Fragile X-related Protein FXR1P Regulates Proinflammatory Cytokine Tumor Necrosis Factor Expression at the Post-transcriptional Level*

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Tumor necrosis factor (TNF) is regulated post-transcriptionally by the AU-rich element (ARE) within the 3′-untranslated region of its mRNA. This regulation modulates translational efficacy and mRNA stability. By using a cRNA probe containing the TNF ARE sequence, we screened a macrophage protein expression library and identified FXR1P. Macrophages that we generated from FXR1 knock-out mice had enhanced TNF protein production compared with wild type macrophages following activation. Expression of several other proteins that are regulated by ARE sequences was also affected by FXR1P deficiency. A GFP-ARE reporter that has green fluorescent protein (GFP) expression under control of the 3′-untranslated region of TNF mRNA had enhanced expression in transfected macrophages deficient in FXR1P. Finally, we found that the ablation of FXR1P led to a dramatically enhanced association of the TNF mRNA with polyribosomes demonstrating the important role of FXR1P in the post-transcriptional regulation of TNF expression. Our data suggest that release of this repression by FXR1P occurs during lipopolysaccharide-induced macrophage activation. Finally, complementation of the knock-out macrophages with recombinant FXR1P resulted in decreased TNF protein production, supporting our findings that FXR1P operates as a repressor of TNF translation.

Tumor necrosis factor α (TNF) is a proinflammatory cytokine produced primarily by macrophages and is crucial for immune responses. The beneficial role of TNF can be perturbed upon its aberrant expression, which is involved in many inflammatory disorders (1). TNF is a major mediator of the systemic inflammatory response syndrome where high TNF levels have been associated with mortality. To protect against the detrimental aspects of TNF, its expression is under strict control.

The production of TNF is controlled both transcriptionally and post-transcriptionally by modulation of translational efficacy and mRNA stability (2). The expression of TNF is induced by various agents including bacterial LPS and IFNγ. Induction of TNF expression depends on two signals; the first signal induces transcription, and the second signal releases the normally operating translational repression that controls TNF protein expression. The sequences responsible for the translational repression and LPS inducibility are located in the 3′-UTR of TNF mRNA and consist of multiple repeats of an AUUUA motif (AU-rich element, ARE).

Previously, we mapped TNF mRNA for RNA-protein interaction and identified two protein-binding regions in the 3′-UTR (3, 4). The first protein-binding region was located inside the main ARE of TNF 3′-UTR (bases 1291–1329), and the second ARE region was 147 bases downstream. Both AREs interacted with several macrophage proteins that play an active role in the post-transcriptional regulation of TNF synthesis. We have also identified HuR as an important RNA-binding protein involved in the post-transcriptional regulation of TNF expression in macrophages (5). Along with HuR, several other proteins have been shown to target the ARE of TNF mRNA influencing its post-transcriptional control, including TTP, TIA-1, TIAR, AUF1, and CBF-A (6–12).

In this study, we demonstrate that another RNA-binding protein, FXR1P, is able to regulate TNF expression in macrophages. FXR1P is a homolog of fragile X mental retardation syndrome protein FMRP and together with FXR2P comprise the FXR (fragile X-related) family of RNA-binding proteins. The FXR proteins RNA-binding domains include a pair of hnRNPK homology (KH) domains and an RGG amino acid motif (RGG box). The proteins also have nuclear localization signal and nuclear export signal domains. Characteristics of

PBS, phosphate-buffered saline; MHC, major histocompatibility complex; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; RNP, ribonucleoprotein; hnRNPK, heterogeneous nuclear ribonucleoprotein; mRNP, messenger ribonucleoprotein; DMEM, Dulbecco’s modified Eagle’s medium; PBS, fetal bovine serum; BSA, bovine serum albumin; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor; FXR, fragile X-related; ELISA, enzyme-linked immunosorbent assay; CMV, cytomegalovirus.
the FXR proteins suggest that they are involved in post-transcriptional regulation, possibly fulfilling mRNA nucleocytoplasmic shuttling function, as well as a role in translation (13). By using macrophages derived from FXR1 knock-out mice and their wild type littermate controls, we clearly demonstrated that FXR1 is involved in the post-transcriptional regulation of TNF expression.

EXPERIMENTAL PROCEDURES

Macrophage Cell Lines—The murine macrophage cell line B10R was grown from the bone marrow of B10A mice and described previously (14). Because FXR1 knock-out mice die shortly after birth,2 newborn FXR1+/−, FXR1−/−, and FXR1+/− littermate mice were delivered by cesarean section, and liver, brain, muscle, and bone marrow cells were isolated. Genotyping to determine Fxr1 knock-out was done by multiplex PCR using the following primers: 5′-TGG TCT TCC TTT ACC AGT GTG-3′, 5′-CTT ATT GGC ACC AGT AAC AAG-3′, and 5′-TGA CTA GGG GAG GAG TAG AAG-3′. We have generated macrophage cell lines from the bone marrow, liver, and brain of FXR1−/− mice, using methods described previously (15). Results presented in this study were generated using FXR1+/− and FXR1−/− bone marrow-derived macrophages. Cells were cultured in DMEM (Invitrogen) supplemented with 7% FBS (HyClone) and maintained at 37 °C in 5% CO2. Otherwise, cells were seeded at 5 × 104/ml prior to treatments with 10 ng/ml LPS (kindly provided by C. B. Thompson) and 10 units/ml IFNγ (Hygrom (Invitrogen)).

