Tumor Necrosis Factor-α-induced Adipose-related Protein (TIARP), a Cell-surface Protein That Is Highly Induced by Tumor Necrosis Factor-α and Adipose Conversion*

Tumor necrosis factor-α (TNFα) is involved in the physiological and biological abnormalities found in two opposite metabolic situations: cachexia and obesity. In an attempt to identify novel genes and proteins that could mediate the effects of TNFα on adipocyte metabolism and development, we have used a differential display technique comparing 3T3-L1 cells exposed or not to TNFα. We have isolated a novel adipose cDNA encoding a TNFα-inducible 470-amino acid protein termed TIARP, with six putative transmembrane regions flanked by a large amino-terminal and a short carboxyl-terminal domain, a structure reminiscent of channel and transporter proteins. Commitment into the differentiation process is required for cytokine responsiveness. The differentiation process per se is accompanied by a sharp emergence of TIARP mRNA transcripts, in parallel with the expression of the protein at the plasma membrane. Transcripts are present at high levels in white and brown adipose tissues, and are also detectable in liver, kidney, heart, and skeletal muscle. Whereas the biological function of TIARP is presently unknown, its pattern of expression during adipose conversion and in response to TNFα exposure as a transmembrane protein mainly located at the cell surface suggest that TIARP might participate in adipocyte development and metabolism and mediate some TNFα effects on the fat cell as a channel or a transporter.

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‡ The abbreviations used are: TNFα, tumor necrosis factor-α; GLUT, glucose transporter; TIARP, TNFα-induced adipose-related protein; STEAP, six transmembrane epithelial antigen of prostate; bp, base pair(s); PBS, phosphate-buffered saline; TM, transmembrane.

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Many in vitro studies also support the view that TNFα has profound effects on lipid metabolism and adipocyte differentiation. TNFα was reported to inhibit lipid storage by reducing synthesis and activity of several proteins essential for lipogenesis, such as lipoprotein lipase (4) and acetyl-coenzyme A carboxylase (5), or by inhibition in the expression and/or function of the insulin-sensitive glucose transporter GLUT4 pathway (6). Otherwise TNFα is able to stimulate lipolysis in adipocytes by different mechanisms (7, 8). In addition to the above effects on lipid storage or mobilization, TNFα potently inhibits adipose conversion and even causes a dramatic de-differentiation of adipocytes in culture (9). Prevention of adipose conversion by TNFα has been essentially related to reduction in C/EBPα and PPARγ expression, two key adipogenic transcription factors (6, 10). These observations underline that TNFα controls the adipocyte phenotype not only by opposite regulations of lipid storage and mobilization, but also through the blockade of adipocyte differentiation. More recently, it has been suggested that TNFα could also inhibit adipose tissue development by inducing preadipocyte and adipocyte apoptosis (11, 12). However, some of the molecular mechanisms at the basis of these potent effects of TNFα are still unknown. Thus, identification of novel genes and proteins that could mediate the effects of the cytokine on the adipose cell remains an important issue.

Under appropriate culture conditions, 3T3-L1 cells differentiate into fat-laden adipocytes (13, 14). Moreover, this murine preadipose cell line has been extensively employed to characterize TNFα effects on adipocyte metabolism and differentiation. Using a differential display approach, we have identified a novel mRNA that is largely induced by TNFα in differentiating and in mature 3T3-L1 cells. This mRNA encodes a new protein of 470 amino acids termed TIARP for “TNFα-induced adipose-related protein.” Sequence analysis predicts that TIARP has a large NH2-terminal domain, followed by six transmembrane domains reminiscent of the general structure of numerous channels. This transmembrane portion of the protein shares a significant homology with STEAP (for six transmembrane epithelial antigen of prostate) and pHyde, two proteins that are highly expressed in human prostate tissues (15, 16). High plasma levels of TNFα likely play an important role in the onset of cachectic states observed during cancer or severe infectious diseases (2). By contrast, more recent studies have indicated that the cytokine is overexpressed in adipose tissue of obese rodents or humans, and that this locally produced TNFα may be involved in the obesity-linked insulin resistance (3). Thus, since abnormalities in its production or action are associated with alterations in body fat mass, TNFα is likely an important effector of adipose tissue development and metabolism in vivo.
The expression of this novel gene dramatically increases during TNFα exposure, but also during the course of 3T3-L1 adipose differentiation. Tissue distribution of TIARP mRNA is detected in liver, kidney, heart, and skeletal muscle. Immunofluorescence and Western blot studies indicate that the TIARP protein is prominently expressed at the level of the plasma membrane. Thus, our results indicate the presence of a new transmembrane protein that is highly regulated by TNFα and by the adipocyte differentiation process. Further investigations will provide new insights into the exact functional properties of TIARP in adipocyte and the physiological and/or pathological relationship with the TNFα-induced expression of this protein.

