Identification of TIAR as a Protein Binding to the Translational Regulatory AU-rich Element of Tumor Necrosis Factor α mRNA*

(Received for publication, October 5, 1998)

Cyril Gueydan‡§, Louis Droogmans‡, Pascale Chalon‡, Georges Huez‡, Daniel Caput§, and Véronique Kruys‡‡

From the ‡Laboratoire de Chimie Biologique, Université Libre de Bruxelles, 67 rue des chevaux, 1640 Rhode Saint Genèse, Belgium and §Sanoﬁ Els Biorecherches, 31676 Labége Cedex, France

In monocyte/macrophages, the translation of tumor necrosis factor α (TNF-α) mRNA is tightly regulated. In unstimulated cells, translation of TNF-α mRNA is blocked. Upon stimulation with lipopolysaccharides, this repression is overcome, and the mRNA becomes efficiently translated. The key element in this regulation is the AU-rich element (ARE). We have previously reported the binding of two cytosolic protein complexes to the TNF-α mRNA ARE. One of these complexes (complex 1) forms with extracts of both unstimulated and lipopolysaccharide-stimulated macrophages and requires a large fragment of the ARE containing clustered AUUUUA pentamers. The other complex (complex 2) is only detected after cell activation, binds to a minimal UUAUUUAUU nonamer, and is composed of a 55-kDa protein. Here, we report the identification of the RNA-binding protein TIAR as a protein involved in complex 1. The RNA sequence bound by TIAR and the cytoplasmic localization of this protein in macrophages argue for an involvement of TIAR in TNF mRNA posttranscriptional regulation.

Tumor necrosis factor-α (TNF-α)1 is a cytokine predominantly produced by macrophages but also by lymphocytes, NK cells, astrocytes, and other cell types. The most powerful inducers of TNF-α production by macrophages are the lipopolysaccharides (LPS), which are membrane components released by Gram-negative bacteria in the course of infection (1). It is now well established that the induction of TNF-α production upon stimulation of macrophages by LPS results from both an enhancement of TNF-α gene transcription and a translational derepression of the mRNA. In unstimulated macrophages, TNF-α mRNA translation is blocked. Upon stimulation with LPS, this repression is overcome, and TNF-α mRNA becomes efficiently translated (2). The key element involved in this regulation is the AU-rich element (ARE) located in the 3′-untranslated region (−UTR) of TNF-α mRNA (3). This 70-nucleotide-long sequence is composed of several repeats of the AUUUUA pentamer. The physiological importance of TNF-α mRNA translational control is demonstrated by the fact that the expression of a TNF-α transgene lacking its 3′−UTR in mouse leads to severe inflammatory disorders (4).

Similar AREs are found in the 3′−UTR of a growing number of mRNAs encoding cytokines, protooncogenes, or other transiently expressed proteins (5). These sequences have also been shown to regulate mRNA stability (6).

In former studies, we reported that TNF-α mRNA ARE can form two complexes with proteins present in cytosolic macrophage extracts. One of these complexes (complex 1) forms with extracts of both unstimulated and LPS-stimulated macrophages and requires a large fragment of the ARE containing clustered AUUUUA pentamers. The other complex (complex 2) is only detected after cell activation, binds to a minimal UUAUUUAUU nonamer, and is composed of a 55-kDa protein (7, 8).

To identify the proteins involved in both complexes, we designed a cloning strategy based on the differential screening of a macrophage cDNA expression library with TNF-α mRNA 3′−UTR riboprobes containing or not the ARE. By this method, we isolated the cDNA encoding the short 40-kDa isoform of the RNA-binding protein TIAR. We show that TIAR specifically binds TNF-α ARE and corresponds to the protein involved in the formation of complex 1. Moreover, analysis of TIAR subcellular localization by immunostaining reveals that TIAR is mainly found in the cytoplasm of murine macrophages.

EXPERIMENTAL PROCEDURES

Materials—Enzymes used in this study were purchased from Boehringer Mannheim and Life Technologies Inc. LPS (Escherichia coli strain 0127:B8), diethyl pyrocarbonate, and anti-actin antibody were obtained from Sigma. Isopropyl-1-thio-b-D-galactopuranoside and oligonucleotides were purchased from Life Technologies Inc. Lysozyme was purchased from Appligene Oncor. DNase I and polyC were purchased from Amersham Pharmacia Biotech.

Goat anti-TIAR polyclonal antibody directed against a C-terminal peptide of TIAR and rabbit anti-NF-κB antibody were purchased from Santa Cruz. The goat IgG control antibody was purchased from Rockland (Gilbertsville, Pa). Mouse actin cDNA cloned in the pBluescript SK− phagemid was purchased from Stratagene.