Screening of Bacteriophage cDNA Protein Expression Library—The cDNA was prepared from mRNA isolated from IFN-γ-stimulated B10R macrophages using oligo(dT) and random primers and cloned into ZAP Express vector (Stratagene). Phage (107) were plated on XL-1 Blue E. coli (Bacteriophage cDNA Protein Expression Library) and screened for the isolated clone, hence sequence encoding the residues was added (boldface).

Transfections—Macrophages were transfected with endotoxin-free plasmids by electroporation. Cells, passaged the day prior to procedure, were resuspended at 5 × 106 in 0.4 ml of DMEM with 20% FBS. Cells were placed in a 0.4 ml electroporation cuvette with 10 μg of plasmid (pGFP-ARE (Hyg)) linearized with FspI placed on ice for 10 min, and subjected to electroporation at 2000 V for 20 ms. Cells were placed on ice for 10 min and plated in 10 ml of DMEM with 7% FBS in a 100-mm plate. The next day, the media were changed. For selection of stable transfectants, 1 μg/ml G418 or 150 ng/ml hygromycin was added to the media 48–72 h following transfection.

Protein delivery was done using the Chariot kit (Active Motif) following the instructions of the manufacturer. Macrophages were plated 1 day prior to delivery. Cells were washed with 1 ml of PBS, and the Chariot-protein complex was incubated with cells in FBS-free media for 1 h, followed by 1 ml of DMEM with 7% FBS for 1 h.

Purification of Recombinant FXR1P—FXR1P was prepared from E. coli transformed with pQE-FXR1. JM109 harboring pDC952, kindly provided by J. Walker (16), a pACYC184-derived plasmid expressing E. coli argU (encodes TargCm), was used to enhance translational efficiency of FXR1P. Log phase culture grown in LB broth at 30 °C for 4 h was induced with 1 mM IPTG for 3 h. Native protein was prepared by resuspending induced bacteria in buffer (20 mM Tris, pH 8, 100 mM NaCl, 1 mM PMSF), followed by a stringent wash with buffer containing 100 mM imidazole to remove truncated products. Fractions were eluted with buffer (20 mM Tris, pH 8, 300 mM NaCl, 10% glycerol, 0.1% Triton X-100, 1 mM PMSF) containing 100 mM imidazole. This resulted in the purification of full-length FXR1P. After dialysis against PBS, the protein was stored at −20 °C in buffer containing 20 mM Tris, pH 8, 1 mM DTT, 0.1 mM EDTA, and 10% glycerol.

The 10-kDa truncated FXR1P was prepared from E. coli M15 transformed with pQE-FXR1T. Denatured protein was prepared by lysing IPTG-induced bacteria in 8 M urea, pH 8, and purified using nickel-nitritroacetic acid resin. Elution was done either with 8 M urea, pH 6.3, 100 mM EDTA or with PBS, pH 4.5, which was brought to pH 7 after elution.

6His-FXR1P was also prepared by baculoviral system (Bio S&T). Fxr1 was subcloned by PCR (confirmed by sequencing) into a baculoviral vector. Expressed FXR1P was purified under native conditions using a nickel-nitritroacetic acid column. After dialysis against 20 mM Tris, the protein was stored at −20 °C in buffer containing 20 mM Tris, pH 8, 1 mM DTT, 0.1 mM EDTA, and 10% glycerol.

RNA Electromobility Shift Assay—RNA-binding assay and generation of cRNA probes done as described previously (3). Assays consisted of 100 ng of recombinant protein incubated with 200,000 cpm of α-32P-labeled cRNA probe in a total volume of 20 μl. Reactions were incubated at room temperature for 15 min, followed by 15 min with RNase T1 (3000 units/ml) and heparin sulfate (5 mg/ml). The cRNA was cross-linked to protein by an 8% polyacrylamide gel in 0.5× TBE, transferred to a nitrocellulose membrane and exposed to X-ray film. Samples were resolved on 4% native PAGE. Recombinant proteins tested include FXR1P, BSA, GAPDH (Sigma), and insulin (Lilly). Generated cRNAs included the following: TNF (GeneBank™ M11731; murine C57Bl/6) 3′-UTR cRNAs including TNF9 (bases 823–1611), TNF10 (bases 1291–1329), TNF11 (bases 1477–1505), 2AU (kindly provided by C. B. Thompson); and c-Fos 3′-UTR (bases 292–422). Northern analysis of 4% native PAGE. Recombinant proteins tested include FXR1P, BSA, GAPDH (Sigma), and insulin (Lilly). Generated cRNAs included the following: TNF (GeneBank™ M11731; murine C57Bl/6) 3′-UTR cRNAs including TNF9 (bases 823–1611), TNF10 (bases 1291–1329), TNF11 (bases 1477–1505), 2AU (kindly provided by C. B. Thompson); and c-Fos 3′-UTR (bases 292–422).
FXR1P Regulates TNF Expression

3698–3908) as described previously (3, 17); the construct containing IRE was kindly provided by Dr. P. Ponka.