**Experimental Procedures**

**Cell Lines and Cell Culture**—Stocks of murine 3T3-C2 fibroblasts and 3T3-L1 preadipocytes were maintained as described (13, 14). For experiments, cells were seeded at a density of 10^4/cm^2 in plastic culture dishes (Falcon), and were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Biomeda, Boussens, France), 100 units/ml penicillin, and 50 μg/ml streptomycin (Life Technologies, Inc.) in a 10% CO2 humidified atmosphere. For 3T3-L1 cells, differentiation was initiated by administration of confluence of methylisobutyl-xanthine (100 μM), dexamethasone (0.25 μM), and insulin (1 μg/ml) for 48 h, then cells were reseeded by Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and 1 μg/ml insulin (17). Using this protocol, with 95% of the cells acquired an adipocyte morphology at day 7 following confluence. The cultured cells were either left untreated or exposed to murine TNFα (Genzyme, Cambridge) for the indicated periods and at the indicated concentrations.

Cultures of murine NIH-3T3 and C3H10T1/2 fibroblastic cell lines were grown by the same protocol as for 3T3-L1 cells. Primary cultures of rat hepatocytes were performed according to Foretz et al. (18). Differential Display—mRNA differential display was performed by the general procedure of Liang and Pardee (19), modified according to Sokolov and Prockop (20). Total RNA was isolated from 2-day post-confluent 3T3-L1 cells exposed or not for 6 h to 0.5 μl TNFα. 0.25 μg of RNA was used for reverse transcription reaction with 50 units of MMLV reverse transcriptase (Promega, Madison, WI) in a total volume of 20 μl containing 50 μg/ml Tris-HCl, pH 8.3, 10 μM random hexanucleotides, and 3 mM MgCl₂. 4 μl of the reverse-transcribed cDNA was used for each PCR reaction. PCR amplification was performed in a 25-μl volume containing 20 μg/ml Tris-HCl, pH 8.3, 16 mM (NH₄)₂SO₄, 2 mM MgCl₂, 125 μM of each dNTP, 500 μM of the two oligonucleotides, and 1 unit of Taq polymerase (Life Technologies, Inc.). A set of arbitrary oligonucleotides of 10–12 mer in length was used. The conditions that gave the differential-expressed PCR product were: 5′-ATGAAAGCCTTCAGGTCCGGTGAG-3′ and 5′-CACAGAGTACTTTGCTATCATT-3′. Parameters for PCR were 28 cycles of denaturating at 94 °C for 30 s, annealing at 57 °C for 1 min, and extension at 72 °C for 1 min, followed by a final extension of 5 min at 72 °C. PCR products were separated on a 2% agarose gel and stained by ethidium bromide. The candidate PCR product was excised from the gel using the Genevac II kit (Bio 101, Inc.), reamplified with the primers, then cloned into TA cloning vector (pGEM-T Easy, Promega Inc.).

