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XBP-1 Couples Endoplasmic Reticulum Stress to Augmented IFN-β Induction via a cis-Acting Enhancer in Macrophages

Ling Zeng,* Yi-Ping Liu,* Haibo Sha, † Hui Chen, † Ling Qi, † and Judith A. Smith*

Perturbation of the endoplasmic reticulum (ER) results in a conserved stress response called the unfolded protein response (UPR). Macrophages undergoing a UPR respond to LPS with log-fold increased production of IFN-β, a cytokine with diverse roles in innate and adaptive immunity. In this study, we found that thapsigargin-induced ER stress augmented recruitment of IFN regulatory factor-3, CREB binding protein/p300, and transcriptional machinery to the murine ifnb1 promoter during LPS stimulation. Although full synergistic IFN-β production requires X-box binding protein 1 (XBP-1), this UPR-regulated transcription factor did not appreciably bind the ifnb1 promoter. However, XBP-1 bound a conserved site 6.1 kb downstream of ifnb1, along with IFN regulatory factor-3 and CREB binding protein only during concomitant UPR and LPS stimulation. XBP-1 physically associates with p300, suggesting a mechanism of multimolecular assembly at the +6.1 kb site. Luciferase reporter assays provide evidence this +6 kb region functions as an XBP-1–dependent enhancer of IFN-promoter activity. Thus, this study identifies a novel role for a UPR-dependent transcription factor in the regulation of an inflammatory cytokine. Our findings have broader mechanistic implications for the pathogenesis of diseases involving ER stress and type I IFN, including viral infection, ischemia-reperfusion injury, protein misfolding, and inflammatory diseases. The Journal of Immunology, 2010, 185: 2324–2330.

Type I IFNs (IFN-α/β) play diverse roles in adaptive and innate immune responses. Although they were first noted for their antiviral properties, type I IFNs also activate macrophages and NK cells, promote T cell survival and dendritic cell maturation, and increase the production of Th1-polarizing cytokines (1). Cells of the innate immune system, such as macrophages and dendritic cells, produce type I IFNs upon detection of pathogens through pattern recognition receptors that include the TLRs (2). These pattern recognition receptors bind conserved motifs found on pathogens, such as LPS (TLR4), dsRNA (TLR3 and RIG-I), and hypomethylated CpG DNA (TLR9). TLRs may also mediate responses to “endogenous” products released during tissue necrosis, such as hyaluronic acid, heparin sulfate, fibrinogen, and heat shock proteins (3).

IFN-β appears to be the primary cytokine that mediates macrophage type I IFN responses to the TLR4 agonist LPS (4). IFN-β-deficient animals were shown to be much more susceptible to lethal sepsis from several strains of pathogenic bacteria, presumably through weakened host inflammatory responses (5). Mice deficient in IFN-β are also more susceptible to particular viral infections, have lower numbers of macrophages and mature B cells, and exhibit reduced bone mass (6–8).

The regulation of IFN-β transcription in the setting of viral infection has been well studied. Briefly, in the uninfected cell, a nucleosome obstructs the +1 start site, preventing transcription. During infection, a group of transcription factors, including NF-κB, AP-1, IFN regulatory factor (IRF)-7 and IRF-3 cooperatively assemble over a 55-bp stretch of DNA, between −102 and −47 bp upstream of the transcriptional start site (9). This grouping, termed the “enhanceosome,” recruits histone acetylases, such as CREB binding protein (CBP/p300), a large flexible transcription coactivator that may interact simultaneously with multiple transcription factors (activating transcription factor [ATF]-2, c-Jun, p65, and IRF-3/7) (10, 11). CBP/p300 thus acts as a signal integrator. Histone acetylation facilitates the recruitment of chromatin modifiers that slide the nucleosome off the TATA box start site, thus enabling transcription (12, 13). Less is known about the induction of IFN-β transcription following LPS stimulation, although it appears slightly different. For instance, although viral infection induces recruitment of IRF-7 to the enhanceosome, LPS-induced IFN-β appears to depend on IRF-3 rather than IRF-7 (14–16).

Our previous studies have shown that macrophages undergoing an intracellular stress response called the unfolded protein response (UPR) respond to LPS and dsRNA with greatly enhanced IFN-β production (17). The UPR is an adaptive response initiated by environmental stressors (hypoxia, nutrient deprivation, hypoglycemia) or internal derangements (increased protein load, misfolding proteins, calcium gradient deregulation) that disrupt endoplasmic reticulum (ER) function. When ER function is perturbed, excess unfolded protein competes with the ER resident proteins, inositol-requiring enzyme (IRE)-1, protein kinase receptor–like ER kinase (PERK), and ATF-6, for binding of the folding chaperone Ig binding protein (BiP/GRP78). IRE-1 is an endonuclease that is activated after release of BiP and that cleaves a 26-bp intron from the X-box binding protein 1 (XBP-1) transcription factor mRNA. This unusual splicing event removes a premature stop