Analysis of mRNA—Cellular RNA was prepared with Trizol reagent (Invitrogen). Northern blot analysis was done using Hybond-XL membrane (Amersham Biosciences) hybridized with DNA probes labeled with [α-32P]dCTP. Exposures were analyzed with ImageQuant (Amersham Biosciences). TNF, GAPDH, and 18 S rRNA probes were generated as described previously (18, 19). Quantitative PCR was done using the Mx4000 Quantitative PCR system and the Brilliant Two-step Quantitative RT-PCR Core and SYBR Green QPCR Reagent kits from Stratagene. Amplification was performed with 40 cycles of the following conditions: 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 30 s. Prior to the preparation of cDNA, RNA was DNase-treated using the DNA-free kit (Ambion). GFP quantitation was done by SYBR Green QPCR assay preparation of cDNA, RNA was DNase-treated using the DNA-free kit (Ambion). GAPDH, TNF, GAPDH, and 18 S rRNA probes were generated against the 10-kDa truncated FXR1P. Initial immunization was done with 100 μg FXR1P expressed in bacteria. Followed by two boosts containing Incomplete Freund’s Adjuvant (Sigma). IgG was purified from sera using Protein G-Sepharose Fast Flow (Amersham Biosciences) and eluted with 0.1 M glycine, pH 2.9, followed by dialysis against PBS. FXR-10 antibody was labeled with Alexa-488 dye (Molecular Probes), as described by the manufacturer, and used for immunofluorescence analysis.

Western Blot Analysis—To analyze the level of proteins by immunoblot, samples (in sample buffer with 50 mM DTT and heated at 70 °C for 10 min) were resolved on 4–12% NuPAGE gel, which was dried and exposed to film.

RESULTS

Screen for TNF mRNA ARE-binding Proteins: Identification of FXR1P—A prominent part of the regulation of TNF expression occurs post-transcriptionally through the ARE sequences located in the 3′-UTR of TNF mRNA that are targeted by regulatory proteins that modulate the translatability and stability of the message. To identify additional macrophage proteins capable of binding the ARE, a protein expression library derived from a murine macrophage cell line was screened by direct binding with polyclonal anti-FXR1P antibodies corresponding to the region of TNF 3′-UTR spanning the main ARE. Candidate clones were subsequently re-screened using Northwestern assay, and only clones that were consistently detected on North-western using the TNF ARE probe were further analyzed. One of several candidates was the clone designated D8, which is described in this paper.

The excised pBK-CMV phagemid from clone D8 was transformed into DH5α IQ bacterial cells and induced with IPTG, and bacterial extract containing the recombinant protein was analyzed by North-western blot analysis (data not shown). The recombinant protein, with an apparent size of 80 kDa, bound the mRNA probe TNF9 containing the 3′-UTR of TNF mRNA. Sequence analysis of clone D8 determined that it encoded the RNA-binding protein FXR1P. FXR1P RNA-binding domains include a pair of KH domains and an RGG box, as depicted in Fig. 1A.

Analysis of FXR1P Expression in Macrophages—Northern blot analysis of RNA isolated from various murine tissues revealed the presence of two transcript species of the Fxr1 gene estimated at 2.4 and 3.2 kb (Fig. 1B). Fxr1 mRNA was expressed ubiquitously in all tissues tested including the spleen, an organ that is resident to a large population of lymphocytes and macrophages. Analysis of Fxr1 gene expression in macrophages revealed two Fxr1 mRNA species (Fig. 1C). This is consistent with previously published observations and represents the several alternative splicing transcripts and differential polyadenylation sites of the Fxr1 gene (22–24). The expression of the Fxr1 mRNA in macrophages did not appear to be modulated by treatments (LPS and IFNγ) that induce TNF gene expression. Next, FXR1P was examined in macrophages by Western blot analysis (Fig. 1D). The analysis revealed the presence of FXR1P with an apparent size of 80 kDa. Treatments that modulate TNF expression did not significantly change the expressed level of FXR1P.
FXR1P was then analyzed in macrophage cells by confocal microscopy using FXR-10 antibody labeled with Alexa-488 green fluorescent dye (Fig. 1E). FXR1P was detected predominantly in the cytoplasm of cells. The antigen specificity of FXR-10 antibody was verified by blocking the antibody with the recombinant 10-kDa truncated FXR1P (data not shown).

Macrophages stably transfected with pGFP-FXR1 were examined by confocal microscopy (Fig. 1F). GFP-FXR1P fusion protein (Fig. 1F, green) was observed in the cytoplasm localized to the vicinity adjacent to the nucleus (Fig. 1F, red). Localization of GFP-FXR1P does not appear to be changed upon LPS treatment (observed for various times up to 12 h).

Recombinant FXR1P Binds RNAs That Contain an ARE—Further characterization of the binding of FXR1P to cRNA was required to establish its specificity for the ARE. Native recombinant FXR1P purified from E. coli was incubated with cRNA probes and analyzed by gel retardation. FXR1P was able to bind both TNF10 and TNF11 cRNAs corresponding to the two AREs present in 3’-UTR of TNF mRNA (delimited in diagram in Fig. 2A), but not to the IRE cRNA probe containing the iron-response element target for the iron-response protein (Fig. 2B). FXR1P-RNA complex formation also occurred with 5AU cRNA (five reiterated AUUU), but not with 2AU cRNA (two reiterated AUUU). In addition, FXR1P was able to bind c-Fos 3’-UTR cRNA probe, another transcript that has an ARE. Attempts to supershift the complex with polyclonal antibodies to FXR1P was unsuccessful, possibly because antibody recognition site was concealed by complex formation.
FIG. 2. **FXR1P binds specifically to ARE cRNA.** **A,** diagram of 3′-UTR of TNF mRNA. Identifications of cRNA probes that contain ARE regions used in the RNA electromobility shift assay are shown. **B,** the ability of FXR1P to bind ARE-containing cRNA derived from TNF was tested using RNA electromobility shift assay. Recombinant His-tagged FXR1P (100 ng) was incubated with radiolabeled cRNA that contained (TNF10, TNF11, 5AU, and c-Fos 3′-UTR) or lacked (2AU and IRE) an ARE. Complexes were resolved on a nondenatured PAGE. **C,** RNA electromobility shift assay of various protein samples incubated with radiolabeled TNF10 cRNA that contained an ARE. Protein samples contained 100 ng of recombinant FXR1P, FXR1P and 10× BSA (1 μg), BSA, insulin, or GAPDH.
FXR1P Regulates TNF Expression

