N-terminal Hydrophobic Amino Acids of Activating Transcription Factor 5 (ATF5) Protein Confer Interleukin 1\(\beta\) (IL-1\(\beta\))-induced Stabilization*§

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Takanori Abe†, Masaki Kojima†, Satoshi Akanuma‡, Hiromi Iwashita‡, Takashi Yamazakî, Ryuichi Okuyama†, Kenji Ichikawa†, Mariko Umemura†, Haruo Nakano§, Shigeru Takahashi†, and Yuji Takahashi‡

From the †Laboratory of Environmental Molecular Physiology, ‡Laboratory of Bioinformatics, and §Laboratory of Extremophiles, School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo 192-0392, Japan

Activating transcription factor 5 (ATF5) is a stress-response transcription factor that responds to amino acid limitation and exposure to cadmium chloride (CdCl₂) and sodium arsenite (NaAsO₂). The N-terminal amino acids contribute to the destabilization of the ATF5 protein in steady-state conditions and serve as a stabilization domain in the stress response after CdCl₂ or NaAsO₂ exposure. In this study, we show that interleukin 1\(\beta\) (IL-1\(\beta\)), a proinflammatory cytokine, increases the expression of ATF5 protein in HepG2 hepatoma cells in part by stabilizing the ATF5 protein. The N-terminal domain rich in hydrophobic amino acids that is predicted to form a hydrophobic network was responsible for destabilization in steady-state conditions and served as an IL-1\(\beta\) response domain. Furthermore, IL-1\(\beta\) increased the translational efficiency of ATF5 mRNA via the 5’UTR and phosphorylation of the eukaryotic translation initiation factor 2α (eIF2α). ATF5 knockdown in HepG2 cells up-regulated the IL-1\(\beta\)-induced expression of the serum amyloid A 1 (SAA1) and SAA2 genes. Our results show that the N-terminal hydrophobic amino acids play an important role in the regulation of ATF5 protein expression in IL-1\(\beta\)-mediated immune response and that ATF5 is a negative regulator for IL-1\(\beta\)-induced expression of SAA1 and SAA2 in HepG2 cells.

Activating transcription factor 5 (ATF5), a transcription factor in the cAMP response element-binding protein/ATF² family, was first identified by protein blots as a protein that bound the CCAAT/enhancer-binding protein γ (1). Newman et al. (2) reported that ATF5 is a CCAAT/enhancer-binding protein γ-binding transcription factor in a comprehensive protein array analysis. ATF5 contains a basic leucine zipper (bZIP) domain and regulates processes involved in cellular differentiation (3), the cell cycle (4), and apoptosis (5, 6). ATF5 is a target of ubiquitin-mediated proteolysis by Cdc34, a G₂ checkpoint gene (4). Recently, Monaco et al. (7) showed that a wide range of carcinomas express ATF5 and that RNA interference of ATF5 causes apoptotic cell death of neoplastic breast cell lines. These findings suggest that ATF5 could be a target for cancer therapy and that studies of ATF5 expression mechanisms could be important in investigating cancer treatments.

Mammalian cells alter their gene expression to adapt to a variety of environmental stresses, including nutrient limitation, oxidative stress, and hypoxia, although the exact molecular events controlling stress responses have not been fully elucidated. We discovered that ATF5 is a stress-responsive transcription factor that responds to amino acid limitation, cadmium chloride (CdCl₂), and sodium arsenite (NaAsO₂) (8, 9). Roach et al. (10) found that LPS induces ATF5 mRNA expression in macrophages. Chuang et al. (11) reported that up-regulation of ATF5 suppresses the expression of signals for lymphocyte activation molecule-associated protein to activate T cells in hemophagocytic syndrome associated with Epstein-Barr virus infection and immune disorders. These results suggest that ATF5 could be important in the immune system.

In mammals, four eIF2α kinases recognize distinct stress signals and phosphorylate eIF2α. Activation of eIF2α kinases inhibits protein translation (12). The four eIF2α kinases are general control nonderepressible-2 (GCN2), which is activated by nutritional limitation; PKR-related ER kinase (PERK)/pancreatic eIF2α kinase (PEK), which is activated by protein misfolding from endoplasmic reticulum (ER) stress; double-strand RNA-activated protein kinase, which is activated in response to viral infection; and heme-regulated inhibitor, which is activated by heme deficiency in the erythroid lineage. Heme-regulated inhibitor is also activated in response to oxidative stress in Schizosaccharomyces pombe (13) and mammalian cells (14). Phosphorylation of eIF2α reduces the amount of eIF2-GTP.

