16 hours. At this time point, dose response studies showed that increased IGFBP-1 expression was clear at 1 µg/ml tunicamycin and maximal at the concentration of 2 µg/ml (data not shown).

The regulation of IGFBP-1 protein synthesis was assayed by Western blot. As shown in figure 2B, after a 24 hours-treatment by the three different ER stress inducers, IGFBP-1 protein levels were highly upregulated in the cells. Moreover, the levels of IGFBP-1 protein in the culture medium were similarly elevated by tunicamycin and thapsigargin treatment. Thus, IGFBP-1 is translated and secreted during ER stress. When cells were treated with brefeldin A, a chemical which disrupts the structure of the Golgi apparatus and blocks protein secretion, no IGFBP-1 protein is detected in the medium. The expression of the ER resident chaperon BiP was also evaluated. As expected, the three ER stress inducers produced an induction of BiP expression, but the increase in IGFBP-1 protein was more potent than that of the BiP protein. Since BiP is localized in the ER and is not secreted, BiP expression was not observed in the medium. The absence of BiP in the culture medium also proves that the medium was not contaminated by cellular proteins. In conclusion, both cellular and secreted IGFBP-1 is highly increased during ER stress.

A distal fragment of the human IGFBP-1 promoter confers ER stress responsiveness. Various cis-elements are known to confer ER stress sensitivity to UPR target genes: ERSE, UPRE and ATF4 composite sites. We searched for these regulatory elements within 10kb upstream the initiation transcription site and within the first intron of the IGFBP-1 gene. As shown in figure 3A, two UPRE sites strictly identical to the consensus UPRE sequence are located at positions -4143/-4136 (UPRE1) and -6629/-6622 (UPRE2) and one ATF4 composite site, very similar to the ATF4 binding consensus sequence, was located at position -6480/-6469 (see sequences on figure 4A). Since other poorly conserved sequences could mediate IGFBP-1 transactivation, we cloned genomic fragments covering 7129 pb of the IGFBP-1 promoter and 642 bp of intron 1 (which contains the hypoxia responsive elements) (37). Each fragment of the human IGFBP-1 gene was subcloned upstream a firefly luciferase reporter gene in either pGL3-basic vector or the pGL3-basic vector containing a TATA box (pTATA-FL, see Material and Methods).

HepG2 cells were transiently transfected with the recombinant plasmids, treated with DMSO or tunicamycin for 16 hours, and luciferase activities were assayed. A plasmid containing the first 339 bp of the BiP promoter was used as a control of tunicamycin effect. As shown in figure 3B, the BiP promoter is activated about 3-fold by tunicamycin. Fragments of IGFBP-1 promoter up to -4429 kb did not mediate activation of the reporter gene by tunicamycin. The +493/+1135 sequence of intron 1 also did not display any significant regulation. Three fragments were able to mediate induction of the reporter gene after tunicamycin treatment: fragments -6819/-4030, -7129/-6288 and -6682/-6384 which produced an increase in promoter activity of 3-, 10- and 18-fold respectively (figure 3B). Interestingly, these three fragments comprised the second UPRE and the putative ATF4 composite site.

We focused our following studies on the -6682/-6384 fragment which mediated the highest induction by tunicamycin. We first determined the functional contribution of the ATF4 composite site and the UPRE site by targeted mutation of these responsive sequences (figure 4B). ER stress was mediated either by tunicamycin treatment or cotransfection with a plasmid encoding the α1-antitrypsin folding-incompetent Null Hong-Kong (NHK) variant. As shown in figure 4C, mutation of the putative UPRE site (p-6682/-6384-UPRE2mut-TATA-FL) did not significantly affect IGFBP-1 transactivation by tunicamycin treatment or α1-antitrypsin NHK expression. In contrast, mutation of the putative ATF4 composite site (p-6682/-6384-mutATF4-TATA-FL) led to the loss of the regulation of the IGFBP-1 promoter by tunicamycin treatment or α1-antitrypsin NHK expression. These data show a critical role of the ATF4 composite site in ER stress induction of the IGFBP-1 promoter.