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Abbreviations used in this paper: ATF, activating transcription factor; CBP, CREB binding protein; Chip, chromatin immunoprecipitation; 2-DG, 2-deoxyglucose; ER, endoplasmic reticulum; GCN5, general control nonderepressible 5; HA, hemagglutinin; HMG, high-mobility group I protein; IRE, inositol-requiring enzyme; IRF, IFN regulatory factor; NT, no ER stress inducer; PERK, protein kinase receptor–like ER kinase; Pol II, RNA polymerase II; qPCR, quantitative PCR; RNAi, RNA interference; siRNA, small interfering RNA; TBP, TATA box binding protein; Tgp, thapsigargin; Tu, tunicamycin; UPR, unfolded protein response; XBP-1, X-box binding protein-1.

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codon through frame shifting the open reading frame, thus allowing for the translation of the full-length XBP-1 transcription factor. Upon release of BiP, PERK transiently inhibits global protein translation apart from selected transcripts (e.g., ATF-4). Finally, ATF-6 leaves the ER and traffics to the Golgi, where it is processed to an active form. UPR target genes aimed at resolving ER stress include folding chaperones and proteins that aid in ER-associated protein degradation. If these and other adaptations fail, the UPR results in apoptosis (18).

The UPR appears to play a physiologic role in highly secretory cells, such as pancreatic acinar cells, hepatocytes, and plasma cells (19). However, the UPR has also been implicated in such diverse pathologic processes as cardiovascular disease, ischemia-reperfusion injury, neurodegenerative diseases, diabetes, viral infections, and cancer (20). It is becoming increasingly apparent that the UPR also plays a role in immune function. For example, the differentiation of B cells into plasma cells requires splicing of the ifnb1 enhancer of IFN-β production that provides a mechanistic link to spontaneous enteritis and increased susceptibility to Listeria (22). Cholesterol-loaded macrophages undergoing a UPR secrete the inflammatory cytokines TNF-α and IL-6 (23). ER stress leads to the proteolytic activation of CREBH (processed similarly to ATF-6), a transcription factor that induces the production of serum amyloid and C-reactive proteins (24).

Understanding how ER stress regulates IFN-β responses may shed light on disease processes in which both UPR and type I IFNs have been implicated, such as ischemia-reperfusion injury and viral infections, as well as diseases where they may be related (HLA-B27-associated spondyloarthritis and inflammatory myopathies) (25–29). Previous work has supported a critical role for the UPR-regulated transcription factor XBP-1 in mediating synergistic IFN-β induction upon TLR stimulation (17). However, the underlying molecular mechanism behind the synergy was not clear. We hypothesized that XBP-1, as a transcription factor, may regulate IFN-β induction by either a direct or epigenetic mechanism during ER stress. In this study, we demonstrate binding of XBP-1, CBP, and IRF-3 to a DNA region 6.1 kb downstream of the ifnb1 gene during conditions of concomitant ER stress and LPS stimulation. Binding of these factors at this +6 kb site correlated temporally with increased recruitment of CBP and IRF-3 to the ifnb1 promoter. Finally, the presence of the +6 kb site significantly enhanced ifnb1 promoter activity. Collectively, these data suggest that this newly described region 6 kb downstream of the ifnb1 gene is a cis-acting XBP-1–dependent enhancer of IFN-β production that provides a mechanistic link between ER stress and augmented IFN-β induction. As a broader consideration, these findings provide an explanation for how ER stress may drive the pathogenesis of type I IFN-related diseases.

**Materials and Methods**

**Cells, reagents, and stimulations**

The RAW 264.7 macrophage cell line (American Type Culture Collection) was maintained in DMEM/high glucose with 4 mM l-glutamine, sodium pyruvate (HyClone Laboratories, Logan, UT) and supplemented with 10% FBS (HyClone Laboratories), 100 U/ml penicillin, and 100 μg/ml streptomycin. C57BL/6 bone marrow macrophages were isolated as previously described (17); briefly, low-density bone marrow cells from C57BL/6 females were isolated on Histopaque 1083 (Sigma-Aldrich, St. Louis, MO) and plated for 3 d in non–tissue culture petri dishes in DMEM (as above) supplemented with 5% M-CSF–containing conditioned supernatant from CMG14-12 cells (30). Adherent cells were detached by 10 mM EDTA and replated in tissue culture dishes with the 5% conditioned supernatant 3 more days prior to stimulation. The University of Wisconsin is accredited by the American Association of Laboratory Animal Care, and mouse experiments were performed with Institutional Animal Care and Use Committee oversight and approval. To induce ER stress, cells were pretreated with 10 μg/ml tunicamycin for 6 h, 20 mM 2-deoxyglucose for 6 h, 10 μM A23187 for 4 h, 1 mM DTT for 2 h, or 1 μM thapsigargin (Tpg) for 1 h, depending on time required for maximal XBP-1 mRNA splicing. Splicing was determined by OD of PCR products separated on a 3–4% agarose gel. *Salmonella enteritidis* LPS (Sigma-Aldrich) was used at 100 ng/ml. ER stress agents and LPS were from Sigma-Aldrich, except for DTT (Thermo Fisher Scientific, Waltham MA). The DMSO vehicle for Tpg and A23187 had no effect on IFN-β mRNA induction (data not shown). Supernatant IFN-β was quantified by ELISA (PBL InterforerSource, Piscataway, NJ) after 1 h of Tpg treatment followed by 6 h of LPS treatment.