Cell Culture—RAW 264.7 mouse macrophages were maintained as described previously (7). LPS was added at a final concentration of 10 ng/ml for 2 h in all experiments.

Expression Library Screening—A RAW 264.7 mouse macrophage cDNA library was prepared according to a previously described method (5) and was inserted into the pUC-19 vector within the PstI and BamHI restriction sites. The library or the pGEX3X-3C7R plasmid-expressing AUF1 (generously provided by Dr. Gary Brewer, Bowman Gray School of Medicine, Wake Forest University, Winston Salem, North Carolina) or the pGEX5X-1-actin plasmid-expressing actin (a gift from V. Dilbeck, University of Brussels) were electroporated into MC1061 bacteria and plated to obtain 10,000 colonies/dish (dish diameter: 13.5 cm). After overnight incubation, 1 replicate/dish was performed on nitrocellulose filters (Schleicher & Schuell). These “master” replicates were used to perform secondary (2 to 5) replicates. The master and the secondary replicates were then placed in dishes containing ampicillin (100 µg/ml).
or ampicillin (100 μg/ml) and isopropyl-1-thio-b-D-galactopyranoside (1 mm), respectively, and incubated overnight at 37 °C. The master replicates were subsequently stored at 4 °C. The secondary replicates were hung up in a sealed tube containing a 1-cm layer of pure chloroform for 35 min. Filters were then transferred into plastic bags (6 filters/bag) and soaked in 200 ml of autoclaved washing buffer (50 mM Tris, pH 8, 150 mM NaCl, 5 mM MgCl₂, 1/1000 (v/v) diethyl pyrocarbonate) containing lysozyme (1 mg/ml) and DNase I (1 mg/ml) for 2 h at room temperature with light shaking. The washing buffer was then replaced by 300 ml of fresh washing buffer, and the incubation was prolonged for 4 additional h. The washing buffer was replaced for a second time, and the filters were soaked overnight. The filters were then rinsed three times for 5, 30, and 60 min, respectively, in 300 ml of binding buffer (40 mM Tris, pH 8, 8 mM EDTA, 200 mM NaCl, 3.6 mM 2-mercaptoethanol, 1/1000 (v/v) diethyl pyrocarbonate) at room temperature with light shaking. For the binding with the RNA probe, each membrane was transferred into a plastic bag containing 35 ml of binding buffer and incubated for 10 min at room temperature. Heparin (0.55 mg/ml) was added, and a subsequent incubation of 10 min was performed. Poly(C) (10 μg/ml) was then added, and the membranes were incubated for an additional 30 min. Finally, 100 x 10⁶ cpm of riboprobe was added per bag, and the bags were incubated overnight at room temperature with light shaking. The membranes were washed twice for 10 min in 100 ml of binding buffer at room temperature with light shaking; dried for 15 min on ice; and thawed. The protein concentration in the extracts was determined by a low absorbance spectrophotometer at 210 nm.

In Vitro Transcription—The DNA constructs used in this study were previously described (7). The riboprobe used for the screening procedure and electrophoretic shift assay (EMSA) were synthesized with the transcription kit purchased from Epicentre (Madison, WI) according to the following method. Briefly, to generate 100 x 10⁶ cpm (approximately 1.5 x 10⁶ cpm/μg), 4 μl of SP6 transcription buffer 5 x, 5 μl of 100 μm dithiothreitol, 1 μl of 10 mM ATP, 1 μl of 10 mM CTP, 1 μl of 10 mM GTP, 3 μl of 1 mM UTP, 80 μCi of [α-32P]UTP (800 Ci/mmole), 3 μl of linearized DNA (0.5 μg/μl), and 1 μl of SP6 RNA polymerase were mixed and incubated for 2 h at 37 °C. The transcription reaction was then treated with DNase for 15 min, brought to a volume of 50 μl with H₂O, and extracted with phenol/chloroform. The riboprobe was then purified on a P10 minicolumn, and the volume was increased to 300 μl. The probes were stored at -70 °C.

DNA Sequencing—DNA sequencing was performed using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Pharmacia Biotech). EMSA—S100 macrophage extracts and EMSA were carried out exactly as described previously (7). Supershifts with anti-TIAR or control antibodies were performed by incubating 15 μg of S100 macrophage extract with 0.2 μg of anti-TIAR antibodies or control antibodies for 25 min on ice in a total volume of 15 μl before the EMSA. The EMSA were electrophoresed on nondenaturing 3.5% polyacrylamide gels.