Role of the transcription factor ATF4 in IGFBP-1 promoter transactivation. ATF4 composite sites present in CHOP, ASNS, Herp
and GADD34 promoters have in common the property to bind the stress sensitive factor ATF4. The ATF4 composite site found in the IGFBP-1 promoter is identical to the consensus sequence except for the first position in which a T is present instead of a G, A or C (see figure 4A). In order to establish whether this site can be activated by ATF4, the p-6682/-6384-FL vector or the mutated vectors were cotransfected with the ATF4 expression vector (pMycATF4) or the mock vector (pCMV5Myc) (figure 5). Expression of ATF4 induced p-6682/-6384-FL promoter activity about 10-fold. Although the mutation of the UPRE2 motif did not alter ATF4 induction of the IGFBP-1 promoter, mutation of the ATF4 composite site completely abolished this transactivation. These data show that ATF4 transactivates the IGFBP-1 gene promoter through the ATF4 composite site.

Binding of the transcription factor ATF4 to the ATF4 composite site of IGFBP-1. To examine whether ATF4 directly binds to the IGFBP-1 promoter upon ER stress, electromobility shift assays were carried out using a probe encompassing the putative ATF4 composite site named ATF4-IGFBP-1 oligonucleotide. The ATF4-IGFBP-1 oligonucleotide was labeled and incubated with nuclear extracts prepared from HepG2 cells treated or not with 2 µg/ml tunicamycin for 5 hours. In untreated cells, diffuse shifted bands could be detected and probably correspond to low affinity complexes (figure 6 lane 1). Upon tunicamycin treatment, one major DNA-protein complex was observed (lane 2). This complex was competed out by a 75-fold excess of homologous unlabeled probe (lane 3) or a 75-fold excess of AARE-CHOP oligonucleotide (which contains the ATF4 composite site of the CHOP promoter) (lane 4). In contrast, the complex was not displaced by an oligonucleotide mutated on the ATF4 composite sequence (lane 5) or by an oligonucleotide that binds the ubiquitous transcription factor SP1 (lane 6). Furthermore, this complex disappeared when the extracts were incubated with an anti-ATF4 antibody and a faint supershifted band appeared (lane 8). These data were confirmed in two additional experiments. We conclude that ATF4 is the major factor able to bind to the IGFBP-1 ATF4 composite site in stressed cells. We next compared the binding properties of the CHOP and IGFBP-1 ATF4 composite sites. The oligonucleotide AARE-CHOP is known to bind different proteins including ATF2 and ATF4 (15,16). Three major DNA-protein complexes were obtained using AARE-CHOP oligonucleotide as a probe and tunicamycin nuclear extracts. But among these complexes, only one was induced by tunicamycin (lane 10, fastest migrating complex). The inducible complex migrates similarly to the complex observed with ATF4-IGFBP-1 probe and is the only one to disappear in the presence of the anti-ATF4 antibody (lane 13). Therefore, in contrast to AARE-CHOP, which binds other proteins even under basal conditions, the IGFBP-1 ATF4 composite site binds mainly ATF4 upon ER stress.