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† To whom correspondence should be addressed: School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan. Tel.: 81-426-76-7018; Fax: 81-426-76-6811; E-mail: shigeru@ls.toyaku.ac.jp.

‡ The abbreviations used are: ATF, activating transcription factor; PERK, protein kinase R-related endoplasmic reticulum kinase; ER, endoplasmic reticulum; uAUG, upstream AUG; Luc/LUC, luciferase; PQC, protein quality control; SAA, serum amyloid A.

Significance: This study provides new insights about the roles of ATF5 in immune response.
available to initiate translation, lowering global protein synthesis and coinciding with the translational induction of stress response genes.

The human ATF5 gene has two exon 1s, exon 1α and exon 1β, and is transcribed from alternative exon 1α and exon 1β promoters into two mRNAs encoding the same single 30-kDa protein. These mRNAs have alternative 5′-untranslated regions (5′ UTRs), ATF5–5′ UTRα and ATF5–5′ UTRβ. These 5′ UTRs differentially determine the mRNA translation efficiency (15). The 5′ UTRα represses translation of the downstream ATF5 ORF. Repression of 5′ UTRα is released by amino acid limitation or Na3AsO3 exposure via elf2α phosphorylation. In contrast to 5′ UTRα, 5′ UTRβ is insensitive to stress conditions. Alignment of the 5′ UTRα sequences of human, mouse, and rat ATF5 shows high identity, two putative translation start sites (uAUG1 and uAUG2), and similar two-uORF configurations. Under nonstressed conditions, the low level of elf2α phosphorylation favors reinitiation of translation at uAUG2 by scanning ribosomes after translation of uORF1. This precludes translation of the ATF5 ORF. Under stressed conditions, highly phosphorylated elf2α decreases ribosome assembly and favors reinitiation of translation at the ATF5 ORF downstream of uORF2, resulting in elevated ATF5 protein production (15).

Many transcription factors, including ATF4, ATF5, and Nrf2, are regulated by modulation of protein stability via the ubiquitin-mediated proteasome pathway (16, 17). We showed that CdCl2 and NaAsO3 exposure stabilizes ATF5 protein and increases ATF5 protein levels (9). Furthermore, we demonstrated that the N-terminal 21 amino acids of ATF5 protein serve as a destabilization domain in steady-state conditions and function as a stress response stabilization domain after CdCl2 and NaAsO3 exposure. Li et al. (18) showed that HSP70 binds to the N-terminal proline-rich activation domain of ATF5 protein, inhibiting ATF5 protein degradation. Consequently, accumulated ATF5 promotes the survival of glioma cells. Liu et al. (19) also showed that nucleophosmin (NPM1) competes with HSP70 for binding to the basic leucine zipper of ATF5 protein and promotes proteasome- and caspase-dependent ATF5 degradation in hepatocellular carcinoma cells. However, the precise mechanism by which the N-terminal amino acids of the ATF5 protein function as a destabilization domain remains to be elucidated.

In this study, we investigated IL-1β-induced stabilization of ATF5 and subsequent accumulation in HepG2 human hepatoma cells. The N-terminal hydrophobic amino acids are responsible for ATF5 protein stability. HSP70 knockdown reduced IL-1β-induced up-regulation of ATF5 expression. Furthermore, IL-1β induced elf2α phosphorylation and elevated the translation efficiency of ATF5 mRNA in HepG2 cells. ATF5 knockdown resulted in the enhancement of the acute phase mRNA response of serum amyloid A1 (SAA1) and serum amyloid A2 (SAA2) induced by IL-1β. This study provides new insights about the roles of ATF5 in the immune response.
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according to the instructions of the manufacturer. The antibodies used were anti-FLAG M2 (1:3000, Sigma-Aldrich, St. Louis, MO), anti-HA (1:3000, Sigma-Aldrich), anti-HSP70 (1:3000, Calbiochem), anti-NPM1 (1:4000, Sigma-Aldrich), anti-elongin 2α (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-elongin 2α (1:1000, Santa Cruz Biotechnology), anti-β-galactosidase (1:5000, Promega), anti-EGFP (1:6000, Clontech, Palo Alto, CA), horseradish peroxidase-coupled antimouse secondary antibody (1:5000, Cell Signaling Technology, Beverly, MA), and goat anti-rabbit secondary antibody (1:5000, Santa Cruz Biotechnology).