**XBP-1 knockdown and immunoblotting**

RAW cells were transfected with 200–300 nM XBP-1 stealth small interfering RNA (siRNA) or control medium GC content RNA interference (RNAi) (Invitrogen, Carlsbad, CA; catalog no. 12935-300) by Amaxa nucleofection (kit V; Lonza, Walkersville, MD). The sequences of the XBP-1–specific sense and antisense strands were 5′-CACGCGGAGACGCTGGAGAATGACCC-3′ and 5′-UUUCUACUCGCGACGCGCUCG-3′. Twenty-four hours posttransfection, cells were stimulated, lysed with RIPA buffer, and whole cell lysates were resolved by 4–12% SDS-PAGE (Invitrogen). Nitrocellulose blots (Whatman, Piscataway, NJ) were probed with anti-XBP-1 (Santa Cruz Biotechnology, Santa Cruz, CA) or β-actin (Santa Cruz Biotechnology), followed by HRP-conjugated secondary Ab (Bio-Rad, Hercules, CA), and proteins were visualized by ECL (Amersham Biosciences, Piscataway, NJ/film).

**Quantitative PCR (qPCR)**

RNA was purified with TRIzol (Invitrogen), treated with DNase I (Invitrogen), and then reverse transcribed using random primers (Promega, Madison, WI). Relative cDNA was quantified by SYBR Green (Bio-Rad), detected by MyQ (Bio-Rad), and normalized to 18S RNA. Primers were designed using Beacon Design software (Premier Biosoft, Palo Alto, CA) and are as follows: 18S RNA: forward, 5′-GGACACGCGACAGATTGAC-3′ and reverse, 5′-ATCGGTCCACAACTAAGAGC-3′. IFN-β: forward, 5′-ACTAGAGGAAAAGCAGAAGGAAAG-3′ and reverse, 5′-CCACCACCCGCTAGCC-3′. ERδ: forward, 5′-GGCGAAAGGCAACAGGGACAACTG-3′ and reverse, 5′-CTCTGCGGTTGCTGTGG-3′. IL-1β: forward, 5′-CCGAGGACAGCAGCCACATAGC-3′ and reverse, 5′-ACGGGAAAGACAGATGATGAGG-3′. IL-6: forward, 5′-CTTCATCCACGAGTTCGGTC-3′ and reverse, 5′-ATTTCGAGTTTCCACCAAC-3′. ERα: forward, 5′-AAGTYGAGGAACTTGGAAG-3′ and reverse, 5′-GCAAGATGAGCAGGACcccG-3′. ERγ: forward, 5′-AGGAAGGATGAGAAAAATCAG-3′ and reverse, 5′-ACTGGTTGTTGCGTTTGGG-3′. +6 kb site: forward, 5′-CGAGAAAGGAAAGAATAATGG-3′ and reverse, 5′-CTCAGGGAAGACTTGTCG-3′. XBP-1: forward, 5′-AACCCTTGGGAAAATGACAC-3′ and reverse, 5′-CCATGGGAAAGATTGCCGGG-3′.

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation (ChIP) was performed as previously described (31); briefly, RAW cells were fixed with 1% formaldehyde, lysed, and sonicated to generate chromatin fragments. After preclearing with normal rabbit serum (Covance Research Products, Princeton, NJ), immunoprecipitations were performed with protein A-Sepharose (Sigma-Aldrich) coupled to anti-IRF-3, CBP/p300, XBP-1, TFIIID/TATA box binding protein (TBP), and RNA polymerase II (Pol II; Santa Cruz Biotechnology). DNA–protein complexes were eluted with 0.1 M NaHCO₃, 1% SDS, cross-links were reversed with 0.3 M NaCl, and protein was degraded with protease K (Promega). Phenol chloroform–extracted DNA samples were analyzed by qPCR as above. Percentage occupancy was derived by comparison with input chromatin.

**Coimmunoprecipitations**

HEK293 T cells were transfected with expression vectors for Flag-tagged XBP1±αs, hemagglutinin (HA)-tagged XBP1s, and HA-tagged P300 by Lipofectamine 2000 (Invitrogen) (32, 33). Eighteen to 24 h later, cells were lysed (150 mM NaCl, 1% Triton X-100, 0.1 mM EDTA, 50 mM Tris [pH 7.5], DTT, protease inhibitor mixture), and lysates were immunoprecipitated with agaose coupled anti-HA or anti-Flag (Sigma-Aldrich). After washing (20 mM Tris, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1% glycerol, 0.5 mM DTT), samples were boiled and proteins were resolved on 6%–8% gels. Western blots were probed with anti-HA-HRP or anti-Flag-HRP (Sigma-Aldrich), developed with ECL substrate (Pierce, Rockford, IL), and exposed to film.