To illustrate further the specificity of FXR1P for the ARE located in the 3′-UTR of TNF mRNA, other recombinant proteins were tested. As shown in Fig. 2C, recombinant FXR1P, but not randomly chosen proteins (BSA, insulin, and GAPDH), was able to bind labeled TNF10 cRNA probe that contained the ARE sequence. In addition, formation of the FXR1P-TNF10 cRNA complex was not disrupted by inclusion of 10-fold excess BSA protein in the assay. These results demonstrate that FXR1P can specifically bind to the ARE present in the 3′-UTR of TNF mRNA, and possibly to other ARE-containing mRNAs.

Generation of FXR1 Knock-out Macrophage Cell Line—To investigate the role of FXR1P in ARE-mediated regulation, we used macrophage cell lines that we immortalized from FXR1 knock-out (KO) mice and their wild type (WT) littermate controls. The Fxr1 gene was disrupted by construction of a vector with an inserted Neo cassette into the Fxr1 gene and homologous recombination in the embryonic stem cells that were employed in the generation of FXR1-KO mice. The FXR1-KO mice died soon after birth from an unidentified cause, which is indicative of the physiological importance of FXR1P. To obtain live homozygous (+/− and +/+ ) embryos, heterozygous (+/−) breeding pairs were established, and cesarean section was performed 1–2 days prior to the expected delivery date. We obtained 10 live embryos that were subsequently genotyped (Fig. 3A). Macrophage cell lines that we derived from the bone marrow of FXR1-KO mice and their WT littermate controls were used in the subsequent studies presented in this work. The genotype of these cell lines was confirmed by PCR typing using primers specific for Fxr1 and Neo genes (Fig. 3B). Disruption of FXR1P expression in the KO macrophage cell line was confirmed by Western blot analysis (Fig. 3C).

Immortalized macrophage cell lines were phenotypically characterized by FACS analysis, as shown in Fig. 3D, using a panel of antibodies against specific surface markers as described previously (14). Both the WT and KO cell lines expressed macrophage markers F4/80, FcR, MAC3, and MHC class II protein (induced with IFNγ treatment) and were negative for expression for the T-cell-specific marker CD3 and B-cell-specific marker CD19.

Effect of FXR1P Ablation on Responsiveness to Stimuli—We analyzed the WT and KO macrophages for their ability to respond to LPS and IFNγ by examining the induction of STAT1 α/β proteins. We found that both cell lines responded in similar fashion to the stimuli. As shown in Fig. 3E, both WT and KO cells similarly expressed low but detectable levels of STAT1 proteins constitutively. Following stimulation with either LPS (Fig. 3E, upper panel) or IFNγ (Fig. 3E, lower panel) for 12 or 18 h, STAT1 proteins levels were induced to a similar extent. There was no modulation at the level of FXR1P following WT macrophage activation with LPS nor with IFNγ. As expected, no FXR1P was detected in KO macrophages.

We further analyzed the WT and KO macrophages responsiveness to LPS by determining p38 MAP kinase phosphorylation state. The phosphorylation of p38 MAP kinase occurs early upon activation of macrophages and leads to activation of the p38 MAP kinase pathway that is important for ARE-mediated regulation. Western blot analysis was done by using an antibody specific for the phosphorylation state of p38 MAP kinase. As shown in Fig. 3F, LPS treatment for 0.5 h induced p38 MAP kinase phosphorylation in both the WT and KO macrophage cell lines.

Effect of FXR1P Ablation on Expression of Genes That Contain an ARE—Next we analyzed the expression of various genes that have an ARE in their 3′-UTR to determine whether FXR1P deficiency has an effect on their expression. The levels of the proteins produced from these ARE-regulated genes were determined following activation of macrophages with 100 ng/ml LPS (Fig. 4A). Our findings showed that FXR1P deficiency resulted in enhanced protein expression of TNF, IL-1α, IL-1β, IL-6, and GM-CSF.

Our initial findings suggest that FXR1P is involved in the specific regulation of ARE-containing mRNA. However, it was possible that FXR1P deficiency results in a general enhancement of translation. Based on the results of the comparative expression of several proteins (F4/80, FeR, MHC II, MAC3, STAT1, p38) in KO and WT macrophages, it was unlikely that the ablation of FXR1P would have a global enhancing effect on the overall translation rate. To further exclude this possibility, we analyzed incorporation of radiolabeled methionine into LPS-treated and -untreated KO and WT macrophages. As shown in Fig. 4B, protein extracts resolved on SDS-PAGE (upper panel), as well as trichloroacetic acid-precipitable counts (lower panel), showed very similar [35S]methionine incorporation into proteins both in KO and WT cells. These results demonstrated that there was no generalized augmentation of translational efficiency in KO compared with WT macrophages.

Analysis of TFN Gene Expression in FXR1-KO and WT Macrophages—We continued our analysis on the mechanism responsible for the enhanced expression of TNF in the FXR1P-deficient macrophages. The macrophages were activated with LPS, IFNγ, or both stimuli, and expression of TNF was measured. As shown in Fig. 5A, following activation, the production of TNF protein was elevated in KO cells relative to WT cells. FXR1P deficiency resulted in an augmentation at the level of TNF protein production of 2.6 times following LPS stimulation (p < 0.001) and 1.8 times following LPS and IFNγ stimulation (p < 0.001). The differences in TNF protein production between activated KO and WT macrophages were consistent in all (more than 10) experiments performed.