Effect of specific inhibition of ATF4 synthesis on ER stress induction of IGFBP-1 using siRNAs. We next assessed the role of ATF4 in upregulation of IGFBP-1 mRNA levels upon ER stress using two different small interfering double-stranded RNA directed specifically against ATF4 mRNA (siRNA A and B) as well as a non silencing mutated A siRNA (siRNA mutA). SiRNAs A, B or mutA were introduced into HepG2 cells using the calcium phosphate method. Twenty four hours later, cells were treated or not with 2 µg/ml tunicamycin for 16 hours and the mRNA levels coding for three genes were measured: ATF4 (the siRNA’s target), IGFBP-1 and EDEM, a gene which is known to be regulated by the transcription factor XBP-1 upon ER stress (7). As shown in figure 7A, both siRNAs A and B knocked-down ATF-4 expression in basal and tunicamycin conditions while siRNA mutA had no effect. Since we have shown that ATF4 is involved in the induction of IGFBP-1 by ER stress, we tested the effect of the various siRNAs on the induction of this gene by tunicamycin. As shown in figure 7B, both siRNA A and siRNA B led to a 2- to 3-fold decrease in the induction of IGFBP-1 by tunicamycin, while siRNA mutA had no effect. Furthermore, anti-ATF4 siRNA did not significantly interfere with the induction of EDEM mRNA levels, which highlights the specificity of the siRNAs effects (figure 7C).

DISCUSSION

In the present study, we have shown that cellular and secreted IGFBP-1 are highly induced upon ER stress in human liver-derived cells. We were particularly interested in this observation because IGFBP-1 is a secreted protein involved in signalling. Indeed, most of the UPR target genes encode intracellular proteins which allow the cell to cope with stressful conditions. Induced proteins are
involved in folding, secretion, ERAD and quality control and increase the capacity of the secretory pathway (43-45). Increased levels of proteins involved in amino acids metabolism, transport and redox control help the cell to adapt to the metabolic consequences of a high ER activity (8). But not all UPR target promote cell survival; indeed, the pro-apoptotic CHOP gene is also induced. Only few UPR target genes encoding secreted or membrane proteins have been characterized. The vascular endothelial growth factor (VEGF), a pro-angiogenic factor, was shown to be induced by ER stress in retinal epithelial cells: VEGF mRNA was induced up to 10-fold but the synthesis and secretion of the protein were modestly induced (up to 1.8 fold) (46). Expression of membrane transporters for cystine and glycine were also shown to be induced at the mRNA level but nothing is known about the protein levels (8). Interestingly, a recent study of gene expression upon diverse stresses (heat shock, ER stress, oxidative stress, crowding) in cultured human cells showed that most of the genes induced by multiple stresses are involved in cell-cell communication suggesting a coordinated global response (47). Since IGFBP-1 acts as an endocrine and an autocrine-paracrine factor, its induction and secretion upon ER stress could contribute to such a response and inform the organism of the stressed state of the liver.

IGFBP-1 was known to be induced during glucose deprivation of human hepatocytes (48). Our study provides mechanistic data for those initial observations. Indeed, proper protein folding requires extensive energy and is intimately coupled with asparagine-linked glycosylation. Since glucose deprivation reduces the amount of energy available and alters N-linked glycosylation, it can lead to protein accumulation in the ER and induce ER stress. Thus, ER stress may be one pathway by which glucose limitation induces IGFBP-1 expression.

Most of ER stress responsive genes are ubiquitously expressed which is in line with the essential function of the UPR in the adaptation to the toxicity of unfolded proteins. IGFBP-1 differs from other UPR target proteins in that it is tissue-specifically expressed; the major sources are the liver and the endometrium (23). The regulation of a tissue specific factor suggests that the UPR displays tissue specific responses and that the consequences of the ER stress on the organism may differ according to the nature of the stressed tissue. Protein secretion is an important function of the liver (due to the large amount of serum proteins synthesized); it is thus possible that specific regulatory mechanisms are required in this organ. Other tissue specific characteristics of the UPR have already been described such as the abundant expression of PERK in secretory and endocrine organs (pancreatic cells and the osteoblasts) and the exclusive expression of the ER transmembrane transducer IRE1β in the gut epithelial tissue (49). However, those tissue specific aspects of the UPR have not been extensively explored so far.