**Luciferase assays**

IFN-β promoter (bp –330 to +9) and +6 kb sequences (305 bp, 27790207–27790507 [GenBank:88161592–88161890 [FASTA]) were cloned from...
RAW 264.7 genomic DNA isolated by a DNeasy kit (Qiagen, Valencia, CA), XBP-1 binding sites (IFR-primal TGCAC [Δ1] or distal TGCAG [Δ2]) were deleted by PCR with Pfu DNA polymerase (Stratagene, La Jolla, Ca). These segments were inserted into the pGL3 basic luciferase reporter plasmid (Promega) using the Kpn/XhoI and Xho/BglIII sites (respectively) upstream of the luciferase gene. To identify required XBP-1 binding sites, RAW 264.7 macrophages were transiently transfected with 2 μg of luciferase reporter and 0.2 μg of Renilla TK (Invitrogen) by Amaxa. Twenty hours later, cells were stimulated for 8 h. Luciferase activity was detected by the Dual-Luciferase reporter assay system (Promega) and read on a Synergy plate reader (BioTek Instruments, Winooski, VT). Transfection efficiency was normalized to Renilla activity. For comparison of XBP-1s and XBP-1u, RAW cells were transfected with 1 μg of pCDNA3.1 vector, XBP-1s, or XBP-1u (provided by Dr. Laurie Glimcher, Harvard School of Public Health, Boston, MA) plus 1 μg of luciferase reporters using TransIT-Neural transfection reagent (Mirus, Madison, WI) (21). Sixteen hours later, cells were stimulated with LPS for 7 h. Luciferase activity was detected using luciferase assay reagent II (Promega) read on a TR717 luminometer (PE Applied Biosystems, Foster City, CA). Results were normalized to total protein (BCA assay; Pierce). All luciferase assay samples were run in duplicate.

**Statistics**

Statistical differences between groups of data were determined by a two-tailed Student t test. All error bars from combined experiments represent SE of the mean. For representative experiments, error bars represent deviations of duplicate determinations.

**Results**

**ER stress augments LPS-induced IFN-β in macrophages**

Our previous studies had shown that pretreatment of primary macrophages with a commonly used pharmacologic inducer of ER stress, Tpg, could significantly augment subsequent LPS-stimulated IFN-β transcription. Similarly, macrophages expressing a misfolding protein (HLA-B27) and undergoing a UPR produced more IFN-β mRNA in response to LPS (17). In this study, Tpg-primed bone marrow macrophages responded to LPS with log-fold synergy of IFN-β mRNA by 4 h with a return to baseline by 8 h (Fig. 1A). Tpg inhibits the SERCA Ca²⁺ pump, which potently and rapidly induces a UPR through disruption of the ER–cytosol calcium gradient (34). Because Tpg could have multiple effects on cell signaling beyond disruption of the ER, we examined the effect of pretreatment with other pharmacologic UPR inducers on LPS-induced IFN-β transcription (Fig. 1B). Pretreatment with these other UPR-inducing agents also significantly augmented LPS-stimulated IFN-β gene expression in primary bone marrow macrophages. IFN-β induction in the absence of LPS was insignificant. Tpg remained the most potent potentiator of IFN-β transcription. The ∼2 log increase in transcript observed with Tpg pretreatment correlated well with the observed increase in protein secretion. In RAW 264.7 macrophages, Tpg rapidly induces XBP-1 splicing (Fig. 1C), and pretreatment for 1 h maximally increased LPS-stimulated IFN-β mRNA by 3 h with a return to baseline by 8 h (Fig. 1D).

**Tpg amplifies factor recruitment to the ifnb1 promoter**

The log-fold increase in IFN-β mRNA and kinetics of synergy suggested that regulation of IFN-β induction by ER stress occurs at the transcriptional level. Preliminary studies with actinomycin D also suggested that ER stress did not prolong IFN-β mRNA half-life (data not shown). To determine how ER stress affected LPS-induced recruitment of transcriptional and other regulatory factors to the ifnb1 gene promoter, occupancy of the ifnb1 promoter was examined by ChIP. Guided by the kinetics of IFN-β transcriptional synergy (Fig. 1D), we examined factor occupancy during the first 4 h following LPS stimulation. To ensure maximal sensitivity in RAW 264.7 macrophages, we used the most potent and rapid inducer of ER stress, Tpg. Tpg pretreatment resulted in a 2- to 3-fold increase in IRF-3 and CBP occupancy and a 7- to 10-fold increase in transcriptional machinery recruitment (Pol II and TBP) compared with LPS alone (Fig. 2). Preliminary evidence suggests a 2- to 3-fold increase in NF-κB (p65) occupancy as well at the 2 h time point. Maximum occupancy occurred for most factors around 2 h, with a significant decrease in transcriptional machinery occupancy by 4 h, correlating with peak IFN-β mRNA kinetics. The enhanced transcriptional machinery recruitment also correlated well with the degree of synergistic mRNA induction typically observed in RAW cells (Fig. 1D).