Immunodepletion of Macrophage Extract before EMSA—EMSAs were carried out with 50 μg of S100 macrophage extract by incubating 0.2 μg of anti-TIAR antibodies or control antibodies for 25 min on ice in a total volume of 15 μl before the EMSA. The EMSA were electrophoresed on nondenaturing 3.5% polyacrylamide gels.

Binding of TIAR to the AU-rich Element of TNF-α mRNA—Expression Library Screening for TNF-α ARE-binding Protein—TNF-α ARE sequence was previously shown to form two different complexes with proteins of macrophage S100 extracts. Two of these complexes differ in their electrophoretic mobilities and in their recognition motifs within the ARE. Moreover, although the complex of low electrophoretic mobility (complex 1) can be detected with S100 extracts from both unstimulated and LPS-stimulated macrophages, the other complex (complex 2) is formed only upon LPS stimulation (7, 8). To clone the cDNAs encoding the proteins involved in these complexes, we developed an expression library screening method. We set up this method with a plasmid encoding AUF1, which has been shown to bind to AREs derived from several mRNAs (11). As negative control, we used a plasmid encoding actin. Bacteria expressing either AUF1 or actin were plated and replicated to perform a binding with TNF-α mRNA 3′-UTR riboprobes containing or not containing the ARE (Fig. 1 and “Experimental Procedures”). Although TNF 3′-UTR riboprobe bound to the replicate of bacteria encoding AUF1, no signal could be detected with bacteria expressing actin. The binding of TNF 3′-UTR riboprobe to AUF1-expressing colonies involves the ARE, because no signal was detectable with the TNF 3′-UTRAU riboprobe (Fig. 2).}

We then screened the RAW 264.7 macrophage cDNA library in the same conditions to identify clones encoding proteins specifically binding TNF ARE. In a first step, clones detected by the binding of TNF 3′-UTR riboprobe were isolated and replated at high density. Two replicates of the resulting plates were prepared as described previously and submitted to a second differential screening with 3′TNF and 3′TNF3AU probes. As illustrated in Fig. 3, one of the clones detected by the first screening specifically bound to 3′TNF probe in the secondary differential screening. The cDNA of five independent colonies detected with the 3′TNF probe were sequenced, and all were encoding the 40-kDa isoform of the RNA-binding protein TIAR.

**TIAR Is Involved in the Formation of Complex 1**—To determine whether TIAR is involved in one of the two complexes that can form with TNF ARE, we analyzed the binding ability of TIAR to the 3′d2 probe (Fig. 1), which has been previously shown to selectively form complex 2 and not complex 1 (Ref. 8 and Fig. 4A). As illustrated in Fig. 4B, the 3′d2 riboprobe binds very weakly to TIAR-expressing colonies, indicating that TIAR might not be involved in complex 2. We next performed EMSAs with TNF 3′-UTR riboprobe and macrophage extracts in the presence of anti-TIAR or control IgG antibodies. Fig. 5A shows that the addition of anti-TIAR antibody in the EMSA markedly alters the electrophoretic mobility of complex 1 in comparison with a control IgG. On the other hand, complex 2 migration is not affected by anti-TIAR antibody, further confirming that this complex does not involve TIAR. To corroborate the involvement of TIAR in complex 1, we performed EMSAs with macrophage extract depleted of TIAR by immunoprecipitation with anti-TIAR antibody (expression library screening method). A significant decrease in complex 1 intensity was observed as compared with the level obtained when the immunodepletion was performed with a control IgG (Fig. 5B). In parallel, we verified by Western blot analysis that immunodepletion of the extract by anti-TIAR antibody correlated with an increase of TIAR present in the immunoprecipitated protein G-Sepharose pellet (data not shown).
Tiar Expression in Macrophages—tiar has been previously described as a widely expressed gene at least at the mRNA level. Moreover, tiar gene can be expressed into two isoforms of 40 and 42 kDa (TIARb and TIARa, respectively), resulting from alternative splicing of the precursor mRNA (12). TIARa contains an additional 17-amino acid stretch in the first of the three RNA recognition motifs. Because of its location in a loop of the first RNA recognition motif, this 17-amino acid peptide has been suggested to be important for the protein RNA binding specificity (13).