We showed that ATF-4 is critical for the induction of IGFBP-1 during ER stress. Indeed, both site-directed mutagenesis of the ATF4 DNA binding site and the knock-down of ATF4 expression potently decreased the induction of IGFBP-1 upon ER stress. Moreover, no ER stress specific element (such as ERSE or UPRE) appears to be involved in IGFBP-1 regulation: among the cloned 7.12 kb of the human IGFBP-1 gene promoter, no ERSE was found and the two identified UPREs were not functional under our conditions. These data show that IGFBP-1 induction upon ER stress involves ATF4; other factors may also contribute to this regulation as has been shown in the case of other genes. Thus, similarly to the GADD34 gene (19), IGFBP-1 induction upon ER stress depends on the UPR pathway shared by several stresses. This contrasts with Herp and CHOP genes regulations which are mediated by both the shared and the ER stress specific pathways of the UPR (18) or with the regulation of genes involved in ER specific functions (EDEM, Erdj4, RAMP4...) which is mediated by the ER stress specific pathway (44,50). Moreover, these data highlight the contribution of a distal region in the regulation of IGFBP-1 by ER stress, which is distinct from other ER stress regulatory elements that are usually located in the proximal promoter region. In the case of the human C/EBPα, the ER stress responsive element is located downstream of the protein coding sequence (51).

IGFBP-1 expression is increased upon amino acid starvation, a stress which also activates the ATF4 pathway. A recent study performed by Averous et al. showed that IGFBP-1 induction upon amino acid depletion involves both mRNA stabilization and transcriptional activation and does not involve the ATF4 pathway (52). Using the CMV-IGFBP1-tag plasmid kindly provided by Dr P.
Fafournoux, we found that ER stress does not stabilize IGFBP-1 mRNA (data not shown). Thus, ER stress and amino acid depletion upregulate IGFBP-1 expression using different mechanisms. Moreover, transcriptional regulation of IGFBP-1 by these two stress conditions relies on different transcription factors since ATF4 appears not be involved in the amino acid depletion effect. Another factor may be more essential in this stress condition.

Genes regulated by the pathway shared by diverse stresses encode a large variety of functions: they are involved in amino acid import as well as in glutathione biosynthesis and resistance to oxidative stress (8). Various observations suggest that the integrated stress response is aimed at providing resistance to stressful conditions and at promoting cell survival (53,54). Liver IGFBP-1 and circulating IGFBP-1 are up-regulated in a number of catabolic conditions: malnutrition, liver disease and critical illness (55-57) but little is known about the physiological implication of stress-related induction of IGFBP-1. Since ATF4-target genes are mainly survival genes, this suggests that IGFBP-1 could have such a function under stressful conditions. Acute elevation of circulating IGFBP-1 levels in rats has been shown to decrease protein synthesis in specific muscles tissues thus saving energy for more essential functions (58). Studies performed in zebrafish suggest a contribution of IGFBP-1 to growth retardation and survival under stressful conditions (59). Hypoxia leads to IGFBP-1 induction as well as to embryonic growth retardation and developmental delay. Growth impairment is significantly reduced in IGFBP-1 knock-out animals. These data suggest that stress-triggered induction of IGFBP-1 may divert important energy ressources from growth towards survival metabolic processes; in zebrafish, this is mediated by inhibition of IGF effects (59). However, one might expect that a sustained increase in IGFBP-1 may lead to detrimental effects.

IGFBP-1 has been suggested to play a role in glucose homeostasis by modulating the bioavailability of IGFs which exert insulin-like metabolic functions (60). Several studies using mutant mice have established a connection between ER stress and glucose homeostasis. PERK knock-out -/- mice display hyperglycemia within several weeks after birth; this is mainly due to pancreatic cell death (61). We do not expect the liver encoded IGFBP-1 to have a significant contribution to such a phenotype. In contrast, the knock-in eIF2α mutant mice display severe hypoglycemia 6-9 hours after birth; they are defective in gluconeogenesis and glycogen storage (62). Indeed, the upregulation of phosphoenol pyruvate carboxykinase (a rate-limiting enzyme in gluconeogenesis) which normally occurs in the liver shortly after birth, is prevented in eIF2α mutant neonates. If, as expected, IGFBP-1 regulation is perturbed in eIF2α mutant mice, this may contribute to disrupt glucose homeostasis.