Screening of cDNA Library and Sequencing—The PCR product obtained from the differential display was about 310 base pairs (bp) in length. This radiolabeled fragment was used to screen for the full-length cDNA in a cDNA library constructed in the pGEM-1Zf(–) plasmid (Promega, Inc.), and derived from mature 3T3-L1 adipocytes exposed for 6 h to 0.5 μg TNFα. Sequencing of the initial PCR product was determined by dideoxysequencing with Sequenase version 2.0 (U.S. Biochemical Corp.). Final sequencing of the full-length cDNA was performed by Genome Express (Montreuil, France). The nucleotide and deduced protein sequences were determined and analyzed for patterns, motifs, domains, or alignments by exploring the available sequence data bases provided by the web tools through the Centre de Ressources Infobiogen.

In Vitro Transcription and Translation of TIARP cDNA—Pureized 3080-bp cDNA was cloned into the pGEM-1Zf(–) vector (Promega Inc.). 1 μg of plasmid DNA was submitted to in vitro transcription and translation using a TNT coupled reticulocyte lysate system (Promega, Inc.), according to the manufacturer’s instructions. [35S]Methionine (1175 Ci/mmol at 10 μCi/ml, ICN Biomedicals, Inc.) was used to label the translated protein. Control translation reactions using empty pGEM-1Zf(–) vector and luciferase control DNA were performed. Proteins were resolved by denaturing 10% polyacrylamide gel electrophoresis (11). Labeled proteins were visualized by fluorography. After fixation the gel was exposed to Amplify reagent (Amersham Pharmacia Biotech) then dried and exposed to Kodak X-Omat for 20 h at −70 °C. Molecular markers were from Bio-Rad.

**RNA Isolation and Northern Blot Analysis**—Total RNA was isolated from cultured cells as described by Cathala et al. (22) and from 12-week-old mice by the acid phenol-chloroform procedure. Ten to twenty μg of RNA were separated in a 1.2% agarose gel containing 2.2 M formaldehyde and transferred onto nylon membranes (Nylon plus, Schleicher and Schuell). Methyline blue staining of blots was achieved to control the similarity in RNA loading. Hybridization with various 32P-labeled probes was performed as described by Church and Gilbert (24). Final washing was carried out in 0.1% sodium dodecyl sulfate in 0.2 × SSC (1 × SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0) for 15 min at 60 °C. The 310-bp fragment initially derived from the differential display procedure was used as a probe to detect TIARP mRNA. The other DNA probes have been described elsewhere (25). Autoradiograms were analyzed by scanning signals on a videosdensitometer (Vilbert-Lourmat Imaging).

**Western Blot Analysis**—Rabbit polyclonal antiserum directed against the TIARP protein (Quantum Appligene, Illkirch, France) was generated against the amino-terminal peptide HADEFLPTDTSSEKGG to keyhole limpet hemocyanin and affinity purified by antigen affinity purification.

For immunofluorescence staining, 3T3-L1 cells were seeded on glass coverslips and grown following the above described procedures. At the times indicated, the attached cells were rinsed three times with phosphate-buffered saline (PBS) and fixed with 2% paraformaldehyde (Sigma) for 30 min. They were rinsed three times with PBS, then permeabilized with 0.1% saponin (Sigma) in PBS for another 30-min period, washed three times with PBS, and post-fixed with 2% paraformaldehyde for 10 min. They were then exposed to 100 μl glycine in PBS for 10 min. After washing in PBS (5 times), the cells were incubated with blocking medium containing 3% bovine serum albumin, essentially fatty acid- and globulin-free (Sigma), then covered with rabbit polyclonal antiserum (anti-TIARP) (1:250 dilution in 1% bovine serum albumin). After incubation for 1 h, the cells were washed with PBS (3 times for 5 min), then incubated in darkness with an anti-rabbit fluorescein isothiocyanate-conjugated IgG (Fab’/ fragment) of goat anti-rabbit IgG (1:150 dilution in 1% bovine serum albumin, Sigma) for 45 min and for a further 15-min period with propidium iodide (1 μg/ml) which colored nuclei in red. After washing (5 times for 5 min), the slides were mounted with Vectashield mounting medium (Vector Laboratories). All steps were performed at room temperature. The slides were examined using a confocal microscope (LSM510, Zeiss, Jena, Germany). Images series of cell slices of 0.7-μm thickness each were obtained. No staining could be detected in areas of the membranes performed by omitting the primary antibody from the dilution buffer or by replacing the primary antibody by the preimmune serum.

