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Identification of ERdj3 and OBF-1/BOB-1/OCA-B as Direct Targets of XBP-1 during Plasma Cell Differentiation

Ying Shen* and Linda M. Hendershot2*†

Plasma cell differentiation is accompanied by a modified unfolded protein response (UPR), which involves activation of the Ire1 and activating transcription factor 6 branches, but not the PKR-like endoplasmic reticulum kinase branch. Ire1-mediated splicing of XBP-1 (XBP-1(S)) is required for terminal differentiation, although the direct targets of XBP-1(S) in this process have not been identified. We demonstrate that XBP-1(S) binds to the promoter of ERdj3 in plasmacytoma cells and in LPS-stimulated primary splenic B cells, which corresponds to increased expression of ERdj3 transcripts in both cases. When small hairpin RNA was used to decrease XBP-1 expression in plasmacytoma lines, ERdj3 transcripts were concomitantly reduced. The accumulation of Ig γ H chain protein was also diminished, but unexpectedly this occurred at the transcriptional level as opposed to effects on H chain stability. The decrease in H chain transcripts correlated with a reduction in mRNA encoding the H chain transcription factor, OBF-1/BOB-1/OCA-B. Chromatin immunoprecipitation experiments revealed that XBP-1(S) binds to the OBF-1/BOB-1/OCA-B promoter in the plasmacytoma line and in primary B cells not only during plasma cell differentiation, but also in response to classical UPR activation. Gel shift assays suggest that XBP-1(S) binding occurs through a UPR element conserved in both murine and human OBF-1/BOB-1/OCA-B promoters as opposed to endoplasmic reticulum stress response elements. Our studies are the first to identify direct downstream targets of XBP-1(S) during either plasma cell differentiation or the UPR. In addition, our data further define the XBP-1(S)-binding sequence and provide yet another role for this protein as a master regulator of plasma cell differentiation. The Journal of Immunology, 2007, 179: 2969–2978.

The endoplasmic reticulum (ER) is the site of synthesis and maturation of secretory pathway proteins, which include surface and secreted proteins as well as resident proteins of the endocytic and exocytic organelles. Nascent proteins are translocated into the ER and fold with the assistance of molecular chaperone complexes. Properly folded proteins exit the ER and are targeted to their final destinations, whereas misfolded proteins are retained in the ER and eventually degraded by ER-associated degradation, which is also mediated by molecular chaperones (1). When eukaryotic cells encounter unfavorable physiological conditions that disrupt normal ER homeostasis, unfolded or misfolded proteins accumulate in the ER and trigger a signaling cascade termed the unfolded protein response (UPR). This leads to the up-regulation of chaperones to prevent the aggregation of proteins during these unsuitable conditions. In addition, protein synthesis is reduced (2) and the degradative capacity of the cell is increased (3) to further alleviate the burden in the ER. Primarily, this response helps cells survive temporary adverse conditions; however, if the stress conditions are not resolved, apoptotic pathways can be activated to eliminate chronically stressed cells.

The UPR in mammalian cells is activated by the following ER-localized transmembrane proteins: Ire1 α/β kinase (4, 5), activating transcription factor (ATF) 6 α/β (6), and PKR-like endoplasmic reticulum kinase (PERK) (7). In response to ER stress, an endoribonuclease activity encoded in the C terminus of Ire1 is activated. This results in the excision of 26 bases from XBP-1 mRNA (8, 9). After relication, the resulting frameshift remodels the C terminus of XBP-1 to encode a fully active transcription factor, the spliced form of XBP-1 (XBP-1(S)), which has both an N-terminal DNA binding domain and a new C-terminal trans activation domain. A second arm of the UPR is initiated by ATF6, a membrane-anchored transcription factor that is transported to the Golgi in response to ER stress and cleaved by the S1P and S2P proteases (10). The liberated cytosolic domain of ATF6 enters the nucleus and transcriptionally activates the expression of ER chaperones as well as XBP-1 (6, 8). The third ER-localized UPR transducer PERK phosphorylates eukaryotic initiation factor 2α, thereby dramatically decreasing cap-dependent translation and reducing the amount of proteins entering the ER. The mechanism of activating the UPR is not completely understood, but involves the disruption of BiP binding to these three ER sensors when unfolded proteins accumulate (11, 12).

During plasma cell differentiation, B cells begin to produce massive quantities of Abs, which require a corresponding increase in ER chaperones, folding enzymes, and components of the quality control machinery. XBP-1 has been shown to be an essential component of plasma cell development (13, 14). When the XBP-1 null embryonic stem cells were used to reconstitute RAG-2-deficient...
mice, the chimeric animals possessed normal numbers of B lymphocytes, but they were unable to differentiate into Ig-secreting plasma cells. These cells could be rescued by expression of XBP-1(S), but not by an unstable mutant of XBP-1, arguing that Ire1 activity is required for plasma cell differentiation (14). Microarray data suggested that at least 48 genes are downstream targets of XBP-1 during plasma cell differentiation (15), and many of these (i.e., ERdj4, EDEM, and p58IPK) were also regulated by XBP-1 in response to a pharmacological-induced UPR (16), although from these studies it was not clear which genes were direct targets of XBP-1 and which might be indirect. Recently, XBP-1 was shown to regulate ERdj4 expression using reporter assays and to bind to an ACGT tetranucleotide in the ERdj4 promoter using an EMSA (17). Although for the most part, specific binding sequences in XBP-1-regulated genes have not been elucidated and in no case has XBP-1(S) been demonstrated to bind to a promoter in cells.