**Immunoprecipitation**—Cells were lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl (pH 7.2), 1 mM EDTA, 1% Nonidet P-40, 10% glycerol, 0.2 M NaCl, 2.5 mM EGTA) and 1×complete™ protease inhibitor mixture. When interactions between ATF5 and HSP70 or NPM1 were detected, 0.6 mg of protein lysate was subjected to immunoprecipitation using 30 µl of protein G-Sepharose (GE Healthcare) and 16.5 µg of anti-HA antibody following the protocol of the manufacturer. When ubiquitination of ATF5 was detected, 0.4 mg of protein lysate was subjected to immunoprecipitation using 30 µl of anti-DYKDDDDK tag beads (Wako Pure Chemical Industries) following the protocol of the manufacturer.

**Three-dimensional Structure Prediction of ATF5**—The three-dimensional structure of the whole chain for wild-type ATF5 was predicted by the Rosetta server (20) with the Rosetta de novo protocol (21, 22). Using this structure as a template, the three-dimensional structure of the ATF5 mutants was predicted by Modeler (23) with homology modeling. All calculations for the structure prediction were carried out with default parameter settings.

**CD Absorption Spectra of ATF5 N-terminal Polypeptides**—CD measurement was carried out using a J-720 spectropolarimeter (Jasco, Hachioji, Japan). The 27-residue peptide (MSLLATGLGELRLLPASGLGWLVY) corresponding to the N-terminal sequence of ATF5 was dissolved in 5 mM Tris-Cl buffer (pH 7.3), 50% (v/v) methanol. The spectrum was recorded from 200–250 nm at 25 °C, and the solvent background was then subtracted. The spectrum is reported as the average of five scans with 2-s signal averaging and using a 1.0-nm bandwidth. A 0.5-cm path length quartz cuvette was used. The peptide concentration was 8.7 µM.

**Analysis of the Translation Efficiency of ATF5 mRNA**—Cells were harvested in 1 ml of phosphate-buffered saline, and 300 µl of cell suspension was centrifuged. Pellets were dissolved in passive lysis buffer for luciferase activity measurement, and 700 µl was used for RNA extraction and quantification of luciferase mRNA.

**Luciferase Assay**—Cells were lysed in passive lysis buffer (Promega), and luciferase activities were determined using a Dual-Luciferase reporter assay system (Promega) and Lumat LB 9501 (EG and G Berthold, Bad Wildbad, Germany). Activities were normalized to Renilla luciferase.

**Preparation of RNA and Quantification of Transcripts**—Total RNA was isolated using a GenElute mammalian total RNA miniprep kit (Sigma-Aldrich). Reverse transcription using 0.5 µg of RNA and oligo(dT) 12–18 primer (Invitrogen) was performed with ReverTra Ace (Toyobo) according to the instructions of the manufacturer. Reverse-transcribed first-strand cDNA was quantified by real-time quantitative PCR (ABI PRISM 7000 sequence detection system) using SYBR® Premix Ex TaqTM II (Takara, Shiga, Japan). For the PCR of SAA1, SAA2, and β-actin, samples received 50 °C for 2 min; 95 °C for 30 s; and 40 cycles at 95 °C for 5 s, 57 °C for 30 s, and 72 °C for 45 s. For PCR of beetle luciferase and Renilla luciferase, samples received 50 °C for 2 min, 95 °C for 30 s, and 40 cycles at 95 °C for 5 s and 60 °C for 31 s. The following oligonucleotides were used for the amplification of the cDNA corresponding to SAA1, SAA2, ATF-β, actin, beetle luciferase, or Renilla luciferase: SAA1, 5′-ctgcaagaaggtagcatcgg-3′ and 5′-atttgctgctgccactcc-3′; SAA2, 5′-ctgcagaaggtgcatcga-3′ and 5′-attatatgccatatctcagc-3′; ATF5, 5′-tggtatctcttttcctttt-3′ and 5′-gtctctattctcctgccaac-3′; β-actin, 5′-aaacctggaacgctgaa-ggtg-3′ and 5′-agagaagtgggtggtgcttttt-3′; beetle luciferase, 5′-acaaggatggatgctgcttc-3′ and 5′-cttcc-aggcggttcaacagt-3′; and Renilla luciferase, 5′-atgggtgatgaaaggtgcttttttcctggtttctt-3′.

**Hydropathy Index**—Kyte-Doolittle hydropathy plots (24) were used to predict hydrophobic regions in proteins. A Kyte-Doolittle score of >0 indicates a hydrophobic region, and <0 indicates a hydrophilic region (25).

**AGGRESCAN**—AGGRESCAN was used to calculate the influence of sequential changes on protein aggregation propensity (26, 27).

**Statistical Analysis**—Statistical analysis was performed using Student’s t test and analyses of variance. A p value of 0.05 was chosen as the threshold for statistical significance.