**The UPR transcription factor XBP-1 regulates the synergistic induction of IFN-β in macrophages**

Our previous studies examining the different signaling pathways initiated by the UPR (originating from PERK, IRE-1, and ATF-6)
had suggested a critical role for the IRE-1–dependent transcription factor XBP-1: synergy was abrogated in XBP-1 knockout mouse embryonic fibroblasts and by XBP-1 RNAi knockdown in LPS receptor expressing 293 cells (17). In this study, to determine whether XBP-1 was required for synergy in macrophages, we used two approaches: we initially transfected the RAW cells with a dominant-negative XBP-1 containing the DNA binding region, but not the trans-activating region (35). Interestingly, we were unable to expand macrophages containing this construct, suggesting a role for XBP-1 in macrophage survival. We then knocked down XBP-1 with siRNA. Transiently transfecting RNAi to achieve a 4- to 5-fold knockdown of XBP-1 mRNA during stimulation did not have an obvious impact on viability or expression of the 18S rRNA housekeeping gene (Fig. 3C). XBP-1 RNAi decreased both baseline and Tpg-induced XBP-1 protein (Fig. 3A). As can be seen in Fig. 3B, XBP-1 RNAi decreased synergistic induction of IFN-β mRNA by an average of ~70%. The decrease in LPS-induced IFN-β in the absence of Tpg was not statistically significant. In comparison, the induction of ERdj4, a known XBP-1–regulated chaperone, was reduced 80% by XBP-1 RNAi during combined Tpg and LPS stimulation (35). In our transient transfection system, XBP-1 knockdown did not impair induction of IL-1β or IL-6 mRNA (Fig. 3C), suggesting relative specificity of XBP-1 regulation for the IFN-β cytokine.

**FIGURE 3.** XBP-1 knockdown decreases synergistic induction of IFN-β in macrophages. A, RAW 264.7 macrophages transfected with 300 nM control or XBP-1 siRNA (XBP-1si) were treated for 1 h with Tpg and then for 3 h with LPS. XBP-1 (top) or actin (bottom) was detected by Western blot. Results are representative of two separate experiments. B, RAW cells transfected with 300 nM control RNAi or XBP-1 siRNA were stimulated as in A, and relative IFN-β (top) and ERdj4 (bottom) mRNA was determined by qPCR. Results were combined from two (ERdj4) and three (IFN-β) independent experiments. *p = 0.029; **p = 0.021. C, RAW cells transfected with 200 nM control RNAi (black) or XBP-1si (gray) were stimulated as in A, and relative expressions of IFN-β, 18S rRNA, IL-1β, and IL-6 mRNA were determined by qPCR. Results are representative of three independent experiments.

**FIGURE 4.** XBP-1 does not bind the ifnb1 promoter. RAW 264.7 macrophages were stimulated as described in Fig. 2, and then ChIP was performed with anti–XBP-1. Relative occupancy of the ifnb1 (left) and ERdj4 (right) promoters was assessed by qPCR by comparison with input sample. For ERdj4 ChIP, occupancies of control IgG were combined for all stimulation conditions. Results were combined from four (ifnb1 promoter) and two (ERdj4 promoter) independent experiments.

The +6 kb XBP-1 binding site enhances ifnb1 promoter activity CBP/p300 occupancy has been proposed as a “gene enhancer signature” (38). Finding CBP bound to the site during concurrent Tpg and LPS stimulation raised the possibility that the +6 kb site may be an ER stress-sensitive enhancer of IFN-β induction. To determine whether this putative enhancer site had any functional relevance for ifnb1 promoter activity, the ifnb1 promoter and +6 kb site were cloned into a vector bearing a luciferase reporter gene. The putative enhancer alone did not induce any luciferase activity over the vector control in the absence of the promoter (data not shown). In the absence of Tpg (LPS only), the +6 kb site augmented promoter activity, consistent with baseline presence of spliced XBP-1 in RAW macrophages (Figs. 1C, 5D). Tpg treatment augmented promoter activity in the presence of the enhancer, consistent with described induction of NF-κB and MAPK signaling by ER stress (23). However, Tpg treatment further increased promoter activity in the presence of the enhancer. When compared with LPS driven promoter activity alone (no ER stress or enhancer), the presence of the enhancer and addition of Tpg pretreatment augmented activity by ~4-fold in these assays. To determine which conserved XBP-1 binding
A sample experiment is shown in Supplemental Fig. 2. Promoter + 6 kb enhancer) independent experiments. Two (AP-1), XBP-1 consensus binding sites are in open boxes. RAW cells were transfected with luciferase reporters containing the ifnb1 promoter alone or promoter +6 kb site with no deletions or with deletions of either or both conserved XBP-1 core binding sites (Δ1, Δ2, or Δ1 + Δ2). Cells were stimulated for 1 h with Tpg and/or for 7 h with LPS. Results were normalized to Tpg plus LPS stimulation (=100%). Results were combined from three (XBP-1) and four (CBP, IRF-3) independent experiments. Promoter activity of the +6 kb site was detected by ChIP. Results were combined from three (XBP-1) and four (CBP, IRF-3) independent experiments.  *p ≤ 0.01. D, RAW cells were transfected with luciferase reporters containing the ifnb1 promoter alone or promoter +6 kb site with no deletions or with deletions of either or both conserved XBP-1 core binding sites (Δ1, Δ2, or Δ1 + Δ2). Cells were stimulated for 1 h with Tpg and/or for 7 h with LPS. Results were normalized to Tpg plus LPS stimulation (=100%). Results were combined from two (Δ1 + Δ2), three (Δ1, Δ2), and six (promoter versus promoter +6 kb enhancer) independent experiments. A sample experiment is shown in Supplemental Fig. 2. *p = 0.00002; **p < 0.04.