Reducing XBP-1(S) expression in Ag8(8) and Ag8.653 plasma cytomtia lines with small hairpin RNA (shRNA) led to the down-regulation of ERdj3 mRNA and protein. Using chromatin immunoprecipitation (ChiP) assays, we found that XBP-1(S) binds to the ERdj3 promoter in both plasma cytomtia lines and splenic B cells. The binding of XBP-1(S) to the ERdj3 promoter increases during LPS-induced differentiation of splenic B cells, which corresponded to an increase in ERdj3 transcripts. Unexpectedly, we found that XBP-1(S) also contributed to H chain expression in Ag8(8) cells by regulating H chain mRNA levels. Using shRNA, we found that XBP-1 contributes to the expression of OBF-1/BOB-1/OCA-B (hereafter referred as OBF-1). OB-1 was confirmed as a direct target of XBP-1(S) by ChiP assays, and in vitro gel shift experiments revealed that it bound to an ACGT tetranucleotide sequence, which constitutes the core of the UPR element (UPRE). Our study identifies the first two direct targets of XBP-1 during B cell differentiation and in response to classical UPR activation. In addition, our discovery that H chains are also regulated by XBP-1(S) broadens the scope of XBP-1(S) functions in plasma cell biology.

Materials and Methods

Cells and Abs

Primary mouse splenic B cells were enriched from spleens of 8- to 10-wk-old female C57BL/6 mice by negative sorting. Single-cell suspensions were incubated with Abs specific for non-B cell surface markers (CD3, CD4, CD8, Mac1, Gr1, and TER-119), and these cells were depleted using autoMACS (Miltenyi Biotec). The efficiency of B cell enrichment was determined by staining a small aliquot with anti-CD19 and found to be >95%. The studies on mouse splenocytes have been reviewed and approved by an institutional review committee. To induce plasma cell differentiation, primary B cells and the 1.29 μ mouse lymphoma cell line were immediately incubated with 50 μg/ml LPS for 3 days at 8% CO2 in RPMI 1640 medium supplemented with 20% FCS (JRH Biosciences), 2 mM t-glutamine, 1% fungizone (Cambrex), 100 μM MEM nonessential amino acids, 1 mM sodium pyruvate, and 55 μM 2-ME (Invitrogen Life Technologies). Splenic B and 1.29 μ cells were also treated with 2.5 μg/ml tunicamycin in the same medium for 6 h to induce the UPR. Ag8(8) and Ag8.653 murine plasma cytoma lines were maintained in RPMI 1640 medium supplemented with 10% FCS (JRH Biosciences), 2 mM t-glutamine, and 1% fungizone (Cambrex). Polyclonal anti-BIP (18) and anti-ERdj3 (19) antibodies were described previously. Rabbit anti-ERdj3 (ac-7160) and sc-7160X), goat anti-heat shock cognate protein 70 (Hsc70), and HRP conjugates of goat anti-rabbit IgG and donkey anti-goat IgG were purchased from Santa Cruz Biotechnology. Goat anti-mouse Ig H chain antisera was purchased from Southern Biotechnology Associates.

Northern analysis

Total RNA was extracted using the RNeasy mini prep kit (Qiagen), and 20 μg of RNA was loaded on a formaldehyde gel and transferred for Northern blotting, as described (20). The probes used for Northern blotting correspond to the coding sequences of human ERdj3 (19), hamster BiP (20), mouse XBP-1 (21), and human G3PDH (BD Clontech). Additional probes were amplified from Ag8(8) cells using the following primer pairs: IgH chain forward, GATGTGGTGAGTAGCCTTG and reverse, GTTGCCTGCGCTGCC; Blimp-1 forward, CCAAGAGTGTCACAAG and reverse, GGAAGATGCTTGGTAAACGCTE; OB-I forward, CTGG GGCATACAGGGTG and reverse, GAGGTTGGTACGCTG; OCT-2 forward, GCCACCTGCGCTTCCTCCG and reverse, GGTGAGGGTCGTAGCG; INF regulatory factor 4 (IRF4) forward, GTC AGGACAAAGCAACG and reverse, GGACAGAGTCCTGCC; and reverse, GGAAGATGCTTGGTAAACGCTE. The probes were radiolabeled with [α-32P]dCTP (GE Healthcare) using the Prime-it II kit (Stratagen) and purified by ProbeQuant G-50 Micro Columns (GE Healthcare). Radioactive signals for each gene were quantified by PhosphorImage analysis (Amersham Biosciences) and ImageQuant software and normalized to the corresponding signal for G3PDH. The signal for the various genes in the neo control cells was set to one, and that in the cells expressing small hairpin XBP-1 (shXBP-1) was calculated as a fraction of the control value.