**RESULTS**

**IL-1β Stabilizes ATF5**—Treatment of HepG2 cells expressing 3×FLAG-ATF5 with IL-1β (0.1 ng/ml) increased ATF5 protein levels within 2 h, with a subsequent ATF5 decrease (Fig. 1A). The up-regulation of ATF5 protein caused by IL-1β increased ATF5 (22–282), which suggests the influence of N-terminal deletion on IL-1β-induced ATF5 protein expression. Compared with full-length ATF5 (1–282), ATF5 with the deletion of the N terminus (ATF5 1–21) showed significantly up-regulation of ATF5 (22–282), which suggests the influence of N-terminal deletion on IL-1β-induced ATF5 protein levels, at least in part, by protein stabilization.

**IL-1β-induced Up-regulation of ATF5 Requires the N Terminus, Not Polyubiquitination**—In a previous study, we showed that the N-terminal region of ATF5 is responsible for both basal and CdCl2-induced expression of the ATF5 protein (9). To examine whether the N terminus was also responsible for up-regulation of ATF5 expression induced by IL-1β, we evaluated the influence of N-terminal deletion on IL-1β-induced ATF5 protein expression. Compared with full-length ATF5 (1–282), ATF5 with the deletion of the N terminus (ATF5 1–21) showed an increase in ATF5 expression in the presence and absence of IL-1β (data not shown). However, the addition of IL-1β did not significantly up-regulate ATF5 (22–282), which suggests the...
importance of the N terminus in stabilization and regulation of ATF5 expression in HepG2 cells. To determine whether IL-1β affected the proteasome-mediated degradation of ATF5, we examined the influence of MG132 pretreatment on IL-1β-induced ATF5 protein expression. MG132 pretreatment significantly decreased the IL-1β-induced up-regulation of ATF5 (data not shown). This finding indicates that IL-1β-induced ATF5 protein expression is mainly controlled by proteasome-mediated degradation. Further, we examined ATF5 ubiquitination after IL-1β treatment. IL-1β increased ATF5 ubiquitination (data not shown). Moreover, deletion of the N-terminal 21 amino acids of ATF5 increased ATF5 ubiquitination. These results indicate that stabilization of the ATF5 protein caused by IL-1β does not result from inhibition of ATF5 ubiquitination.

**HSP70 Is Responsible for IL-1β-induced ATF5 Up-regulation**—Liu et al. (19) showed that ATF5 interacts with HSP70, that this interaction stabilizes ATF5, and that NPM1, a nucleolar chaperone protein, antagonizes the protective effect of HSP70 to promote ATF5 degradation by competition with HSP70 for ATF5 binding. Thus, we examined the effect of HSP70 knockdown on ATF5 expression. HSP70 knockdown by HSP70 siRNA significantly decreased IL-1β-induced up-regulation of ATF5 (Fig. 2A). However, the expression of endogenous HSP70 protein was not induced by IL-1β (Fig. 2B). Furthermore, IL-1β did not decrease the expression of endogenous NPM1 protein (Fig. 2B). These findings prompted us to investigate whether interactions of these proteins and ATF5 were involved in IL-1β-induced ATF5 up-regulation. Immunoprecipitation (Fig. 2C, *bottom panels*) showed that the levels of the ATF5-HSP70 and ATF5-NPM1 complexes were equivalent to the levels of the ATF5 protein (Fig. 2C, *top panels*). This result suggests that the affinity between ATF5 and HSP70 or NPM1 does not change after IL-1β stimulation. This indicates that IL-1β increases the stability of ATF5 by mechanisms other than reinforcing the binding between ATF5 and HSP70.