For Western blot analysis, the cells were rinsed with PBS, harvested in ice-cold lysis buffer (20 mM Hepes, pH 7.4, 1 mM EDTA, 250 mM sucrose, containing a protease inhibitor mixture (Roche Molecular Biochemicals), and broken in a Dounce homogenizer (20 strokes, pestle B). Cell extracts were centrifuged at either 15,000 × g for 200,000 × g for 90 min at 4 °C, as indicated. The resulting pellet fractions were resuspended in 30 mM Hepes, pH 7.4. Plasma membranes were isolated as previously described (26) from the 15,000 × g pellet fraction dispersed in lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 3 mM ATP, 250 mM sucrose, supplemented with a protease inhibitor mixture) and then layered on a linear sucrose gradient (27.6–51.4% w/v) containing 1 mM EDTA, 1 mM ATP in 10 mM Tris-HCl, pH 7.4. The gradient was centrifuged in a Beckman L3 centrifuge at 60,000 × g for 60 min at 4 °C. The upper zone under the meniscus (ρ = 1.13–1.14) was aspirated and diluted with 10 volumes of 5 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, and centrifuged at 30,000 × g for 15 min at 4 °C. The resulting pellet was resuspended in 5 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, and 50 μg protease inhibitors (1:20 dilution of the solution of Lebey et al. (27). Heat-denatured samples (30 μg of protein) of total cell extracts, supernatant, pellet, or plasma membrane fractions were resolved on a 10% polyacrylamide gel in the presence of SDS and β-mercaptoethanol (21) and proteins were transferred onto a polyvinylidene fluoride membrane (Biorace, Gelman Sciences). The membrane was saturated with 5% delipidated milk in 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1%
Experimental Procedures. Total RNA was isolated, and mRNA differential display reactions were carried out as described under "Experimental Procedures." RNA was treated (+) or not (−) with reverse transcriptase (RT) to ensure that subsequent DNA amplification did not derive from contaminating DNA. PCR products were separated on an agarose gel and visualized by ethidium bromide staining. Molecular weight markers are shown on the right margin. Arrow indicates the position of the candidate 310-bp DNA fragment that is up-regulated by TNFα. B. Northern analysis of total RNA (10 μg/lane) derived from day 2 post-confluent 3T3-L1 cells treated (+) or not (−) by TNFα. The membrane was hybridized with the 32P-labeled 310-bp DNA fragment. The apparent size of the transcript indicated on the right. Methylene blue staining of the 28 S and 18 S ribosomal RNAs is shown on the lower panel.

RESULTS

Identification of TIARP cDNA and Deduced Amino Acid Sequence—To seek new genes that are differentially regulated by TNFα in differentiating preadipocytes, we employed a mRNA differential display procedure (19, 20). RNA was prepared from day-2 post-confluent cells, cultured for 6 h in the absence or presence of 0.5 nM TNFα. cDNAs derived from the mRNAs were subsequently assayed for differential display PCR reaction in the presence of different sets of primers arbitrary in sequence. Among various PCR products detected on agarose gels, we identified a DNA fragment of about 310 bp that was present only in TNFα-treated cells (Fig. 1A). Using this DNA fragment as a probe in Northern analysis, we confirmed that the related transcript, with an apparent size of 3.1 kb, was highly expressed in TNFα-exposed cells, but was also present at much lower levels in untreated cells (Fig. 1B).