Real-time PCR

Total RNA samples were subjected to real-time PCR, and reactions were done in triplicate using a TaqMan One-Step PCR Master Mix kit (Applied Biosystems) and a standard thermal cycler. Amplification of the corresponding genes was achieved with specific primers and measured continuously with an ABI 7900HT Sequence Detection System. The signal was compared with an 18S RNA internal control supplied with the Pre-Developed TaqMan Assay Control Kit (Applied Biosystems). The primers and 5′-FAM-3′TAMRA modified probes used were: IgH chain forward, AG CACCAAAAGTGGGAGAAGA and reverse, ATGGTGAGCACATCTTG TGG primers; IgH chain probe, CAACCAAAATCCCTGGGCA; ERdj3 forward, TGGAGAAAGTGACTCCAGGA and reverse, GA CGGTGTCACTCTCGTTTGGT; ERdj3 probe, CTTGTTGCCC GCAGCGCTCTCT; OB-1 forward, CCTCCTC CGGTTGACACGAT; and reverse, GGGTTGAGCTGGTTCTTG GCGTTGACACGAT; OB-1 probe, TC CACCACTCATCAATAGTGCAGC; BiP forward, AGGAGACT GCTTAGGGCTAT and GCTGGGCATTTGAAGTAAGT; and BiP probe, CTGCTAGGAACTCCTTCCCA. Again, the normalized value for each transcript in the control cells was set to one, and the normalized value for the corresponding transcript in the XBP-1(S)-inducing cells was expressed as a fraction of the control. SD and p values were calculated from three independent experiments. In the case of splenic B and 1.29 μ cells, the transcript level for each gene in the untreated cells was set to one, and the corresponding levels in tunicamycin- and LPS-treated samples were expressed as a fold increase over untreated cells. SD and p values are calculated from two separate experiments.

Retroviral infection

Viruses that express the neomycin-resistant gene alone or together with the XBP-1 shRNA (22) were provided by L. Glumcher (Harvard University, Boston, MA). Ag8(8) and Ag8.653 cells were infected with these viruses and selected with 1 mg/ml G418 to obtain stable lines.

Pulse-chase labeling and immunoprecipitation

Two million Ag8(8) Neo- and Ag8(8) shXBP-1-expressing cells were metabolically labeled for 40 min with 100 μCi/ml [35S]Translabel (ICN) and chased in complete RPMI 1640 medium for 0, 4, and 8 h. Cells were lysed in 1 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% Nonidet P-40, and 0.5% deoxycholic acid), and protein concentration was determined by Bio-Rad protein assay. Then 400 μg of each sample was incubated with protein A-Sepharose for 1 h to isolate the H chain. Precipitated proteins were analyzed on SDS gels under reducing conditions, and signals were enhanced with Amplify (GE Healthcare) for radiographic visualization.

ChiP

Cells were incubated in RPMI 1640 medium containing 1% formaldehyde at room temperature for 10 min to cross-link protein-chromatin complexes. The cell pellets were lysed with cell lysis buffer I (1 mg/ml SDS, 50 mM HEPES pH 6.5, 10 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100) first, then again with cell lysis buffer II (10 mM HEPES pH 6.5, 1 mM EDTA, 0.5 mM EGTA, and 200 mM NaCl). Nuclear pellets were obtained by centrifugation; lysed in 50 mM Tris-Cl (pH 8.0), 10 mM EDTA, and 1% SDS, and then sonicated with a Branson Sonifier 250 (VWR), as described (23). Either 1% (v/v) of input cell lysates or 5% (v/v) of each sample of each was saved and used for the input control. The remaining samples were split in half and immunoprecipitated with either 2 μg of rabbit anti-XBP-1 polyclonal antiserum (Santa Cruz Biotechnology; sc-7160X) or a rabbit
anti-BiP polyclonal antiserum, which served as a negative control. The immunoprecipitated protein-DNA complexes and input controls were treated with proteinase K (25 μg/ml) to remove the protein components. The remaining DNA was purified, and 10-fold serial dilutions of DNA were made and subjected to PCR amplification using primer pairs that correspond to the indicated regions of the Erdj3 and Obf-1 promoter sequences. The following were used: mOBF-1 ACAT core forward, CACCGGGCTGTGTGCAAAATG and reverse, CCTACAGCAACACCGCCTGTCA; mOBf-1 1 kb upstream forward, GCCAACAGGGTGTTGTTGCTGCA and reverse, GCCCCCCGGCCGGCCGGACG; mErdj3 3XERE core forward, CTCACGGCCCTGTGAAG and reverse, GCCCCCCGGCCGGCCGGACG; mErdj3 3 kb upstream forward, CACCCTACCTAGCTGCCTGCA and reverse, GCTCTAACACAGTCACCATAGGC.