**FIGURE 5.** Identification of an XBP-1–dependent enhancer site 6.1 kb downstream of ifnb1. A, Genomic region containing ifnb1, XBP-1 binding site, and contiguous genes. Ifnb1 gene: 27796544–27797313 (GenBank)/88168698–88167929 (FASTA). B, Nucleotide sequence of the +6 kb site containing base pairs 27790246–27790479 (GenBank)/88161631–88161864 (FASTA). Conserved nucleotides between mouse and human are in bold type. Predicted ifnb1 enhanceosome component binding sites (80–90% consensus identity) are denoted by gray box (IRF), dotted line (NF-kB), and underscoring (AP-1). XBP-1 consensus binding sites are denoted by gray box (IRF), dotted line (NF-κB), and underscoring (AP-1). Results were combined from three (XBP-1) and four (CBP, IRF-3) independent experiments. *p ≤ 0.01. D, RAW cells were transfected with luciferase reporters containing the ifnb1 promoter alone or promoter +6 kb site with no deletions or with deletions of either or both conserved XBP-1 core binding sites (Δ1, Δ2, or Δ1 + Δ2). Cells were stimulated for 1 h with Tpg and/or for 7 h with LPS. Results were normalized to Tpg plus LPS stimulation (=100%). Results were combined from two (Δ1 + Δ2), three (Δ1, Δ2), and six (promoter versus promoter +6 kb enhancer) independent experiments. A sample experiment is shown in Supplemental Fig. 2. *p = 0.00002; **p < 0.04.

**FIGURE 6.** XBP-1s associates with CBP/p300 and enhances ifnb1 promoter activity via the +6 kb enhancer

XBP-1 is a CREB family basic leucine zipper transcription factor that can form heterodimers (e.g., with c-fos and ATF-6) (39, 40). As a CREB family member, it was possible that XBP-1 interacted with CREB binding protein CBP/p300. CBP/p300 has been shown to associate directly with phosphorylated IRF-3 following viral stimulation (41). Thus, an interaction between XBP-1 and CBP/p300 might explain the increased recruitment of both CBP and IRF-3 to the putative enhancer site in a multimolecular complex during concomitant ER stress and LPS stimulation. Spliced XBP-1s encodes the 371-aa ER stress-induced active transcription factor, whereas the 267-aa unspliced XBP-1u has the DNA binding N-terminal domain, but not the trans-activating C-terminal domain (18). In overexpression studies (Fig. 6A), XBP-1s, but not XBP-1u, coprecipitated with p300. Thus, the CBP/p300 coactivator may associate with the active XBP-1 transcription factor during ER stress. The predicted molecular mass of the unspliced XBP-1 is ~30 kDa, so the higher molecular mass products in lane 2 may represent ubiquitinated protein visualized as a result of the overexpression system (21).

To determine whether XBP-1s or XBP-1u binding regulated enhancer activity, RAW 264.7 macrophages were transfected with XBP-1 expression vectors and the above luciferase reporter constructs. XBP-1s (but not XBP-1u) increased ifnb1 promoter activity in the presence of the +6 kb enhancer element (Fig. 6B). This increase was abrogated when the IRF-proximal XBP-1 core sequence was deleted (Δ1 Enh-Pro). In the absence of XBP-1, deletion of this core sequence also decreased enhancer-related luciferase activity to the level observed with the promoter alone, suggesting that the background enhancer activity in the