In human, some liver diseases are believed to be accompanied by ER stress. One example is a form of α1-antitrypsin deficiency due to the PiZ variant. The PiZ α1-antitrypsin variant forms large aggregates which are retained in the ER and can induce cirrhosis and liver failure (63,64). Elevated IGFBP-1 levels, if confirmed, may participate in metabolic and signalling perturbations associated with ER stress-related liver diseases.

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The abbreviations used are: ER, endoplasmic reticulum; UPR, unfolded protein response; ERAD, ER-associated degradation; IGFBP-1, insulin-like growth factor binding protein-1; IGF, insulin-like growth factor; ATF, activating transcription factor; AARE, amino acid response element; NSRE, nutrient sensing response element; UPRE, UPR element; ERSE, ER stress response element; XBP1, X-box binding protein 1; BiP, immunoglobulin binding protein; GRP, glucose-regulated protein; ALS, acid labile subunit; DMSO, dimethyl-sulfoxide.; ASNS, asparagine synthetase gene.

FIGURES LEGENDS

Fig. 1. Specific induction of IGFBP-1 mRNA content by tunicamycin in primary cultures of human hepatocytes. Human hepatocytes were treated or not treated with DMSO or 2 µg/ml tunicamycin for 24 h. A. Northern Blot analysis. The blot was hybridized with probes labeling the mRNAs of IGFBP-1, BiP, GRP94 and Herp (three markers of the unfolded protein response) and 18S ribosomal RNA. B. Real time quantitative RT-PCR analysis of components of the IGF/IGFBP system in human hepatocytes. Messenger RNAs encoding IGFBP-1, IGFBP-2 and IGFBP-4 (two other members of the IGFBP family expressed in human hepatocytes), IGF-I, IGF-II, IGF receptor type I (IGF-IR), acid-labile subunit (ALS) and four UPR target genes were studied. The values were corrected using ribosomal protein L13a as the reference gene. Fold induction of mRNA levels in tunicamycin treated cells versus DMSO treated cells is presented. Data shown are the means +/- S.E.M. of three independent RT-PCR experiments.

Fig. 2. Effect of different ER stress inducers on IGFBP-1 expression in HepG2 cells. Human hepatocarcinoma HepG2 cells were treated or not with 2 µg/ml tunicamycin, 0.25 µM thapsigargin, 0.25 µg/ml brefeldin A or their solvents (DMSO for tunicamycin and thapsigargin, ethanol for brefeldin A) for 24 h. A. Levels of IGFBP-1 mRNA (Northern Blots). The blot was hybridized with IGFBP-1, BiP, Herp and GRP94 and with 18S ribosomal RNA probes. B. Levels of intracellular and secreted IGFBP-1 protein levels (Western blots). HepG2 cells were treated with ER stress inducers in a serum free medium so that medium IGFBP-1 solely reflected secreted IGFBP-1. Cellular extracts (40 µg/lane) or culture medium (20 µg/lane) were loaded and the blot was hybridized with anti-human IGFBP-1 and anti-BiP IgG. The experiment was repeated twice with the same results.

Fig. 3. Induction of IGFBP-1 gene expression by ER stress is mediated by the -6682/-6384 fragment of the promoter. A. Schematic representation of the 5’upstream region and the first intron of IGFBP-1 gene. The putative ER stress sensitive sequences (UPRE and ATF4 binding site) are indicated. The different genomic fragments subcloned upstream the firefly-luciferase gene are also represented. B. HepG2 cells were transiently transfected with luciferase reporter gene constructs containing different fragments of the IGFBP-1 promoter or part of IGFBP-1 intron 1. Thirty hours after transfection, cells were treated with DMSO or with 2µg/ml tunicamycin for 16 h and then luciferase activity was assayed. The fold induction of promoter activity by tunicamycin are presented. Data shown are the means +/- SEM of three independent experiments.