**The Hydrophobic N Terminus Is Responsible for ATF5 Destabilization and Stress Response**—The N terminus of ATF5 is important for both basal and inducible expression. We ana-
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lyzed the primary structure of this region and found strong hydrophobicity, including leucine and valine (hydropathy indexes of 3.8 (Leu) and 4.2 (Val)) (Fig. 3A). Next, the three-dimensional structure of the whole chain for wild-type ATF5 was predicted by the Robetta server with the Rosetta de novo protocol. For all the five models produced by Robetta, the N-terminal region corresponding to residues 1–15 in each model was well superimposed on each other (root mean square for Cα atoms was ranged from 0.28–2.2 Å). According to the secondary structure prediction by the Jpred server (supplemental Fig. S1) (28), residues 3–15 were assigned to the helical structure. Actually, the modeled structure of this region consists of two helices. One consists of residues 2–7 and the other of residues 11–15. As illustrated in Fig. 3B, the two helices were located almost perpendicular to each other. Leu-3 and Leu-7 in the former helix and Leu-11 and Leu-15 in the latter were aligned along the helical cylinder to form a hydrophobic environment on one side of the helix sphere. Along with the interaction of Leu-4 with both Leu-9 and Leu-11, this hydrophobic network may stabilize the tertiary structure of this region. In addition, residues 18–27 formed a helical structure in all predicted models. In this helix, Leu-21 and Val-25 were also located on one side of the helical cylinder, suggesting the hydrophobic interaction of the helix with another portion or molecule. The far-UV CD spectrum of the fragment peptide of residues 1–27 indicated a high content of helical structures (Fig. 3C), which is consistent with the predicted model. The mutant structure predicted with homology modeling was almost the same as that of the wild type, with an root mean square for Cα atoms of the whole chain of 0.52 Å (data not shown). The results suggest that, upon mutation of Leu or Val to Ala, the secondary structure of the whole molecule was hardly changed and that only the hydrophobic network inside the N-terminal region was loosened to destabilize its tertiary structure. This prompted us to investigate whether strongly hydrophobic amino acid residues in the N terminus of ATF5 were responsible for basal and inducible protein stability control. We used a transient transfection system to express point mutants (M1–M6) in the N-terminal ATF5 region, substituting leucine and valine with the low-hydrophobicity amino acid alanine (hydropathy index, 1.8) (Fig. 3A). Without a stimulus, the protein levels of ATF5 mutants M1–6 were increased compared with wild-type ATF5. In particular, the ATF5-M1 mutant, in which all leucines and valines were substituted with alanines, was increased dramatically (Fig. 3D). To exclude the possibility that leucine was important for ATF5 protein stabilization regardless of the hydrophobicity potential, we used N-terminal point mutants, ATF5-M7 and ATF5-M8, that substituted the first and second

FIGURE 2. HSP70 is responsible for IL-1β-induced up-regulation of ATF5. A, HepG2 cells were transfected with HSP70 siRNA or control scramble (Scr) siRNA 24 h before transfection with 2 μg of pSV40–3×FLAG-ATF5 and 0.5 μg of pSV40–3×FLAG-Luciferase as a control. 48 h after transfection, cells were treated with IL-1β (0.1 ng/ml) for 4 h. 3×FLAG-ATF5, 3×FLAG-Luciferase, or HSP70 were analyzed by Western blot analysis using anti-FLAG or anti-HSP70. Relative ATF5 was the ratio of 3×FLAG-ATF5 to 3×FLAG-Luciferase. ATF5 in untreated cells was set at 1.0. Each value is the mean ± S.E. of five independent experiments. **, p < 0.01 versus IL-1β (0 h). B, HepG2 cells were treated with IL-1β (0.1 ng/ml) for the indicated times. HSP70, NPM1, and β-actin were analyzed by Western blot analysis using an anti-HSP70, anti-NPM1, or anti-β-actin. Relative protein was the ratio of HSP70 or NPM1 to β-actin. Protein in untreated cells was set at 1.0. Each value is the mean ± S.E. of three independent experiments. C, HepG2 cells were transiently cotransfected with 10 μg of pSV40–3×FLAG-ATF5 and 8 μg of pSV40-HSP70-HA (left panel) or transfected with 10 μg of pSV40–3×FLAG-ATF5 alone (right panel). After 48 h, cells were treated with IL-1β (0.1 ng/ml) for 4 h. Immunoprecipitation (IP) was carried out, and 3×FLAG-ATF5 and HSP70-HA or NPM1 were analyzed by Western blot analysis. IB, immunoblot.
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N-terminal leucines for more hydrophobic amino acids, isoleucine or valine (hydrophathy indexes of 4.5 (Ile) and 4.2 (Val)), on expression plasmids. The M7 and M8 mutations did not increase ATF5 protein levels. We next examined the stress response of the ATF5 point mutants. Protein levels of the ATF5 wild type and ATF5-M1, M2, and M7 increased in the presence of IL-1β or CdCl₂ (Fig. 3, E and F). ATF5-M1 and ATF5-M2 increased with IL-1β or CdCl₂ but to a lesser extent than the ATF5 wild type and ATF5-M7. We next confirmed the importance of strong hydrophobic amino acid residues in the N-terminus of ATF5 for basal and inducible protein stability using a transient transfection system to express the ATF5 N-terminal region (amino acids 1–27) with alanines substituted for leucines and valines. Mutations are shown in red. C, CD absorption spectra of ATF5 N-terminal polypeptides. CD absorption spectra were measured as described under "Experimental Procedures." D, HepG2 cells were transiently cotransfected with 2 μg of pSV40–3×FLAG-ATF5 (1–282) or a pSV40–3×FLAG-ATF5 point mutant and 0.5 μg of pSV40–3×FLAG-Luciferase as a control. 3×FLAG-ATF5 and 3×FLAG-Luciferase were analyzed by Western blot analysis as in Fig. 1A. Relative ATF5 protein levels were the ratio of 3×FLAG-ATF5 to 3×FLAG-Luciferase. ATF5 in cells transfected with 3×FLAG-ATF5 (Wt) was set at 1.0. Each value is the mean ± S.E. of at least four independent experiments. **, p < 0.01 versus 3×FLAG-ATF5 (Wt); **, p < 0.05 versus 3×FLAG-ATF5 (Wt); E, and F, HepG2 cells were transiently cotransfected with 2 μg of pSV40–3×FLAG-ATF5 (1–282) or a pSV40–3×FLAG-ATF5 point mutant (M1, M2, and M7) and 0.5 μg of pSV40–3×FLAG-Luciferase as control. 48 h after transfection, cells were treated with IL-1β (0.1 ng/ml) for 2 h or CdCl₂ (50 μM) for 5 h (F). 3×FLAG-ATF5 and 3×FLAG-Luciferase were analyzed by Western blot analysis as in Fig. 1A. Relative ATF5 protein levels were the ratio of 3×FLAG-ATF5 to 3×FLAG-Luciferase. ATF5 in untreated cells transfected with 3×FLAG-ATF5 (Wt) was set at 1.0. Fold induction is the ratio of ATF5 protein in IL-1β-treated cells to untreated cells. Each value is the mean ± S.E. of at least four independent experiments.