A full-length cDNA clone was subsequently obtained by screening a cDNA library from TNFα-treated mature 3T3-L1 adipocytes with the 310-bp partial cDNA clone. The complete nucleotide sequence of the resulting cDNA was composed of 3080 bp. Blastn searching of the nucleic acid data bases at the National Center for Biotechnology Information (NCBI) to look for sequence homologies (28) revealed that cDNA sequence displayed significant alignments with a human clone from chromosome 7q21 (GenBank accession number AC003991) (90% identity). The first ATG codon was found at position 76 from the 5′-end of the cDNA. This codon initiates an open reading frame extending up to position 1486. The deduced amino acid sequence of 470 amino acids (molecular weight = 52,937) (Fig. 2A) was used as a query for searching at various servers. It consists in a long NH2-terminal sequence of 200 amino acids residues followed by five or six transmembrane-spanning domains (positions 201–434) and a COOH-terminal sequence. According to PROSITE analysis results, this protein was mainly characterized by the presence of an ATP/GTP-binding site motif as the P-loop at the NH2-terminal sequence (positions 26–33), an hemopexin domain signature at the junction between the cytosolic and membrane domains (positions 196–210), and three putative N-glycosylation sites. Blast search results (28) revealed significant homologies of the NH2-terminal sequence between positions 21 and 173 with several NAD/NADPH oxidoreductases characterized in Archaea and bacteria (for instance, the sequences with accession numbers: sp/Q58896, sp/O26350, embl/CAB61935.1, dbj/BAA29608, gb/AAF10566.1, and pir/IC71165) (30% identities). Especially, the sequence of TIARP matches domains characteristic of the NAD/NADPH-dependent acetoxyhydroxy acid isomeroreductase described in Archaea, bacteria, and plants (29) (positions 21–79), of the ubiquitous NAD/NADH-dependent glycerol-3-phosphate dehydrogenase (positions 22–41) and, in positions 22–124, of the KTN NAD-binding domain (Pfam analysis at St. Louis, MO; Pfam accession number PF02254) present in Drosophila and bacteria (30, 31) and in a variety of proteins, including potassium channels, phosphoesterases, and other transporters. Interestingly, this domain was characterized in Escherichia coli as a putative potassium channel protein (Swiss-Prot accession number P31069) with the features of an integral membrane protein.

Depending on the architecture research tools used for prediction of protein secondary structure, the amino acid sequence spanning from residue 201 to 434 defines either five (according to the SOSUI system at Kyoto Encyclopedia of Genes and Genomes, Kyoto University, Japan) or six transmembrane helices (according to Tmpred, Das Transmembrane Prediction, Pred-TMR, or HMMTOP analysis programs consulted through the Centre de Ressources Infobiogen, France). Interestingly, SMART (Simple Modular Architecture Research Tool) (32, 33) resource release predicts five transmembrane segments (shown as TM1–4 and TM6 on Fig. 2A) and another possible transmembrane domain (TM5 on Fig. 2A). The entire transmembrane sequence 201–434 presents homologies with the NH2-terminal sequences of STEAP characterized in epithelia of the human prostate tissue (15) (GenBank™ accession number AF198249) (38% identity). Otherwise, a high degree of homology was found between the entire sequences of the protein TIARP and of the recently described “tumor suppressor pHyde,” the product of a novel tumor suppressor gene that inhibits growth of prostate cancer (16) (GenBank™ accession number AAK00361.1) (44% identity). It is noteworthy that the homology between amino acid sequences of TIARP, pHyde, and STEAP is higher in their transmembrane parts than in their NH2-terminal portion (Fig. 2B) (34). In an in vitro transcription and translation system, the TIARP cDNA generated a protein of ~52,000 Da in size (Fig. 3), in agreement with the 52,937 Da molecular mass predicted from the cDNA sequence.

Pattern of TIARP mRNA Expression in Various Rat Tissues—To examine the tissue distribution of TIARP mRNA, we performed Northern analysis using various rat tissues (Fig. 4). A single mRNA species with a size of 3.1 kilobases was ex-
pressed both in white and brown adipose tissue. These high expression levels were not restricted to adipose tissue, but were also found in heart, liver, kidney, and skeletal muscle. Lower levels of TIARP transcripts were observed in lung and spleen, while TIARP mRNA appeared undetectable in brain and intestine.