The level of TNF mRNA was compared between the macrophage cell lines following treatment with LPS for 3 h and/or IFNγ for 24 h (Fig. 5B). We found slightly higher levels (relative, 1.3 ± 0.2) of TNF mRNA in KO macrophages treated with LPS than in WT macrophages; however, it did not achieve statistical significance from analysis of seven experiments. The differences in the levels of TNF mRNA between WT and KO macrophages treated with LPS and IFNγ were even smaller. We obtained similar results when TNF mRNA levels were examined in cytoplasmic and nuclear fractions of macrophages treated with LPS (Fig. 5C). The analysis also indicates that the nucleocytoplasmic transport of TNF mRNA was operational in macrophages deficient in FXR1P. Overall, the larger effect that we observed on TNF protein production in macrophages lacking FXR1P suggests that the FXR1P role is at the level of translational control.

To verify that the affected TNF regulation was at the post-transcriptional level, an ARE-controlled reporter was used. The pGFP-ARE reporter contained the 3′-UTR of TNF mRNA cloned 3′ to GFP. This places the GFP expression under the control of both the ARE in the 3′-UTR and the constitutive CMV promoter, as illustrated in Fig. 6A. Stably transfected macrophages were characterized by confocal microscopy and FACS. A wild type macrophage cell line transfected with pGFP had high GFP expression, which was not significantly affected by LPS treatment. When the macrophage cell line was transfected with pGFP-ARE, the expression of GFP was very low, demonstrating the post-transcriptional influence of the ARE. The repression of GFP expression occurring in the macrophages transfected with pGFP-ARE was released following macrophage activation with LPS.

The WT and KO macrophage cell lines were stably transfected with the pGFP-ARE reporter to ascertain the effect of
FXR1P ablation on the post-transcriptional regulation of TNF. As observed in Fig. 6B, both cell lines showed the repressive effect of the ARE on GFP expression in nonactivated conditions that was released with activation with LPS. Most strikingly, the level of GFP expression from the pGFP-ARE reporter following activation was enhanced to a much higher level in KO compared with the WT macrophages (8.4-fold higher GFP fluorescence; \( p < 0.001 \)). Most interestingly, even in nonactivated KO macrophages, there was significant expression of GFP that was not observed in WT cells. These results indicate that a repressive control operating through the 3'-UTR of TNF mRNA is at least partially disrupted in the absence of FXR1P.

To obtain further understanding of the repressive control that FXR1P exerts, we ascertained the level of GFP-ARE mRNA in the pGFP-ARE transfectants, as presented in Fig. 6C. KO transfectants had higher levels of GFP-ARE mRNA.
compared with WT transfectants, which were enhanced with LPS treatment. The level observed in LPS-treated KO cells stems to a large extent from the basal level of GFP-ARE mRNA, which was 2.5-fold higher ($p < 0.001$) in KO compared with the WT transfectants. This suggests the possibility that FXR1P ablation causes some stabilization of the ARE-regulated mRNA, particularly in nonactivated macrophages. The differences observed at the level of mRNA are still minor compared with the large increase in GFP expression caused by FXR1P ablation that was observed at the protein level both in unstimulated and stimulated cells. Specifically, even with the observed 2.5-fold elevated GFP-ARE mRNA level in the nonactivated KO transfectants, there was a 12.4-fold elevated ($p < 0.001$) level of GFP protein in this condition, compared with the WT transfectants. Most interestingly, the level of GFP protein expression observed in the nonactivated KO transfectants was even higher than the level observed in LPS-treated WT transfectants. Overall, the results indicate that FXR1P has a repressive effect on ARE-mediated translational regulation and potentially on message stability in nonactivated macrophages.

**Analysis of the Mechanism of FXR1P in the Post-transcriptional Regulation of TNF Expression**—There are two components of ARE-mediated regulation that impact on TNF expression, the stability and translatability of its message. To ascertain whether FXR1P deficiency has an effect on the stability of TNF mRNA, message half-life was measured. Macrophages were treated for 3 h with LPS, and TNF mRNA levels were determined by Northern blot analysis at various times following blocking transcription with actinomycin D, as shown in Fig. 7A. Determination of the TNF mRNA half-life following 3 h of LPS stimulation revealed no significant difference between WT and KO macrophages (Fig. 7B). However, we have observed that KO macrophages had a slightly higher level of steady state TNF mRNA following 3-h treatment with LPS than WT macrophages. In addition, our transfection studies with the pGFP-ARE reporter showed an elevated level of GFP-ARE mRNA in KO compared with WT transfectants. These findings suggest that FXR1P deficiency had some impact on the
stability of ARE-regulated mRNA. No significant difference observed at the level of TNF mRNA half-life at 3 h of LPS treatment as used in our experiment may be due to the fact that the potential destabilizing effect regulated by FXR1P may occur prior to or very early during the course of LPS stimulation, resulting in a lack of appreciable difference at the time of measurement. To address this possibility, we measured the half-life of TNF mRNA following a shorter treatment with LPS of 1 h, and the results are displayed in Fig. 7C. As before, the stability of the TNF mRNA is similar in both WT and KO cell lines. Although the results suggest that FXR1P does not have a significant effect on TNF mRNA stability, examination of the kinetics of TNF mRNA decay following the addition of actinomycin D may suggest that there was a slight increase in transcript stability in KO macrophages early after LPS stimulation. Any potential destabilizing effect on ARE-regulated messages would occur in nonactivated macrophages, and the effect may be diminished upon activation of the macrophages with LPS, which would initiate the program that releases the repressive control on TNF expression.