Local Hydrophobicity and Aggregation Propensity Are Key Features for the Stability of ATF5 Protein—We evaluated the changes in hydrophobicity of ATF5 protein after substituting alanine for leucine and valine in the N-terminal region. Kyte-Doolittle hydrophathy values (24) were used to evaluate local hydrophobic regions in the ATF5 27-amino acid sequence (window size set to five) (Fig. 5). The plots showed that the hydrophathy scores of ATF5 amino acid residues 1–10 and 10–25, individually or together, decreased with mutations M1–6 compared with the wild type (Fig. 5). Next, we evaluated the aggregation propensity of ATF5 (amino acids 1–282) using the AGGRESCAN program (26, 27) (Fig. 6). Hot spot areas
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A

EGFP  EGFP

EGFP  ATFS (1-100) EGFP

ATFS (1-100) EGFP  ATFS (1-100) M1-EGFP

B

|        | EGFP | ATFS (1-21)-EGFP | ATFS (1-100)-EGFP | ATFS (1-100) M1-EGFP |
|--------|------|-----------------|-------------------|---------------------|
| IL-1β  | +    | -               | -                 | -                   |
| IL-1β  | -    | +               | -                 | -                   |
| IL-1β  | -    | -               | +                 | -                   |
| IL-1β  | -    | -               | -                 | +                   |

C

|        | IL-1β | CdCl₂ | MG132 |
|--------|-------|-------|-------|
| EGFP   | -     | +     | +     |
| 3×FLAG-LUC | + | +     | +     |

Fold induction

IL-1β  0.8
CdCl₂  0.9
MG132  0.9

IL-1β  1.6
CdCl₂  1.5
MG132  3.7
indicated aggregating regions of ATF5. Mutations M1–6 decreased the first and the second hot spots (amino acids 1–8 and 18–29), individually or together, compared with the wild type. The M7 and M8 mutations did not significantly decrease hydropathy scores or aggregating hot spots. Amino acid substitution from leucine or valine to alanine in the ATF5 N terminus.

**FIGURE 4.** Two hydrophobic regions of the ATF5 N terminus are essential for destabilization and inducible expression. Kyte-Doolittle hydropathy values for the N-terminal region of ATF5. The window size was set to five amino acid residues. Red, hydropathy scores of wild-type ATF5; black, point mutants (M1–M8).