In vitro translation and EMSA
The spliced form of XBP-1 was cloned by RT-PCR from Ag8(8) cells using the following primer pairs: forward primer, CCAAGCTTGGCCCGCTGGCGTATGTTGTTGAC and reverse, TCAAGAGCTGGTGTGGCCTTATAG; the amplified DNA was inserted into pBluescript-SK downstream of the T7 promoter, and the identity of the clone was confirmed by DNA sequencing and Western blotting. XBP-1(5′)-protein was produced using the T7 TNT Coupled Reticulocyte Lysate (Promega). Two micrograms of lysates containing either in vitro translated XBP-1(5′) or no-exogenous protein (as the negative control) was incubated with the indicated oligonucleotide-radiolabeled probes (−0.01 pmol; 10,000 cpm) in a 20-μl reaction containing 1 μl of salmon sperm DNA, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 4% glycerol, 0.5 mM EDTA, and 0.5 mM DTT at 4°C for 1 h, as described previously (17). When indicated, 1000- to 2000-fold excess of unlabelled oligonucleotide probe was used to compete against the [γ-32P]ATP-labeled wild-type (WT) probe, or 2 μg of anti-XBP-1 (sc-7160X) was used for the recognition of the protein complex. At the end of the incubation, 3 μl of a stop solution (80% glycerol, 10 mM EDTA (pH 8.0)) was added. Samples were loaded onto 5% (v/v) non-denaturing TGE polyacrylamide gels and electrophoresed in 1X TGE buffer (25 mM Tris-HCl (pH 8.0), 192 mM glycine, and 1 mM EDTA) at 150 V for 120 min at 4°C. The double-stranded synthetic oligonucleotide sequences used for the EMSA: probe 1 forward, 5′-CACCGGCTGGTGTGGCCTTATAGGGCGTTGCAC-3′; probe 1 reverse, 5′-GGGCGGTGGTGTGGCAACACCGCATTG-3′; probe 2 forward, 5′-GGGCGGTGGTGTGGCAACACCGCATTG-3′; probe 2 reverse, 5′-CTTCGACAAACCGCCCTCTGTGTTGAC-3′; probe 3 forward, 5′-GGGAGAAGGGTATGAGCTGCTGGCCT-3′; probe 3 reverse, 5′-CTCAGAAGGGCCACACATCCTC-3′; probe 4 forward, 5′-ATGCGATGAAGGGCCACACATCCTC-3′; probe 4 reverse, 5′-CACGGAATTCGGTATCACTCCTC-3′; probe 5 forward, 5′-GGGATTATGGTTCTTAAATGGCCGGAGGC-3′; probe 5 reverse, 5′-CTCAGAAGGGCCACACATCCTC-3′.

Results
XBP-1(5′) is highly expressed in H chain-only plasmacytoma cells in which the protein level of Erdj3 is up-regulated
Previously, we reported that the BiP cofactor, Erdj3, appears to bind directly to unassembled Ig H chains in vivo (19, 24). Consistent with its substrate-binding/cochaperone function, Erdj3 is highly expressed in secretory tissues and is up-regulated by agents that affect protein folding in the ER (19, 25). Although XBP-1(5′) regulates Erdj3 during the classical UPR (16), it is not known whether it also controls Erdj3 levels during plasma cell differentiation or in stable plasmacytoma cells. To address the latter point, XBP-1(5′) expression was investigated in Ag8(8) and Ag8.653 cells. Ag8(8) cells accumulate unassembled, nontransported γ H chains due to the lack of L chains, and Ag8.653, which is a derivative of the Ag8(8) cell line, no longer synthesizes either L chains or H chains (26). We found that XBP-1(5′) protein expression was remarkably greater in Ag8(8) cells (Fig. 1) that express free H chains than in the Ig− Ag8.653 cells (Fig. 1). As previously reported (19), protein levels of both Erdj3 and BiP were also higher in Ag8(8) cells than in Ag8.653 cells (Fig. 1), although the difference in Erdj3 expression is much less impressive than that of XBP-1(5′).

XBPl(5′) binds to the Erdj3 promoter in vivo
Although the XBP-1(5′) consensus binding site has not been well defined, XBP-1 was identified, along with ATF6, in a one-hybrid screen using an ER stress response element (ERSE) that regulates BiP expression during the UPR (27). When the promoter regions of human and mouse Erdj3 were examined, we found three potential ERSEs between −200 and −400 bp that could provide an XBP-1 binding site (Fig. 2A, boxed and italicized), as well as one conserved ACAT tetranucleotide element that was identified as an XBP-1 binding site in the ERdj4 promoter (Fig. 2A, boxed and in bold face) (17). To determine whether XBP-1 directly trans activates Erdj3 in these cells, we performed a ChIP assay. When the protein-chromatin complexes from Ag8(8) cells were immunoprecipitated with an Ab specific for XBP-1(5′), a band could be amplified with primers that flank this region, but not with a nonspecific Ab (Fig. 2B), demonstrating that XBP-1(5′) binds to the Erdj3 promoter in vivo. Thus, although many genes are regulated by XBP-1, Erdj3 is the first direct target of XBP-1(5′) to be identified in vivo. The specific promoter region is specified locally within 1 kb of the conserved region, because the vast majority of shared chromatin was <1 kb in length (data not shown). However, it is not clear from this analysis whether XBP-1(5′) binds to the ERSE or to the ACAT core because both are found in this region. When a primer pair that corresponded to a sequence ~3 kb upstream of the conserved region was similarly used, it did not amplify a band (Fig. 2B). The evenness of the input controls indicates that the quality and the amount of the chromatin used are equal between samples.

To determine whether XBP-1 also regulates Erdj3 expression during plasma cell differentiation, we examined primary splenic B cells and I.29 μc cells. First, XBP-1(5′) protein expression was determined by Western blot analysis of lysates from cells that were untreated, tunicamycin treated to activate the UPR, or LPS stimulated to induce plasma cell differentiation (Fig. 2C). The expression of XBP-1(5′) in both splenic B and I.29 μc cells was up-regulated by tunicamycin and LPS treatment, although the increase of XBP-1(5′) was much greater with LPS treatment in splenic B cells and with tunicamycin treatment in I.29 μc cells. When ChIP experiments were applied to splenic B cells (Fig. 2D), we found that the increased binding of XBP-1 to the Erdj3 promoter was considerably greater in response to LPS-induced plasma cell differentiation than that observed with tunicamycin treatment, whereas binding of XBP-1(5′) was enhanced with both tunicamycin and LPS in the I.29 μc cells (data not shown).