To explore whether the other component of ARE-mediated regulation, translatability of the TNF mRNA, is affected by FXR1P deficiency, polysome profile analysis was performed. A representative polysome profile is illustrated in Fig. 8A. Initially, we prepared fractions from untreated and LPS-treated WT macrophages by using sucrose gradient centrifugation, and RNA-protein complexes were precipitated with ethanol. The samples were subsequently analyzed by Western blot using antibodies against FXR1P and ribosomal protein S6. The result presented in Fig. 8B clearly demonstrated FXR1P in polysome fractions, indicating that FXR1P is able to interact with the translational machinery. The distribution of FXR1P following

![Fig. 5](http://www.jbc.org/)
FXR1P Regulates TNF Expression

GFP expression levels were similar when both cell lines were stably transfected with a GFP reporter lacking the TNF 3′-UTR. C, analysis of GFP-ARE mRNA levels in transfectants. The transfected macrophages were cultured with no treatment (Media) or treated with 100 ng/ml LPS for 3 h, and RNA was extracted. The level of GFP-ARE mRNA was measured by Northern blot and densitometry analyses, and relative GFP-ARE mRNA levels (mean ± S.E.) are depicted. Also, the relative values of GFP-ARE mRNA levels measured by reverse transcription-quantitative PCR analysis using a quantitative PCR standard curve of GFP gene template (equality of template corrected with GAPDH mRNA measurement) are inserted above the respective bars. The experiment was done in triplicate. Statistical significance was determined by one-way analysis of variance test.

**DISCUSSION**

Post-transcriptional regulation operating on the ARE has a major impact on TNF expression. The regulation involves the translatability and stability of the TNF mRNA. Expression of the TNF protein that occurs upon macrophage activation depends largely upon the release from the translational repression and to an extent on mRNA stabilization. Agents that permit delivery of active protein to cells in culture. To test the Chariot delivery procedure with macrophages, recombinant β-galactosidase was delivered to KO macrophage cells. Two hours following protein delivery, cells were washed extensively and fixed, and β-galactosidase was detected by X-gal staining (Fig. 9A). The delivery procedure was successful, with ~60% of the macrophage cells showing β-galactosidase staining in the cytoplasm.

FXR1P was next delivered with Chariot peptides to KO macrophages. As shown in Fig. 9B, 2 h following delivery of FXR1P, Western blot analysis of the cell extract revealed FXR1P in the KO macrophages. By taking into account a 60% delivery efficiency, the level of FXR1P detected would be roughly half as seen in the WT macrophages. To determine whether reconstitution of KO macrophages with FXR1P has functional significance, expression of TNF protein was measured. FXR1P or β-galactosidase protein (control) were delivered to KO macrophages, and 2 h later macrophages were activated with LPS for 3 h. As shown in Fig. 9C, restoration of FXR1P to the KO macrophages resulted in reduced production of TNF protein. The level of TNF produced from reconstituted macrophages was 63% that of control FXR1P-deficient cells (p < 0.001). Overall, our results demonstrate that FXR1P is specifically involved in the post-transcriptional regulation of TNF in macrophages and acts as a negative regulator of TNF protein translation.

**FIG. 6.** Analysis of pGFP-ARE reporter to monitor post-transcriptional regulation of TNF. A, macrophage B10R was stably transfected with pEGFP-N1 or pGFP-ARE (G418R). As diagrammed, both reporter constructs have GFP under the control of the constitutive CMV promoter (P_CMV), whereas the pGFP-ARE has the 3′-UTR of TNF mRNA that contains the ARE inserted between GFP coding region and the polyadenylation signal. The cells were cultured at 3.5 × 10^6/ml with no treatment (Media) or treated with 100 ng/ml LPS for 3 h. The expression of the GFP reporter protein was monitored by confocal microscopy (upper panel) and FACS (lower panel). FACS data are representative of at least three experiments and analyzed with WinMDI. B, FXR1-WT and FXR1-KO macrophages were stably transfected with pGFP-ARE (HygR). Expression of GFP is under the control of constitutive CMV promoter and 3′-UTR of TNF mRNA. Following hygromycin selection, more than 100 individual colonies were pooled for both transfections, and the equality of plasmid load was verified by using quantitative PCR measurement of the GFP gene using genomic template. The transfected macrophages were cultured with no treatment (Media) or treated with 100 ng/ml LPS for 3 h. The confocal microscopic images show representative expression. Results (from three experiments) are tabulated in the bar chart for the level of GFP fluorescence (mean ± S.E.), which was calculated from FACS data dot blots (as depicted in the figure for B10R transfected) by multiplying % total cells that shift to the delimited region (R2) by mean fluorescence in FITC channel (FL1). The values of GFP fluorescence are also shown above the respective bars. In both confocal microscopy and FACS, a red channel was used to control for possible autofluorescence of the cells.

LPS treatment was not significantly affected. Next, WT and KO macrophages were induced with LPS for 3 h, and the cellular extracts were fractionated using sucrose gradient centrifugation. RNA extracted from the fractions was blotted, and TNF mRNA levels were determined. As shown in Fig. 8C, the level of TNF mRNA was elevated on ribosomes in the KO macrophage. Most importantly, the elevated TNF mRNA in the KO macrophages was even more pronounced in all fractions associated with the higher molecular weight polyribosomes (p < 0.001). This indicates that FXR1P deficiency results in an enhancement in the translatability of the TNF mRNA. This suggests that FXR1P is active in translational repression of TNF mRNA, which is released in a restricted fashion upon macrophage activation.