**FIG. 2. Amino acid sequence of TIARP protein and alignment with other cloned members of the family.** Amino acid sequence showing the putative transmembrane spanning domains TM1 to TM6 (double-underlined), according to SMART analysis. The G-rich amino-terminal sequence is underlined. Predicted ATP/GTP-binding site motif A (P-loop) is in italic boldface type and hemopexin domain signature is in boldface type. The asterisks denote the position of three potential N-glycosylation sites. B, alignment of the amino acid sequence of TIARP with the proteins STEAP from the prostate (*Homo sapiens*) (339 amino acid residues) (GenBank™ accession number AF188249) and tumor suppressor pHyde (*Rattus norvegicus*) deduced from cDNA libraries derived from Dunning rat prostate cancer cell lines (488 amino acid residues) (GenBank™ accession number AAK00361.1). Sequences were aligned using MultiAlin version 5.4.1 at INRA, France (34).

**FIG. 3. In vitro transcription and translation of TIARP cDNA.** 1 μg of plasmid cDNA template was submitted to coupled transcription/translation in the presence of [35S]methionine. The translation products were analyzed by polyacrylamide gel electrophoresis and fluorography. Lane 1, empty pGEM-1Zf(+); lane 2, 3080-bp TIARP cDNA cloned into pGEM-1Zf(+); lane 3, luciferase control cDNA. A 35S-labeled band of about 52,000 Da in size was specifically generated with TIARP cDNA template (position indicated by an arrow). The molecular weight standards are shown in the right margin.

**FIG. 4. Tissue distribution of TIARP mRNA.** 20 μg of total RNA prepared from various tissues of 12-week-old Wistar rats were subjected to Northern blot analysis. WAT, white adipose tissue; BAT, brown adipose tissue. The membrane was hybridized with the radiolabeled 310-bp DNA fragment as mentioned under "Experimental Procedures." Methylene blue staining of 28 S and 18 S ribosomal RNAs was performed to assess the equivalence in RNA loading (lower panel).

**Regulation of TIARP mRNA and Protein Expression during Adipocyte Differentiation**—The presence of low but significant levels of TIARP mRNA in differentiating 3T3-L1 cells (Fig. 1B, lane 1) and its strong expression in white and brown adipose tissue (Fig. 4) prompted us to examine TIARP expression during the course of differentiation of 3T3-L1 cells. Total RNA was harvested from cells at various intervals before and after confluence. As shown in Fig. 5, TIARP mRNA was undetectable in growing 3T3-L1 preadipocytes (1 day before confluence), and...
was barely expressed at confluence (day 0) and at day 1 following confluence. Thereafter, its expression dramatically increased at day 3 and reached a maximal level at day 8 following confluence. TIARP mRNA was virtually absent in the fibroblastic 3T3-C2 cells cultured under the same culture conditions. This confirmed that the spontaneous emergence of TIARP mRNA, i.e., in the absence of TNFα addition, is a differentiation-linked event. Moreover, the kinetics of expression of TIARP mRNA appeared very similar to that of the adipocyte lipid-binding protein and glycerol-3-phosphate dehydrogenase mRNAs, two well characterized markers of the adipocyte conversion process (25) and indicates that TIARP mRNA induction is a late event of differentiation.

Using an antibody directed against the amino-terminal sequence of the TIARP protein, we studied by confocal microscopy image analysis the expression, subcellular localization, and the fate of this protein during the 3T3-L1 adipose conversion process. Immunocytochemical analysis performed on permeabilized cells (Fig. 6) revealed a faint signal within a cytoplasmic area close to the nucleus of some preadipocytes, suggestive for a Golgi localization (day 0, panel A). However, at day 2 following confluence, 3T3-L1 cells acquired TIARP immunoreactivity scattered within the cytoplasm as punctuate patterns and also at the cell periphery (panel B). Then the cell surface-associated staining progressively increased in fluorescence during adipose conversion as shown at days 5 (panel C) and 10 (panel D). In addition, at these time periods, TIARP protein also accumulated in the Golgi area (panels C and D). Reconstitution of cells in three-dimension performed from an image series of 0.7-μm thickness each indicated that in these fully differentiated cells (day 10), immunostaining was clearly associated with the entire area of the plasma membrane as discontinuous clusters, with small vesicle-like structures approaching the plasma membrane and with the Golgi area (panels E-H). Orthogonal cut-off of the cells often show strongly stained invaginations of the plasma membranes (panel H). Thus, in agreement with the profile of TIARP mRNA expression during adipose conversion, signals were virtually undetectable in confluent preadipocytes and protein expression increased during the adipose conversion