We characterized the expression of tiar gene in unstimulated and LPS-stimulated macrophages at the RNA and protein levels. The Northern blot analysis of TIAR mRNA accumulation shows that TIAR mRNA is equally expressed as a 1.6-kilobase transcript in both unstimulated and LPS-stimulated RAW cells (Fig. 6A). The resolution of the gel electrophoresis did not allow the separation of the mRNA species encoding the two isoforms. At the protein level, we observed that the 40-kDa TIARb is significantly more expressed than the 42-kDa TIARa. As at the RNA level, TIAR proteins are equally expressed in unstimulated and LPS-stimulated macrophages (Fig. 6B).

Subcellular Localization of Tiar in Macrophages—Tiar, which is a protein related to TIA-1, has been initially described to trigger DNA fragmentation in permeabilized thymocytes (14) and to be localized mainly in the nucleus. However, upon Fas-mediated apoptosis, Tiar has been shown to be translocated to the cytoplasm (15). Because we identified Tiar as a...
component of complex 1, which is formed upon incubation of TNF ARE with macrophage S100 cytosolic extracts, we determined TIAR subcellular localization in macrophages by an immunostaining analysis. This experiment showed that similarly to NF-κB, TIAR is predominantly found in the cytoplasm of RAW macrophages (Fig. 7). This cytoplasmic localization of TIAR correlates with its ability to form complex 1 from S100 cytosolic extract.

**DISCUSSION**

It is now well established that AREs play a major role in the post-transcriptional regulation of several transiently expressed genes. These elements have been classified in two main categories according to the number and the distribution of AUUUA pentamer repeats. Class I AREs contain one to three copies of the AUUUA pentamer in a U-rich context. Prototypes of these class I AREs are found in the 3′-UTR of c-Myc and c-Fos mRNAs and confer mRNA instability. Class II AREs are defined by the presence of multiple clustered pentamers and are preferentially found in the 3′-UTR of cytokine mRNAs (16). Depending on the systems considered, these AREs mediate mRNA instability and/or translational blockade. Several proteins have been reported to bind AREs (reviewed in Refs. 17 and 18). However, based on their molecular mass and sequence affinity, none of these factors seemed to correspond to the proteins involved in the complexes formed with TNF ARE that we previously described. The expression library screening method with RNA probes has been successfully used to clone cDNAs of proteins specifically binding to RNA through different types of RNA binding domains (19, 20). Therefore, we developed a similar but simplified strategy based on the direct screening of bacterial colonies transformed with cDNA-expressing plasmids. This method led to the identification of TIARb as a protein specifically binding TNF ARE. Moreover, we have confirmed by supershift experiments that TIAR corresponds to the constitutive TNF ARE-binding protein present in macrophage cytosolic extract previously described as complex 1. Indeed, EMSA performed in the presence of anti-TIAR antibody markedly alters the electrophoretic mobility of complex 1. However, it should be noted that the band corresponding to complex 1 is not totally supershifted and that increasing the amount of anti-TIAR antibody does not modify the supershift ratio (data not shown). This might result from a limited affinity of anti-TIAR antibody. Alternatively, the remaining band could correspond to another complex co-migrating with complex 1. The supershift of complex 1 by anti-TIAR antibody markedly alters the electrophoretic mobility of complex 1. However, it should be noted that the band corresponding to complex 1 is not totally supershifted and that increasing the amount of anti-TIAR antibody does not modify the supershift ratio (data not shown). This might result from a limited affinity of anti-TIAR antibody. Alternatively, the remaining band could correspond to another complex co-migrating with complex 1.

**FIG. 4.** Comparison of the formation of complexes 1 and 2 and the binding specificity of TIAR to TNF ARE. A, EMSA performed with cytosolic (cyto.) extract of RAW cells induced with LPS (10 ng/ml, 2 h) and 3′TFN or 3′d2 RNA probe. B, comparison of the binding specificity of TIARb to the 3′TFN and 3′d2 probes. The probes were labeled with [32P]UTP, and an equal amount of cpm for each probe was used in the binding reactions.

**FIG. 5.** Identification of TIAR as a component of complex 1. A, supershift of complex (Compl.) 1 by anti-TIAR antibody. EMSA experiment was performed with cytosolic extract from noninduced (NI) or LPS-activated RAW cells and the 3′TFN probe in the presence of anti-TIAR antibody or control IgG. The figure is representative of four independent experiments. B, EMSA was performed with macrophage extract immunodepleted with either an anti-TIAR antibody or a control IgG (expression library screening method). The figure is representative of two independent experiments.