Reconstitution of FXR1-KO Macrophage Cell Line with Recombinant FXR1P—Our results demonstrate that macrophages deficient in FXR1P have an alteration in ARE-mediated regulation and indicate that FXR1P is involved in the translational repression of TNF. As support for this functional role, FXR1P was restored to KO macrophages. This was accomplished with Chariot peptides (Active Motif) that permit delivery of active protein to cells in culture. To test the Chariot delivery procedure with macrophages, recombinant β-galactosidase was delivered to KO macrophage cells. Two hours following protein delivery, cells were washed extensively and fixed, and β-galactosidase was detected by X-gal staining (Fig. 9A). The delivery procedure was successful, with ~60% of the macrophage cells showing β-galactosidase staining in the cytoplasm.

FXR1P was next delivered with Chariot peptides to KO macrophages. As shown in Fig. 9B, 2 h following delivery of FXR1P, Western blot analysis of the cell extract revealed FXR1P in the KO macrophages. By taking into account a 60% delivery efficiency, the level of FXR1P detected would be roughly half as seen in the WT macrophages. To determine whether reconstitution of KO macrophages with FXR1P has functional significance, expression of TNF protein was measured. FXR1P or β-galactosidase protein (control) were delivered to KO macrophages, and 2 h later macrophages were activated with LPS for 3 h. As shown in Fig. 9C, restoration of FXR1P to the KO macrophages resulted in reduced production of TNF protein. The level of TNF produced from reconstituted macrophages was 63% that of control FXR1P-deficient cells (p < 0.001). Overall, our results demonstrate that FXR1P is specifically involved in the post-transcriptional regulation of TNF in macrophages and acts as a negative regulator of TNF protein translation.
FXR1P Regulates TNF Expression

FXR1P is a member of the FXR proteins, which also includes FMRP and FXR2P. The FXR proteins bind RNA by means of two KH domains and an RGG box and may specifically interact with a subset of mRNAs (25–28). The FXR proteins are components of mRNP complexes that are localized predominantly in the cytoplasm and have been found in association with polyribosomes (20, 26, 29–32). Two additional functional domains conserved by the FXR proteins are the nuclear localization signal and the nuclear export signal (30). The characteristics of FXR proteins suggest a role in translation regulation as well as in nucleocytoplasmic shuttling. The predicted behavior of FXR1P correlates with the characteristics of the TNF mRNA-binding proteins that are present in ribonucleoprotein complexes that have association with polyribosomes. Observation of FXR1P in macrophages showed perinuclear localization, and its capability of interacting with polyribosomes may indicate that it is bound to ribosomes on the rough endoplasmic reticulum, where TNF protein is produced and is then routed to the Golgi apparatus for processing and secretion. The regulation of TNF may involve interaction of FXR1P with TNF mRNA via its ARE to form an mRNP, which would transport from the nucleus to the cytoplasm, where the mRNP would localize to particular ribosomes located on the endoplasmic reticulum for TNF translation.

Recently, FMRP was shown to recognize the G quartet RNA motif (33, 34). The G quartet motif is recognized by the RGG box of FMRP, which potentially allows the KH domains to define additional target RNA motifs. It remains to be ascertained whether the specificity for the G quartet extends to the other FXR proteins. Earlier experiments to define the specificity of the FXR proteins using homopolymer RNA showed they had affinity in vitro for both poly(G) and poly(U) RNA (28, 35), perhaps predictive of the G quartet and ARE specificity, respectively. This is supported by recent evidence that FMRP can also target uridine-rich sequences (36, 37). Thus, it seems that the FXR proteins recognize various RNA motifs, targeting the corresponding transcripts to the post-transcriptional regulatory machinery.

Most interestingly, FMRP was shown to inhibit the translation of a subset of mRNA to which it binds in an in vitro translation system (38, 39). Repression of translation was also observed in cells for FMRP and Drosophila dFXR protein (40, 41). Our studies demonstrated that FXR1P was able to bind TNF mRNA, and macrophages deficient in FXR1P had enhanced TNF expression coupled with increased translatability of the TNF mRNA due to more efficient association of TNF mRNA with polyribosomes, as well as increased expression of an ARE-controlled GFP reporter. Therefore, we hypothesize that FXR1P functions in a similar repressive role in the regulation of TNF gene expression. This is further supported by the transient complementation of the FXR1-KO macrophages with FXR1P that resulted in decreased TNF protein induction, suggesting the restoration of the translational repressive control on TNF expression. Hence, the repression of TNF translation would involve FXR1P, with the release of this repression during macrophage activation. The repressive function of FXR1P may be inactivated following macrophage activation via the p38 or JNK kinase pathways that are involved in TNF post-transcriptional regulation.

Two uridine-rich binding proteins, TIA-1 and TIAR, were shown to be involved in the repression of TNF translation (11, 12). Activated macrophages deficient in TIA-1 have 2–3-fold elevated TNF protein expression, without a significant change in TNF mRNA. This suggests that the repression of TNF translation is a complex event, involving more than one repressive mechanism or a single regulation in which FXR1P and TIA-1 are components. Possible functional association of FXR1P and TIA-1 was suggested by results that show that FMRP, together with FXR1P and TIA-1, can be detected in cytoplasmic stress granules that appear to also contain mRNAs that are translationally silenced (40). This suggests a means for the translational repression by FXR1P, at least during cellular stress.