Fig. 4. Mutational analysis of the UPRE and ATF4 composite site in the IGFBP-1 gene promoter. A. Alignment of ATF4 composite sites sequences found in different promoters and the C/EBP-ATF consensus site. B. Sequences of wild type and mutated IGFBP-1 ATF4 composite site and UPRE2. C. Transfection of HepG2 cells with pTATA-FL, the native p-6682/-6384-FL or this construct mutated in either UPRE2, p-6682/-6384 UPRE2mut-FL or in the ATF4 composite site, p-6682/-6384 ATF4mut-FL. Thirty hours after transfection, cells were treated with DMSO or with
IGFBP-1 regulation by ER stress is mediated by ATF4

2μg/ml tunicamycin for 16 hours and then luciferase activity was assayed. Similar experiments were performed without tunicamycin but by cotransfecting the pA1ATΔTC encoding α1-antitrypsin NHK variant for 40 h to generate ER stress. The fold induction by either tunicamycin (versus DMSO) or α1-antitrypsin NHK variant overexpression (versus plasmid-FL alone) are presented. Data shown are the means of +/- SEM of four independent experiments.

Fig.5. Role of ATF4 in IGFBP-1 promoter transactivation. Two micrograms of p-6682/-6384-FL vector or of the mutated vectors were cotransfected with 500 ng of ATF4 expression vector (pMycATF4) or the mock vector (pCMV5Myc) in HepG2 cells. Twenty four hours after transfection, luciferase activity was assayed. The fold induction of promoter activity obtained with pMycATF4 compared to promoter activity obtained with the mock vector are presented. Data shown are the means +/- SEM of three independent experiments.

Fig.6. ATF4 binding to the IGFBP-1 ATF4 composite site. Electrophoretic mobility shift assays were performed using a 32P-labeled oligonucleotide containing the ATF4 composite site of the IGFBP-1 gene (ATF4-IGFBP-1) or of the CHOP gene (AARE-CHOP) and 10 μg of HepG2 nuclear extracts (N.E.) prepared after DMSO or tunicamycin (2 μg/ml, 5 h) treatment of the cells. Competition experiments were performed with a 75-fold excess of unlabeled ATF4-IGFBP-1 or AARE-CHOP or mutATF4-IGFBP-1 or SP1-oligonucleotide (which specifically binds the ubiquitous transcriptional factor SP1). The binding reaction was also carried out in the presence of either 1 μg antibody directed towards the transcription factor ATF4 (anti-ATF4) or 1 μg control IgG (non-immune). Three independent experiments were performed with the same results. The arrow to the right indicated a supershifted band.

Fig.7. Effect of ATF4 knock-down on IGFBP-1 gene regulation by ER stress. Two micrograms of anti-ATF4 siRNA (siRNA A, siRNA B) or the non silencing siRNA mutA were introduced into HepG2 cells using the calcium phosphate precipitation technique. Calcium phosphate precipitates containing no siRNA (no) were also applied on cells. The day after transfection, cells were treated with DMSO or tunicamycin for 16 hours and mRNA levels were measured using real time quantitative RT-PCR with RPL13a as the reference gene. DMSO-treated cells which received no siRNA were considered as controls and fold induction was calculated as a function of these control cells. A. Effect of ATF4 knock-down on basal and induced levels of ATF4 mRNA. B. Effects of siRNA A, B and mutA on IGFBP-1 induction upon ER stress. C. Effects of siRNA A, B and mutA on EDEM induction upon ER stress. EDEM mRNA levels were measured as a negative control since EDEM ER stress induction is believed not to depend on ATF4. Data shown are the means +/- S.E.M. of three independent experiments.
Figure 1