FIGURE 1. The XBP-1(5′) is highly expressed in Ag8(8) cells, in which Erdj3 is up-regulated. Cells from Ag8(8) and Ag8.653 plasmacytoma cell lines were collected and lysed. The protein samples (2 μg each) were run on 10% reducing gels and subjected to Western blotting with antisera specific for BiP, Erdj3, γ H chain (γHc), and XBP-1(5′), as indicated. Hsc70 served as the loading control.

FIGURE 2. The XBP-1(5′) and Erdj3 expression are induced in Ag8(8) cells. The XBP-1(5′) and Erdj3 expression were determined by ChIP experiments to confirm the results obtained by Western blotting. A, The XBP-1(5′) is highly expressed in Ag8(8) cells, in which Erdj3 is up-regulated. Cells from Ag8(8) and Ag8.653 plasmacytoma cell lines were collected and lysed. The protein samples (2 μg each) were run on 10% reducing gels and subjected to Western blotting with antisera specific for BiP, Erdj3, γ H chain (γHc), and XBP-1(5′), as indicated. Hsc70 served as the loading control.

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A low basal level of XBP-1(S) binding to the ERdj3 promoter was consistently observed in unstimulated splenic B cells, which may reflect the small amount of XBP-1(S) protein detected in these cells (Fig. 2C). Consistently, real-time PCR revealed that the ERdj3 transcripts were increased by ~1.5- and 9-fold in splenic B cells in response to tunicamycin and LPS treatment, respectively (p < 0.05) (Fig. 2E). In keeping with patterns of XBP-1(S) protein expression in I.29 µ⁺ cells, ERdj3 transcripts were increased by ~5- and 1.5-fold, respectively, by these treatments.

The expression of ERdj3 and H chain is compromised when XBP-1 is down-regulated

Due to the essential role of XBP-1 during plasma cell differentiation, it is impossible to obtain XBP-1 null plasma cells to examine the effect on ERdj3 expression. Thus, to further investigate the role of XBP-1(S) in regulating ERdj3 expression in plasmacytoma cells, we introduced XBP-1-specific shRNA (22) into Ag8(8) and Ag8.653 cells and obtained stable bulk cultures. Total RNA (Fig. 3A) and cell lysates (Fig. 3C) were examined for XBP-1 expression. Due to the minor difference (26 bases) in length, the full-length unspliced and the spliced XBP-1 mRNA cannot be separated by mobility. Thus, the single band shown includes both forms and represents the total amount of XBP-1 mRNA. To our surprise, we observed that the level of the total XBP-1 mRNA (Fig. 3A) is actually higher (~2- to 3-fold by Northern and semiquantitative PCR) in Ag8.653 cells than in Ag8(8) cell (Fig. 3A and data not shown), although Ag8(8) cells express significantly more XBP-1(S) protein (Figs. 1 and 3C). When primer pairs were designed to specifically recognize and amplify only either the spliced or unspliced form of XBP-1, we found that Ag8(8) and Ag8.653 cells contained similar amounts of the spliced form of XBP-1,
whereas the Ag8.653 cells expressed appreciably more of the unspliced form of XBP-1 than Ag8(8) cells did (data not shown). Thus, the ratio of spliced to unspliced XBP-1 differs between the two lines. Recent data demonstrate that the unspliced XBP-1 protein can bind to XBP-1(S) and destabilize it (28), which may account for the very different amounts of XBP-1(S) protein in these two lines. We found that expression of XBP-1 transcripts was reduced by up to 70–80% in both cell lines after introducing the XBP-1 shRNA vector (Fig. 3, A and B). More importantly, we confirmed that XBP-1(S) protein expression was also reduced to below 20% in both lines (Fig. 3 C).

We next investigated the effect of lowering XBP-1(S) levels on ERdj3 and BiP expression. Using real-time PCR, we found that the expression of ERdj3 and BiP transcripts was reduced in both cell lines to 50% of the value obtained in their respective neo control lines (p < 0.05) (Fig. 3 B). These values are similar to data obtained by Northern blot analyses (Fig. 3 A). Reducing ERdj3 transcripts resulted in decreased expression of ERdj3 protein to ~60% of that present in the neo control Ag8(8) line and to ~70% in Ag8.653 cells (Fig. 3 C). Because H chain is a substrate of ERdj3 (19, 24), we examined the resulting effects on H chain expression in these cells. Similar to ERdj3 protein, we found that the accumulation of H chain protein was reduced to ~50% in the shXBP-1-expressing Ag8(8) cells (Fig. 3 C). Coupled with the results in Fig. 2, our data strongly suggest that XBP-1(S) directly regulates the ERdj3 promoter and contributes to the expression of ERdj3 transcripts during both a classical UPR, as well as in response to the modified UPR that occurs during plasma cell differentiation.

The t½ of H chain is not decreased in Ag8(8) cells that express shXBP-1; however, the synthesis of H chain is reduced.