Another intriguing manner in which translational repres-
tion may work was the observation that FMRP phosphorylated by casein kinase II was found associated with stalled polyribosomes (42, 43). This mechanism would gear up for translation but then keep it on hold until the required cellular signal was generated. Also, this stalling could eventually lead to the delivery of the mRNP to the stress granule. The phosphorylation site found in FMRP that controls the stalling of the polyribosomes was found to be conserved in FXR1P. This suggests that FXR1P may have similar control on translation of the mRNA with which it associates.

In addition to the involvement of FXR1P in the control of translational repression, our experiments suggest the possibility that it also has a potential destabilizing effect on ARE-containing mRNA, particularly in nonactivated macrophages.

**Fig. 8. Analysis of TNF mRNA translatability in FXR1-KO macrophages.** A, representative polysome profile. B, FXR1P association with polysomes. WT macrophages were either untreated or LPS treated for 3 h and cellular extract fractionated by sucrose gradient centrifugation, and 400-μl fractions were ethanol-precipitated. RNP complexes were solubilized in 80 μl of sample buffer, and equal aliquots were analyzed by Western blot for FXR1P and ribosomal protein S6. C, polysome profile of TNF mRNA in FXR1-WT and FXR1-KO macrophages. Macrophages were treated with LPS for 3 h, and cellular extract was fractionated on a sucrose gradient. RNA was isolated from the fractions and used to do Northern slot blot analysis. The blots were probed for TNF and GAPDH mRNA. Levels of TNF mRNA (quantified by densitometry), corrected to GAPDH, were plotted (mean ± S.E.). The experiment was repeated four times. *p < 0.001* WT fractions 7–10 compared with corresponding KO fractions. Statistical significance was determined by a one-tailed Student’s *t* test.
FXR1P Regulates TNF Expression

Fig. 9. Reconstitution of the FXR1-KO macrophage cell line with recombinant FXR1P. A, delivery of active protein to macrophages using Chariot peptides. KO macrophages seeded at 3 × 10^5 cells in a 35-mm plate were delivered with 1 μg of β-galactosidase (β-gal) complexed with Chariot peptides. Cells were washed and fixed, and the β-galactosidase (stained blue with X-gal) was observed (inset, ×10). B, delivery of FXR1P to KO macrophages. KO macrophages seeded at 1 × 10^5 cells in a 24-well plate were delivered with 1 μg of recombinant native FXR1P (baculovirally expressed) or β-galactosidase that was complexed with Chariot peptides. Cells were washed and boiled in SDS lysis buffer, and the β-galactosidase that was complexed with Chariot peptides. KO macrophages seeded at 1 × 10^5 cells in a 24-well plate were delivered with 1 μg of recombinant native FXR1P (baculovirally expressed) or β-galactosidase that was complexed with Chariot peptides. Cells were washed and boiled in SDS lysis buffer, and the β-galactosidase (stained blue with X-gal) was observed (inset, ×10). C, effect on TNF expression following FXR1P reconstitution of KO macrophages. KO macrophages were delivered with 1 μg of protein complexed with Chariot peptides. Following protein delivery, macrophages were treated with LPS for 3 h. Supernant was tested for TNF production by ELISA. Results (mean ± S.E.) for the experiment were done in quadruplicate, and differences in TNF induction are representative of three experiments. Statistical significance was determined by a one-tailed Student’s t test.

This is supported by the slight increase in TNF mRNA in FXR1-KO compared with WT macrophages, possibly resulting from the slight increase in stability of TNF mRNA as suggested by the actinomycin D chase experiment, as well as by the pGFP-ARE reporter studies. FXR1P may function directly in control of mRNA stability, or it may interact with and influence the function of other proteins that regulate TNF mRNA stability such as HuR, TTP, AUF1, or CBF-A.

The importance of ARE-mediated regulation on TNF expression was shown using mice in which the ARE is deleted. These mice showed high levels of serum TNF and inflammatory disorders such as arthritis (44). High levels of TNF are problematic in several chronic inflammatory diseases. Deregulation of the ARE-regulatory machinery may be underlying many of these diseases. For instance, TTP knock-out mice have high serum TNF levels leading to an inflammatory disorder (9, 45). High levels of TNF from the FXR1-KO macrophages suggest that a similar inflammatory outcome may result; however, because FXR1-KO mice die, this could not be determined. However, less pronounced alterations of FXR1P may have detrimental effects on the inflammatory process. A suggestion that FXR1P is involved in chronic inflammation in human was the discovery that FXR1P is an autoantigen in systemic sclerosis, a rheumatic disease (46). Systemic sclerosis patients have elevated levels of serum TNF that may contribute to the inflammatory disorder (47). Defining the proteins involved in the regulation of TNF expression will permit the unraveling of inflammatory diseases and provide targets for treatments.

Overall, our findings demonstrate that FXR1P is involved in the post-transcriptional regulation of TNF expression through its interaction with the ARE located in 3′-UTR of TNF mRNA. Our results demonstrate that the presence of FXR1P is required for the efficient translational repression of TNF. This highlights the prime importance of the ARE-mediated regulation of TNF, where TNF expression is maintained in a repressed state by translational repression. This repressive control of FXR1P would be released upon macrophage activation. These findings are of importance for understanding the regulation of ARE-containing genes and elucidating the functional involvement of the FXR family of proteins.

Acknowledgments—We thank Dr. G. Dreyfuss for providing embryos derived from the FXR1 knock-out mice and littermate controls. We also thank Drs. P. Ponka, J. Walker, and C. B. Thompson for kindly providing plasmids.

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