**FIGURE 5.** Identification of an XBP-1–dependent enhancer site 6.1 kb downstream of ifnb1. A, Genomic region containing ifnb1, XBP-1 binding site, and contiguous genes. Ifnb1 gene: 27796544–27797313 (GenBank)/88168698–88167929 (FASTA). B, Nucleotide sequence of the +6 kb site containing base pairs 27790246–27790479 (GenBank)/88161631–88161864 (FASTA). Conserved nucleotides between mouse and human are in bold type. Predicted ifnb1 enhanceosome component binding sites (80–90% consensus identity) are denoted by gray box (IRF), dotted line (NF-κB), and underscoring (AP-1). XBP-1 consensus binding sites are in open boxes. RAW cells were transfected for 1 h with Tpg followed by LPS for the times indicated. Factor occupancy of the +6.1 kb site was detected by ChIP. Results were combined from three (XBP-1) and four (CBP, IRF-3) independent experiments. *p ≤ 0.01. D, RAW cells were transfected with luciferase reporters containing the ifnb1 promoter alone or promoter +6 kb site with no deletions or with deletions of either or both conserved XBP-1 core binding sites (Δ1, Δ2, or Δ1 + Δ2). Cells were stimulated for 1 h with Tpg and/or for 7 h with LPS. Results were normalized to Tpg plus LPS stimulation (=100%). Results were combined from two (Δ1 + Δ2), three (Δ1, Δ2), and six (promoter versus promoter + 6 kb enhancer) independent experiments. A sample experiment is shown in Supplemental Fig. 2. *p = 0.00002; **p < 0.04.

**FIGURE 6.** XBP-1s associates with CBP/p300 and enhances ifnb1 promoter activity via the +6 kb enhancer

XBP-1 is a CREB family basic leucine zipper transcription factor that can form heterodimers (e.g., with c-fos and ATF-6) (39, 40). As a CREB family member, it was possible that XBP-1 interacted with CREB binding protein CBP/p300. CBP/p300 has been shown to associate directly with phosphorylated IRF-3 following viral stimulation (41). Thus, an interaction between XBP-1 and CBP/p300 might explain the increased recruitment of both CBP and IRF-3 to the putative enhancer site in a multimolecular complex during concomitant ER stress and LPS stimulation. Spliced XBP-1s encodes the 371-aa ER stress-induced active transcription factor, whereas the 267-aa unspliced XBP-1u has the DNA binding N-terminal domain, but not the trans-activating C-terminal domain (18). In overexpression studies (Fig. 6A), XBP-1s, but not XBP-1u, coprecipitated with p300. Thus, the CBP/p300 coactivator may associate with the active XBP-1 transcription factor during ER stress. The predicted molecular mass of the unspliced XBP-1 is ~30 kDa, so the higher molecular mass products in lane 2 may represent ubiquitinated protein visualized as a result of the overexpression system (21).

To determine whether XBP-1s or XBP-1u binding regulated enhancer activity, RAW 264.7 macrophages were transfected with XBP-1 expression vectors and the above luciferase reporter constructs. XBP-1s (but not XBP-1u) increased ifnb1 promoter activity in the presence of the +6 kb enhancer element (Fig. 6B). This increase was abrogated when the IRF-proximal XBP-1 core sequence was deleted (Δ1 Enh-Pro). In the absence of XBP-1, deletion of this core sequence also decreased enhancer-related luciferase activity to the level observed with the promoter alone, suggesting that the background enhancer activity in the
unstimulated conditions reflected baseline ER stress and the presence of spliced XBP-1 in RAW cells (Fig. 1C). In the LPS-stimulated conditions, XBP-1s increased enhancer activity by ~2-fold over baseline ER stress and increased luciferase activity 6- to 7-fold over the promoter alone. Taken together, these data support a role for the +6.1 kb site as an XBP-1–dependent enhancer of ifnb1 promoter function.

Discussion

We have identified an enhancer site 6 kb downstream of the ifnb1 gene that exhibits significantly increased binding of XBP-1 only during concomitant Tpg and LPS treatment. Furthermore, enhancer activity of the +6 kb site was responsive to the active XBP-1s transcription factor, not XBP-1u, and was dependent on a predicted IRF-proximal XBP-1 core binding sequence. This +6 kb region also bound the key IFN-β enhanceosome components IRF-3 and CBP/p300 during concomitant LPS stimulation and ER stress. The kinetics of XBP-1, CBP, and IRF-3 binding to the enhancer site showed a striking synchronicity with the increased recruitment of CBP and IRF-3 to the ifnb1 gene promoter, with maximal occupancy after 2 h of LPS treatment. The physical interaction between XBP-1 and CBP/p300 (which is known to associate with phosphorylated IRF-3 following LPS stimulation) provides a mechanistic explanation for the presence of all of these factors together at the enhancer site: one could hypothesize the formation of a multimolecular complex whereby XBP-1 associates with CREB/p300, which in turn associates with IRF-3, thus allowing for cooperative and synchronous binding of XBP-1 and IRF-3 to the enhancer. This model reflects the need for both ER stress and TLR stimulation to promote simultaneous binding. Precedence for such cooperative mechanism may be found at the ifnb1 promoter itself (16).