In addition to their ability to stimulate the ATPase activity of their heat shock protein 70 partners, some DnaJ homologues possess chaperone activity themselves by binding directly to unfolded substrates through their C-terminal domains (29, 30). Because our previous data suggested that ERdj3 may also bind directly to unassembled H chains (19) (our unpublished data), it was plausible to speculate that the down-regulation of the ERdj3 expression could affect the stability of the H chain. Thus, we examined the t½ of H chains in Ag8(8) cells with normal and reduced levels of ERdj3. The cells were metabolically labeled and chased for the indicated times, and H chains were isolated for electrophoretic analyses. The radiographic signals of the 35S-labeled H chain did not disappear faster in the Ag8(8) shXBP-1-expressing line (Fig. 4), demonstrating that the turnover rate of the H chain was not increased with

FIGURE 3. ERdj3 and H chain are both modestly down-regulated in plasmacytoma cells that express XBP-1 shRNA. A, Total RNA from Ag8(8) and Ag8.653 cells stably expressing an empty vector (neo) or shRNA specific for XBP-1 (iXBP-1) was subjected to Northern blotting with the indicated probes. Two transcripts for ERdj3 (~1.9 and ~1.6 kb) were detected, which represent mRNA products with different lengths of 3′ UTR (19, 25). G3PDH levels served as a control for loading. The two panels on the right show a comparison of the normalized quantification of each gene, which was done by PhosphorImager. B, Total RNA from the indicated cells was subjected to real-time PCR, and relative mRNA levels were analyzed, quantified, and expressed as a fraction of the control value. SDs are indicated; p < 0.05. C, Whole cell lysates (5 μg each) were analyzed by Western blotting with antisera against XBP-1(S), γ H chain (γHC), or ERdj3, as indicated. Hsc70 was used as a loading control. Relative signals for each protein were quantified by densitometry and normalized to Hsc70.

FIGURE 4. The t½ of H chain is not decreased in Ag8(8) cells that express shXBP-1; however, the synthesis of H chain is reduced.

In addition to their ability to stimulate the ATPase activity of their heat shock protein 70 partners, some DnaJ homologues possess chaperone activity themselves by binding directly to unfolded substrates through their C-terminal domains (29, 30). Because our previous data suggested that ERdj3 may also bind directly to unassembled H chains (19) (our unpublished data), it was plausible to speculate that the down-regulation of the ERdj3 expression could affect the stability of the H chain. Thus, we examined the t½ of H chains in Ag8(8) cells with normal and reduced levels of ERdj3. The cells were metabolically labeled and chased for the indicated times, and H chains were isolated for electrophoretic analyses. The radiographic signals of the 35S-labeled H chain did not disappear faster in the Ag8(8) shXBP-1-expressing line (Fig. 4), demonstrating that the turnover rate of the H chain was not increased with
lower levels of ERdj3. However, we noticed that the radioactive signal of H chains at time = 0 in Ag8(8) shXBP-1 cells was consistently weaker than that observed in control cells, indicating a decreased expression of H chains. This did not appear to be due to increased aggregation of H chain as determined by examining the Nonidet P-40 insoluble fraction by Western blotting (data not shown). Because the lower levels of ERdj3 are mitigated in this line due to a similar decrease in H chain expression, it was not possible for us to establish whether inadequate amounts of ERdj3 would affect H chain stability.

**XBP-1 regulates H chain transcription and the OBF-1, a H chain transcription coactivator**

To determine whether the lower level of H chain expression in the shXBP-1 line was due to decreased transcription of H chains, the same Northern blot membrane shown in Fig. 3A was hybridized with a probe specific for γ H chain (Fig. 5A). We observed that expression of shXBP-1 resulted in an obvious decrease in transcripts encoding the secretory form of the H chain (γs) (Fig. 5A, C, and D), which explains the decreased expression of H chain proteins in these cells. The membrane form of the H chain (γm) did not appear to be affected (Fig. 5A). To determine how XBP-1(S) might be regulating H chain transcription, we examined the expression of several transcription factors that are either induced downstream of XBP-1 (17), although no data were provided to support this possibility. It is important to note that other than our data on ERdj3 (Fig. 2), the binding of XBP-1 to the promoter of any gene in cells has not been demonstrated, and therefore, a bona fide consensus-binding sequence has not been easy to determine.

**XBP-1 binds to the OBF-1 promoter in vivo**

Using synthetic probes, one-hybrid screens, and reporter assays, XBP-1 was shown previously to bind to both UPRE (3, 8, 37) and ERSE-II (37) sequences in response to ER stress, but only poorly to ERSE sites (8, 37). In addition, XBP-1 can bind to CRE-like sequences that contain an ACGT core (38, 39), which is also a central element of the 8-bp UPRE. As reported recently, although the ERdj4 promoter does not have either a UPRE or ERSE-II element, it does contain an ACGT tetranucleotide sequence, and EMSA studies demonstrated that rXBP-1 could bind to this sequence (17). Alignment of the human and mouse OBF-1 promoters revealed one potential UPRE site TAGCTTGGA, which is conserved in both (Fig. 6A, bold). However, the human OBF-1 promoter has a T→C substitution in the ACGT core of the UPRE site (Fig. 6A). We also found one potential ERSE site, GGTGG-N9-ATTGG, in the mouse OBF-1 promoter (Fig. 6A, italicized), but it is not present in the corresponding region of the human OBF-1 promoter. In addition, we found a TGCA core that was conserved in both the human and mouse OBF-1 promoters, which represents a reversion of the ACGT sequence (Fig. 6A, bold). This sequence was speculated to serve as a putative XBP-1 binding site in the EDEM promoter, another UPR target that is downstream of XBP-1 (17), although no data were provided to support this possibility.
To determine whether XBP-1(S) binds and directly transactivates the OBF-1 promoter in vivo, we performed ChIP assays using cell extracts from Ag8(8) cells (Fig. 6B), as well as from tunicamycin-treated and LPS-stimulated mouse primary splenic B and I.29 μ^+ cells (Fig. 6C), as described above. Chromatin was immuno precipitated with either rabbit anti-XBP-1 or a nonspecific rabbit control antiserum. When the primers located in the conserved region of the mouse promoter (Fig. 6A, indicated by arrows) were used for amplification, a product was obtained only from the DNA precipitated with anti-XBP-1, demonstrating the interaction of XBP-1(S) with the OBF-1 promoter (Fig. 6B and C) in vivo. The control primers did not amplify the chromatin immunoprecipitated with either the Ab to XBP-1(S) or the negative control Ab, demonstrating the specificity of XBP-1 binding to the conserved region (Fig. 6B). Similar to the binding of XBP-1 to the ERdj3 promoter (Fig. 2D), the association of XBP-1 with the OBF-1 promoter was elevated in response to UPR activation by tunicamycin and by LPS-induced differentiation (Fig. 6C). The increased binding of XBP-1(S) to the OBF-1 promoter with tunicamycin and LPS stimulation corresponded to similar increases in OBF-1 transcripts in both lines (Fig. 6D).