**FIGURE 5.** IL-1β up-regulates ATF5 expression. A, schematic of ATF5 N-terminal region-EGFP fusions. Mutations are shown in black. B, HepG2 cells were transiently cotransfected with 2 μg of pEGFP, pATF5 (1–21)-EGFP, pATF5 (1–100)-EGFP, or pATF5 (1–100)M1-EGFP and 0.5 μg of pSV40-3×FLAG-Luciferase as a control. EGFP and 3×FLAG-Luciferase were analyzed by Western blot analysis as in Fig. 1A. C, HepG2 cells were transiently cotransfected with 2 μg of pEGFP or pATF5 (1–100)-EGFP or pATF5 (1–100)M1-EGFP and 0.5 μg of pSV40-3×FLAG-Luciferase as a control. 48 h after transfection, cells were treated with IL-1β (0.1 ng/ml) for 2 h, CdCl2 (50 μM) for 5 h, or MG132 (20 μM) for 6 h. EGFP and 3×FLAG-Luciferase were analyzed by Western blot analysis using antibodies against FLAG or EGFP. Relative EGFP protein was the ratio of EGFP to 3×FLAG-Luciferase. EGFP in cells transfected with pEGFP was set at 1.0. Fold induction was the ratio of relative EGFP in IL-1β-, CdCl2-, or MG132-treated cells to untreated cells. Each value is the mean ± S.E. of at least three independent experiments. *, p < 0.05 versus IL-1β (–), CdCl2 (–), or MG132 (–).
decreased the hydrophobicity and aggregation propensity. The decreases of these indexes influenced both basal and inducible ATF5 protein expression.

**Inhibition of Autophagic Protein Degradation Does Not Up-regulate ATF5**—Aggregation propensity in exposed protein regions correlates with autophagic protein degradation (29). Thus, we examined whether autophagic protein degradation was involved in ATF5 expression. We examined the effect of autophagic inhibition on ATF5 expression using 3-methyladenine and chloroquine (supplemental Fig. S2). 3-Methyladenine inhibits PI3K and blocks autophagosome formation. Chloroquine neutralizes the pH value inside the lysosome and inhibits lysosomal protease activities. Chloroquine induced the expression of endogenous LC3-II and p62, presumably by blocking the lysosomal degradation of LC3-II and p62 (supplemental Fig. S2). Furthermore, 3-methyladenine inhibited the
expression of endogenous LC3-II and up-regulated p62 expression, which indicated an inhibition of LC3-II-positive autophagosomes. These findings indicate that chloroquine and 3-methyladenine effectively inhibit the autophagic protein degradation. However, these inhibitors failed to up-regulate ATF5 expression, whereas the proteasome inhibitor MG132 effectively up-regulated ATF5 expression. These results show that autophagy is not the main catabolic pathway for the ATF5 protein in HepG2 cells.

**IL-1β Increases Translation Efficiency of ATF5 via 5’-UTRα and eIF2α Phosphorylation**—We showed that translation of ATF5 mRNA is regulated by the 5’-UTRα of ATF5α mRNA and phosphorylation of eIF2α after stress (15, 30). Oliver et al. (31) showed that IL-1β induces eIF2α phosphorylation in C-28/12 cells. We investigated whether IL-1β increased the translational efficiency of ATF5 mRNA via eIF2α phosphorylation. Treatment of HepG2 cells with IL-1β induced phosphorylation of eIF2α time-dependently (Fig. 7A). To examine the effect of IL-1β treatment on translational control of ATF5 mRNA, we performed luciferase assays and quantitative PCR using expression plasmids ATF5–5′-UTRα–LUC. In these plasmids, human ATF5–5′-UTRα and ATF5–5′-UTRα mt2-LUC with a point mutation for the second uAUG in ATF5–5′-UTR were subcloned upstream of the luciferase coding region (15). Luciferase activity from expression plasmids with LUC alone (5′-UTRαΔ) or ATF5–5′-UTRα mt2-LUC (5′-UTRαmt2) were not changed by IL-1β (Fig. 7B). However, luciferase activity from ATF5–5′-UTRα-LUC (5′-UTRα) was increased by IL-1β. The mRNA levels from all luciferase constructs examined were unchanged by IL-1β. These results indicate that IL-1β increases the translational efficiency of ATF5 mRNA via ATF5 mRNA 5′ UTRα and eIF2α phosphorylation.

**ATF5 Knockdown Boosts IL-1β-induced SAA1 and SAA2 Expression**—A DNA microarray analysis using mRNAs from the livers of ATF5 knockout mice indicated that SAA1 and SAA2 mRNA expression levels increased with ATF5 gene deficiency.3 Thorn et al. (32) reported that IL-1β induces SAA1 and SAA2 in HepG2 cells. We examined whether ATF5 knockdown influenced IL-1β-induced SAA1 and SAA2 mRNA expression in HepG2 cells. As shown in Fig. 8, IL-1β significantly induced SAA1 and SAA2 mRNA 79-fold and 44-fold at 4 h. ATF5 knockdown boosted IL-1β-induction of SAA1 and SAA2 mRNA 141-fold and 94-fold at 4 h. These results indicate that ATF5 down-regulates SAA1 and SAA2 expression. However, ATF5 mRNA expression was increased only 1.3-fold after 8 h of IL-1β administration.