A. Western blot showing the expression of IGFBP-1, BiP, GRP94, Herp, and 18S under DMSO and tunicamycin treatment.

B. mRNA levels fold induction by tunicamycin:

- IGFBP-1
- IGFBP-2
- IGFBP-4
- IGF-I
- IGF-II
- IGF-IR
- ALS
- BiP
- GRP94
- Herp
- CHOP
Figure 2

A. untreated DMSO ethanol tunicamycin thapsigargin brefeldin A

B. cellular extracts

culture medium

untreated DMSO ethanol tunicamycin thapsigargin brefeldin A

18S GRP78 Herp Bip IGFBP-1
Figure 3

A.

-7400  -6629  -6480  -4143  ATG  +165  +514  +2059

UPRE2  ATF4

-2644  +102

-3966  -2523

-4429  -3778

-6819  -4030

-7129  -6288

-6682  -6384

B.

Luciferase activity fold induction

- 5  10  15  20  25

p-399/+41-BiP-FL
pGL3-Basic
p-2644/+102-FL
pTATA-FL
p+493/+1135-TATA-FL
p-3966/-2523-TATA-FL
p-4429/-3778-TATA-FL
p-6819/-4030-TATA-FL
p-7129/-6288-TATA-FL
p-6682/-6384-TATA-FL
A. TTTCATCA TCCCC IGFBP-1
   ATTGCATCA TCCCC CHOP
   GTTTTCATCA TGCCCT ASNS
   GTTGCATCA GCCCG Herp
   GTGACGTCA GCACG GADD34
   RTTRCRTCA consensus
   \[ R = G \text{ or } A \]

B. TTTCATCA IGFBP-1 ATF-4 (-6481/-6473)
   TAGCGCAAC IGFBP-1 mutATF-4 (-6481/-6473)
   TGACGTGA IGFBP-1 UPRE 2 (-6629/-6622)
   GTGACCTC IGFBP-1 mutUPRE 2 (-6629/-6622)

C. Luciferase activity
   Fold induction
   
   pTATA-FL
   p-6682/-6384-TATA-FL
   p-6682/-6384-mutATF4-TATA-FL
   p-6682/-6384-mutUPRE2-TATA-FL
   
   \[ \text{tunicamycin} \]
   \[ \alpha_1-\text{antitrypsine NHK variant} \]
Figure 5

[Bar chart showing luciferase activity fold induction for different constructs: pTATA-FL, p-6682/-6384-TATA-FL, p-6682/-6384-mutATF4-TATA-FL, p-6682/-6384-mutUPRE2-TATA-FL]
| probe               | ATF4-IGFBP-1 | AARE-CHOP |
|---------------------|--------------|-----------|
| lane 1              | 1            | 9         |
| lane 2              | 2            | 10        |
| lane 3              | 3            | 11        |
| lane 4              | 4            | 12        |
| lane 5              | 5            | 13        |
| DMSO N.E.           | +            | +         |
| tunicamycin N.E.    | - +          | - ++      |
| ATF4-IGFBP-1 x75    | -            | - ++      |
| AARE-CHOP x75       | -            | - +       |
| mutATF4-IGFBP-1 x75 | -            | -         |
| SP1 x75             | -            | -         |
| non immune          | -            | -         |
| anti-ATF4           | -            | -         |

Figure 6
Figure 7

A.

ATF4 mRNA levels fold induction

siRNA: no mutA A B

B.

mRNA levels fold induction by tunicamycin

siRNA: no mutA A B

mRNA: IGFBP-1

C.

mRNA levels fold induction by tunicamycin

siRNA: no mutA A B

mRNA: EDEM
Endoplasmic reticulum stress induction of insulin-like growth factor binding protein-1 involves ATF4
Alexandre Marchand, Céline Tomkiewicz, Laurent Magne, Robert Barouki and Michèle Garlatti

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