In previous studies, Tpg pretreatment had no effect on the induction of the IRF-3–regulated chemokine RANTES (CCL5) by LPS. Also, XBP-1 RNAi did not affect the induction of the IRF-3–regulated chemokine IL-8 in TLR4-bearing 293 cells (17). These data would suggest that Tpg (and by extension the UPR) does not generally activate all IRF-3–regulated genes and that the effect on IFN-β is more specific. Indeed, by gene expression microarray in primary mouse macrophages, the only chemokine or cytokines showing more specific. Indeed, by gene expression microarray in primary mouse macrophages, the only chemokine or cytokines showing more specific induction were IFN-β and IL-23 (42).

Looping of chromatin has been proposed as a mechanism that brings gene promoters and distal regulatory sites into physical apposition (43). Following synchronous binding of XBP-1, IRF-3, and CBP/p300 to the +6 kb region during ER stress and LPS stimulation, the enhancer may then loop around to provide increased CBP and IRF-3 delivery to the ifnb1 promoter (Fig. 7). Given the cooperative assembly of factors at the ifnb1 promoter, a small amount of "extra" IRF-3 (even 2- to 3-fold) early in the sequence could be greatly magnified during the successive recruitment of histone modifiers and transcriptional machinery to result in the observed log-fold synergy. According to this looping enhancer theory, the enhancer would only come into play during combined ER stress and LPS stimulation; thus, the comparison between LPS-stimulated promoter function and enhancer-promoter function during concomitant Tpg/XBP-1s plus LPS would be the most relevant. Because XBP-1 was not detected on the promoter, XBP-1 may dissociate following looping. The compressed kinetics makes it difficult to assess this possibility. Otherwise, the cross-linking may have been insufficient to detect factors indirectly associated with the promoter.

An alternative explanation for the role of XBP-1 in synergy is that XBP-1 induces an unknown factor that binds the ifnb1 promoter. However, the induction of a negative regulator of IFN-β transcription by LPS precluded more direct evaluation of this hypothesis using cyclohexamide (44). The time frame, with 1 h of Tpg pretreatment sufficient to detect synergy after 2 h of LPS treatment, would argue against the involvement of a newly transcribed XBP-1 gene target.

In this study, the kinetics of promoter occupancy following LPS stimulation was greatly compressed compared with what has been described for viral infection, with a significant decrease in the transcriptional machinery by 4 h (12). This decreased promoter occupancy correlated well with the disappearance of IFN-β mRNA transcript. Other transcription factors and chromatin modifiers, besides the ones mentioned in this study, have been reported to bind the ifnb1 promoter during viral infection. However, we were unable to detect significant binding (>0.002 occupancy) of IRF-1, IRF-7, or ATF-2 transcription factors, general control nonderepressible 5 (GCN5) histone acetyltransferase, or the HMG-I-Y architectural factor (data not shown) (13, 16). The HMG-I-Y DNA binding protein has been proposed as a chaperone that facilitates and stabilizes assembly of the enhanceosome, although it is not likely to be present in the final structure (9, 45). Acetylation of the HMG-I-Y structural protein at Lys17 by GCN5 promotes association of HMG-I-Y with enhanceosome components and protects against destabilization. CBP-mediated acetylation of Lys65 decreases the affinity of HMG-I-Y for DNA and destabilizes the enhanceosome (46). Thus, sequential activity of GCN5 followed by CBP appears to be critical for sustained transcription. With regard to this study, the brief duration of transcription machinery occupancy following LPS stimulation may reflect CBP predominance and insufficient acetylation of HMG-I-Y by GCN5 activity.

The luciferase results support a functional role for the newly identified XBP-1 binding enhancer in regulating the ifnb1 promoter. However, the luciferase assay may greatly underestimate the effect of the +6 kb enhancer site on promoter activity in situ for the following reasons: (1) The regulation of IFN-β transcription is highly chromatin sensitive. During unstimulated conditions, a nucleosome blocks access of the transcriptional machinery to the TATA box start site. The orchestrated sequential and cooperative recruitment of various factors to the ifnb1 promoter culminates in sliding this nucleosome upstream, thus enabling transcription (13). This event has been described as a regulatory "on–off" switch for ifnb1 transcription. The luciferase construct would not recapitulate this nucleosomal sliding event. (2) The 6-kb distance between enhancer and promoter might be required for looping of the chromatin and optimal orientation of enhancer and promoter. (3) Finally, there may be a cooperative opening/modification of chromatin by histone acetylation that is simply not captured in a luciferase-
implications for understanding the pathogenesis of protein misfolded proteins may enhance recruitment of IRF-3 and CBP/p300 to the XBP-1 binds a potential enhancer element 6 kb distal to the IFN-β gene that may enhance recruitment of IRF-3 and CBP/p300 to the XBP-1 enhancer. These findings have significant mechanistic implications for understanding the pathogenesis of protein misfolding and ER stress-related inflammatory diseases.

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**Disclosures**

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**References**