**DISCUSSION**

In this study, we investigated the effect of IL-1β treatment of HepG2 cells on ATF5 protein levels. We showed that IL-1β increased ATF5 protein levels, at least in part, by enhancing protein stability. ATF5 regulated SAA1 and SAA2 mRNA levels in HepG2 cells treated with IL-1β. Strongly hydrophobic amino acid residues in the N-terminal region of ATF5, which are concentrated on one side of the α-helix, were responsible for basal destabilization and for stabilization in response to IL-1β.

Kyte-Doolittle hydropathy values and AGGREGSCAN analysis showed that local hydrophobicity and the aggregation propensity of the N-terminal region were important for basal ATF5 protein stability and induced expression. This region might be an ATF5-binding site to which unidentified trans_factors that control protein stability are recruited. Abundant ubiquitinated ATF5 appeared in the presence of IL-1β (data not shown) concomitant with increased ATF5 stability. Our results suggest that ubiquitinated ATF5 might be the result of accumulation of ubiquitinated proteins. Our results also suggest that IL-1β-induced ubiquitination of ATF5 could enhance protein stability prior to degradation.

Li et al. (18) showed that HSP70 interacts with ATF5 and stabilizes ATF5 protein in glioma cells by protecting ATF5 from both proteasome-dependent and caspase-dependent protein degradation. NPM1, a nucleolar chaperone protein, antagonizes the protective effect of HSP70 to promote ATF5 degradation by competition with HSP70 for ATF5 binding. NPM1 and ATF5 interact through their C-terminal regions. Therefore...

3 T. Abe, M. Kojima, S. Akanuma, H. Iwashita, T. Yamazaki, R. Okuyama, K. Ichikawa, M. Umemura, H. Nakano, S. Takahashi, and Y. Takahashi, manuscript in preparation.
nuclear ubiquitin ligases promyelocytic leukemia IV (PMLIV) and UHRF-2 have been implicated in mammalian nuclear PQC degradation (37–39). Therefore, the human nuclear PQC ubiquitin ligase might interact with hydrophobic amino acid residues in the N-terminal region of ATF5.

We showed that IL-1β increases the translational efficiency of ATF5 mRNA via ATF5 mRNA 5′ UTR and eIF2α phosphorylation. Zhou et al. (30) showed that thapsigargin-induced translation of ATF5 mRNA requires PERK-mediated eIF2α phosphorylation and ATF4 expression. IL-1β is responsible for pancreatic apoptosis and cell death, which are common features of diabetes (40, 41), and we showed that knockdown of ATF5 reduces arsenite-induced death of HepG2 cells (42). Recently, Chan et al. (43) showed that proinflammatory cytokines induce ER stress as increased phosphorylation of PERK and eIF2α, up-regulating ATF4 expression in islets cells. IL-1β induces ER stress through JNK, which determines pancreatic cell death (44). Therefore, IL-1β-induced translational efficiency of ATF5 mRNA could be regulated by PERK-mediated eIF2α phosphorylation in a JNK-dependent manner in liver and pancreatic cells. Studies on the mechanism by which ATF5 expression is regulated in pancreatic cells might be important for the development of diabetes treatment.

We also showed that ATF5 knockdown up-regulates IL-1β-induced SAA1 and SAA2 gene expression in HepG2 cells. Human SAA has four isotypes: SAA1, SAA2, SAA3, and SAA4. SAA1 and SAA2 are acute-phase isotypes generated in response to inflammatory stress and are known as acute-phase serum amyloid A proteins (45). SAA3 is a pseudogene (46). SAA4 is a constitutive isotype (C-SAA) (47). SAA1 and SAA2 expression increased several hundred-fold in response to inflammatory stress (48, 49). Acute-phase serum amyloid A proteins induce the synthesis of several cytokines that function as chemoattractants for neutrophils and mast cells, resulting in the induction of lipolysis by regulating lipid metabolism-related genes (50–53). Acute-phase serum amyloid A proteins promote cholesterol export from macrophages for recycling and reuse of cholesterol during tissue injury (54). SAA is thought to be involved in the onset of chronic inflammatory diseases such as rheumatoid arthritis and atherosclerosis, and SAA is a potential target for treatment of these diseases (55). From these reports and our results, ATF5 could be involved in chronic inflammation and lipid metabolism by regulating SAA gene expression.

Our results revealed the importance of N-terminal hydrophobic amino acids by which ATF5 expression is regulated in the IL-1β-mediated immune response. Our data support the conclusion that ATF5 is a negative regulator for IL-1β-induced SAA1 and SAA2 expression in hepatoma cells, suggesting the importance of ATF5 in inflammation.

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