**XBP-1 regulates OBF-1 expression through a UPRE**

To identify the specific binding sites for XBP-1(S) in the OBF-1 promoter, four double-strand probes (as underlined in Fig. 6A) were synthesized and tested by EMSA. Probe 1 (−340 to −311 bp) contains the TGCA element (speculated to serve as an XBP-1 binding site) (17) probe 2 (−234 to −197 bp) corresponds to a region with very high sequence homology between the two species (but which has not been suggested to serve as an XBP-1-binding sequence in any published study), probe 3 (−168 to −140 bp) includes the potential UPRE site, and probe 4 (−302 to −274 bp) represents a potential ERSE site in the mouse promoter. Either control rabbit reticulocyte lysates or lysates containing in vitro translated XBP-1(S) were incubated with [γ-32P]ATP-labeled probes (Fig. 7A). We observed an XBP-1-specific band shift (indicated with an arrow) only with the

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**FIGURE 6.** XBP-1 binds to the OBF-1 promoter in vivo. A. Alignment of a portion of the promoter sequences of the human and mouse OBF-1 genes. Potential XBP-1 binding sites are framed as follows: the ERSE site is italicized, whereas TGCA and UPRE are in bold face. A primer pair flanking the potential XBP-1 binding sites in the mouse OBF-1 promoter is indicated with arrows. Probes 1–4 used in Fig. 7A are underlined. The start codon ATG is capitalized and in bold face. B. A portion of the samples obtained from Ag8(8) cells in Fig. 2B was amplified with a primer pair corresponding to the potential XBP-1 site in the OBF-1 promoter as well as with a primer pair corresponding to a region 1 kb upstream. C. Samples from splenic B cells or I.29 μ^+ cells were amplified with a primer pair corresponding to the potential XBP-1 site in the OBF-1 promoter. D. Total RNA from the indicated B cell samples was subjected to real-time PCR, and the normalized OBF-1 mRNA levels were expressed relative to that found in untreated B cells. SDs are indicated; p < 0.05.
FIGURE 7. XBP-1(S) binds to an ACGT/C element in OBF-1 promoters. A. The four radiolabeled probes (indicated in Fig. 6A) were incubated with rabbit reticulocyte lysate containing in vitro translated XBP-1(S) or no exogenous protein and subjected to nondenaturing electrophoresis to separate the protein-oligonucleotide complexes. A specific protein-probe complex observed in the presence of XBP-1(S) and probe 3 was pointed by a solid arrow. The other protein-probe complexes that do not contain XBP-1(S) are indicated by asterisks. B. A 2000-fold excess of various unlabeled oligonucleotide probes or an anti-XBP-1(S) Ab was incubated with [γ-32P]ATP-labeled probe 3 (32P ACGT WT) and reticulocyte lysates containing XBP-1(S) or nothing (control), as indicated. A specific XBP-1-containing protein-probe complex is indicated by a solid arrow.

Discussion

XBP-1(S) is a central component of the classical UPR (8, 16) and an essential regulator of plasma cell differentiation (15). Despite this, XBP-1 has not been shown to directly interact with any promoter in vivo and the XBP-1(S) binding site has not been clearly elucidated. We demonstrate in this study that XBP-1(S) binds to the ERdj3 and OBF-1 promoters in primary splenic B cells and L29 μ− cells when the UPR is induced by tunicamycin, even though tunicamycin usually produces a very strong activator of the UPR and XBP-1 splicing. This is most likely to be due to the fact that splenic B cells cannot be maintained in culture for even short amounts of time without mitogenic growth factors. In keeping with this possibility, similar experiments using the I.29 μ− murine B cell line revealed that XBP-1(S) protein was more potently up-regulated in response to tunicamycin treatment (Fig. 2C), as were the ERdj3 and OBF-1 transcripts (Fig. 2E).