**FIG. 6.** Analysis of TIAR expression in RAW macrophages. A, Northern blot analysis of total RNA from noninduced (NI) or LPS-activated RAW cells using a TIAR or actin cDNA probes. The size of the corresponding mRNAs is indicated. B, Western blot analysis of total protein extract from noninduced or LPS-activated RAW cells using an anti-TIAR or anti-actin antibody. The molecular mass of the proteins is indicated. The figure is representative of three independent experiments.
polypeptide in Western blot. This 50-kDa protein is not detected in Western blot experiments performed with RAW 264.7 cell extract and an anti-TIAR antibody directed against a C-terminal peptide of TIAR (Fig. 6 and “Experimental Procedures”).

Although TIAR was known as a RNA-binding protein (13), this study identifies the first RNA regulatory sequence recruiting TIAR. We have previously reported that a cluster of five overlapping AUUUA pentamers is the minimal sequence within TNF ARE required for complex 1 formation. We have also shown that complex 1 can form with TNF and granulocyte-macrophage colony-stimulating factor class II AREs and not with c-myc class I ARE. Therefore, TIAR being a component of complex 1 can be considered as a class II ARE-specific binding protein.

TNF mRNA is both unstable and translationally regulated in macrophages (2, 22). Recently, tristetrapolin, a CCCH zinc finger protein, has been reported to mediate TNF mRNA destabilization in macrophage by interacting with TNF mRNA ARE (18), but the proteins involved in the translational control of TNF mRNA are not identified yet. As TIAR is located in the cytoplasm of macrophages and binds to TNF ARE independently of LPS treatment of the cells, it could mediate TNF translational repression. Activation of macrophages with LPS leads to the release of the translational blockade and is accompanied by the binding of a 55-kDa protein on TNF ARE, which could be responsible for this effect (8).

The identification of the 55-kDa protein along with further characterization of TIAR function in macrophage will provide more insight into the mechanism of cytokine mRNA translational control mediated by AREs.

Acknowledgments—We thank P. Defrance for excellent technical assistance, T. De Smedt and B. Pajjak for their expertise with the immunostaining technique, M. Kaggad for help in bioinformatic analysis, T. Lewis, and D. Discon for helpful discussion.

REFERENCES

1. Beutler, B. (1992) Tumor Necrosis Factors: The Molecules and Their Emerging Role in Medicine, pp. 485–513, Raven Press, Ltd., New York

2. Han, J., Brown, T., and Beutler, B. (1990) J. Exp. Med. 171, 465–475

3. Han, J., and Beutler, B. (1990) Eur. J. Immunol. 20, 71–73

4. Kefler, J., Puchert, L., Carlaw, H., Georgopoulos, S., Kaslaris, E., Kiousis, D., and Kollias, G. (1991) EMBO J. 10, 4025–4031

5. Captur, D., Beutler, B., Hartog, K., Thayer, R., Brown Shimer, S., and Cerami, A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1670–1674

6. Chen, C. Y., and Shyu, A. B. (1995) Trends Biochem. Sci. 20, 465–470

7. Gueydan, C., Houzet, L., Marchant, A., Sels, A., Huez, G., and Kruys, V. (1996) Mol. Med. 2, 479–488

8. Lewis, T., Gueydan, C., Huez, G., Teulon, J., and Kruys, V. (1998) J. Biol. Chem. 273, 13781–13786

9. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

10. Hanbru, C., Lafon, I., Audigier, S., Gensac, M. C., Vagner, S., Huez, G., and Prats, A. C. (1997) J. Biol. Chem. 272, 32061–32066

11. DeMaria, C. T., and Brewer, G. (1990) J. Biol. Chem. 265, 12179–12184

12. Beck, A. R., Medley, Q. G., O’Brien, S., Anderson, P., and Streuli, M. (1996) Nucleic Acids Res. 24, 3829–3835

13. Dembner, L. M., Kim, N. D., Liu, K. Q., and Anderson, P. (1996) J. Biol. Chem. 271, 5783–5788

14. Kawakami, A., Tian, Q., Duan, X., Streuli, M., Schlossman, S. F., and Anderson, P. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8681–8685

15. Taupin, J. L., Tian, Q., Kedersha, N., Robertson, M., and Anderson, P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1629–1633
Identification of TIAR as a Protein Binding to the Translational Regulatory AU-rich Element of Tumor Necrosis Factor α mRNA

Cyril Gueydan, Louis Droogmans, Pascale Chalon, Georges Huez, Daniel Caput and Véronique Kruys

J. Biol. Chem. 1999, 274:2322-2326.
doi: 10.1074/jbc.274.4.2322

Access the most updated version of this article at http://www.jbc.org/content/274/4/2322

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 19 references, 11 of which can be accessed free at http://www.jbc.org/content/274/4/2322.full.html#ref-list-1