A combination of one-hybrid screens, reporter assays, and EMSA has suggested that XBP-1(S) binds several different UPR-inducible elements with the following preferences: UPRE>ERSE-II>ERSE (3, 8, 37). ERSE sequences appear to have a higher affinity for ATF6 than XBP-1 (8, 37). The presence of multiple ERSE sites in the ERdj3 promoter may suggest that ATF6 is largely responsible for the remaining expression of ERdj3 in our Ag8(8) cells after shRNA transfection (Fig. 3) and in XBP-1 null mouse embryonic fibroblasts (data not shown). In support of this, overexpression of ATF6 in NIH3T3 cells up-regulates ERdj3 without inducing XBP-1 splicing (40). Although we did not investigate the exact XBP-1 binding site in the ERdj3 promoter, we focused on finding the target sequence of XBP-1 in the promoter of our second direct, and unexpected target of XBP-1, OBF-1. The OBF-1 promoter contained one ERSE and one UPRE, as well as a TGCA sequence that was previously suggested to serve as a putative XBP-1(S) binding site (17). The UPRE sequence TGACGTGG/A has a tetranucleotide core (ACGT), which was first identified as an optimal binding sequence for XBP-1 in an oligonucleotide screen (39) and recently was demonstrated to be served as an XBP-1(S) site in the ERdj4 promoter using EMSA (17). Our data demonstrated that the ACGT core of the UPRE was required for XBP-1(S) association and eliminated the ERSE and the TGCA sequences as likely binding sites (shown in Fig. 7). In addition, we found that the single change in this sequence found in the human OBF-1 promoter did not prevent its association with XBP-1(S), thus refining the UPRE sequence as TGACGTCC/G/A. Our demonstration that XBP-1(S) binds to the OBF-1 promoter, coupled with our EMSA studies provide reasonable support for the UPRE as a primary XBP-1-binding sequence and warrant further mutagenesis studies to fully define critical residues in this element.
Somewhat unexpectedly, we found that XBP-1(S) regulated the expression of H chain at the transcriptional level. A previous microarray study suggested that both Blimp-1 and XBP-1 contribute to H chain expression; however, because Blimp-1 regulates XBP-1 and both the Blimp-1 and the XBP-1 null cells fail to fully differentiate into plasma cells, it has been somewhat difficult to assess the relative contribution of these two transcription factors to H chain expression (15). Our studies reveal that OBF-1 is a direct target of XBP-1 and that reducing XBP-1(S) levels resulted in decreased expression of OBF-1 in cells. This is in contrast to the previous microarray study, which suggested that OBF-1 was downstream of Blimp-1, but not XBP-1, during differentiation (15). It is possible that the contribution of XBP-1(S)-to-OBF-1 expression is fairly modest compared with that of Blimp-1, and was therefore not detected in the microarray study. It is important to note that although decreased levels of OBF-1 corresponded to reduced expression of H chain, our experiments do not allow us to unequivocally conclude that OBF-1 levels per se are entirely responsible. It was surprising that the effect of lowering XBP-1 was greater for γc than for γm. However, this is consistent with a previous report showing that Blimp-1 is responsible for the developmentally regulated switch from μc to μm (31). Because XBP-1 is downstream of Blimp-1, it is possible that this occurs at least partially through XBP-1. A second possibility is that XBP-1 directly transactivates the H chain promoter. Inspection of the H chain promoter did not reveal any obvious UPRE sites, but a further delineation of the complete UPRE sequence may be required to draw this conclusion.

Although a modified UPR has been shown to be activated during the differentiation of B cells to plasma cells, and at least some elements of this signaling cascade are essential to the process, it was not known whether this pathway remained active in cells that stably produce large quantities of Ab molecules such as plasmacytoma, long-lived plasma cells, and hybridomas. In this study, we examined plasmacytoma cells as a model and found that they appear to continuously activate at least some arms of the UPR. The fact that XBP-1(S) is very abundant in Ag8(8) cells implies that Ire1 is likely to remain activated in these cells, because Ire1 is the only known protein that is capable of splicing XBP-1. The high levels of total XBP-1 mRNA and BiP expression in this line imply that the ATF6 pathway is also activated. A previous study reported that ATF6 is cleaved during LPS-induced differentiation of CH12 cells (41) and enforced expression of a dominant-negative ATF6 mutant in splenic B cells decreased Ab secretion (42). Similar to what has been shown in differentiating CH12 cells (41), we did not find evidence of C/EBP homologous protein (CHOP) expression in the plasmacytomas (data not shown), suggesting that whatever suppresses the PERK pathway during differentiation continues to do so in stable Ig-expressing lines, which could have implications for long-lived plasma cells. The signal for activating the ATF6 and Ire1 branches remains unclear. Previous studies show that XBP-1 splicing and up-regulation of ATF6 targets during plasma cell differentiation precede or coincide with increased IgM synthesis (41, 43); thus, Ig H chain is therefore unlikely to be the signal for their activation. However, our data showing higher levels of XBP-1(S) in the Ag8(8) cells that produce H chain are consistent with a previous report showing that μ H chain-deficient splenic B cells from the B1–8m mouse have a lower level of XBP-1(S) in response to LPS (14) and suggest a possible amplification of this loop later in the differentiation process by the increased synthesis of Ig proteins.

In summary, we found that stable plasmacytoma lines continue to activate a modified or partial UPR, as evidenced by high levels of XBP-1(S), ERdj3, and BiP in the absence of detectable CHOP expression. We identified ERdj3 and OBF-1 as the first direct targets of XBP-1 during plasma cell differentiation and in response to a classical UPR. Furthermore, we found that XBP-1(S) also contributes to H chain transcription, which is likely to occur indirectly through its regulation of a H chain transcription factor. Our studies strongly suggest that XBP-1 regulates genes via UPRE sequences and extend the already vast role of XBP-1 in plasma cells.

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Disclosures

The authors have no financial conflict of interest.

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