**FIG. 5.** Induction of TIARP mRNA levels during adipose differentiation of 3T3-L1 cells. Total RNA was extracted from 3T3-L1 or 3T3-C2 cells at different intervals relative to confluence arbitrarily considered as day 0. 10 μg of total RNA were loaded for each RNA sample. Northern blots were sequentially hybridized to 32P-labeled cDNA probes corresponding to TIARP, adipocyte lipid-binding protein (aP2), and glycerol-3-phosphate dehydrogenase (G3PDH). Methylene blue staining of 18 S ribosomal RNAs is shown under the autoradiograms for each cell line.

**FIG. 6.** Immunocytochemical localization of TIARP protein during 3T3-L1 adipocyte differentiation. 3T3-L1 cells were fixed at different time intervals relative to confluence (day 0). Cells were processed for labeling of TIARP using rabbit anti-TIARP polyclonal antibody and fluorescein isothiocyanate-conjugated goat anti-rabbit Ig (colored in green) in combination with counterstaining of nuclei with propidium iodide (red color). Cells were viewed by confocal microscopy. A faint green fluorescence was visible close to the nuclei in undifferentiated cells at day 0 (A), then staining progressively increased. At day 2 (B) it was abundant as punctuate pattern throughout the cytoplasm and discrete at the cell periphery. Staining localized to the periphery and in the Golgi area at day 5 (C) and increased in intensity at day 10 (D). Observations at various focal planes (E-G, at 7, 2.5, and 0.5 μm distance from the substratum-attached surface of the cell, respectively) and of cut-offs of the same cell (H) showed staining at the plasma membrane as discontinuous clusters, small intracytoplasmic vesicle-like structures, a large Golgi-like compartment close to the nucleus. In E and H, intense staining on plasma membrane invaginations. These views are representative of observations of several preparations from four independent series of cell cultures. Control assays were systematically performed either using preimmune serum or without the primary antibody and gave negative results. Bars, 30 μm (A-D) or 10 μm (E-H).
cells, TNFα increased TIARP mRNA expression in a dose-dependent manner. This effect was detectable at 3 pm and was maximal at 30 pm, giving a half-maximal response between 3 and 10 pm (Fig. 8). The effect of TNFα (0.5 nm) on TIARP transcripts was maximal after a 6-h exposure to the cytokine and persisted at high levels 24 h after initiation of the treatment (not shown). Likewise, TIARP protein expression was enhanced following TNFα exposure of post-confluent 3T3-L1 cells (Fig. 9). Treatment of day 2 post-confluent cells for 24 h with 0.5 nm TNFα resulted in a strong increase of immunostaining concerning the cytoplasm and overall the plasma membrane (compare the TNFα-treated cells in panel B to untreated cells in panel A). In agreement with the pattern of transcript regulation, these changes were observable after TNFα exposure for only 6 h (not shown). Reconstitution of cells in three-dimension showed that the entire cell surface was immunoreactive (illustrated in panels C-E). Staining was particularly intense at contact with the neighbouring cells and with the culture support (panels D and E). The presence of intracytoplasmic immunoreactive vesicle-like structures in the vicinity of the plasma membrane suggests that TNFα could elicit the translocation of vesicle-associated TIARP to the cell periphery and association and/or fusion with the plasma membrane. Interestingly, similar distributions of TIARP were observable on the day-3 TNFα-treated cells (Fig. 9, panels B-E) and on the above described untreated cells at day 10 (Fig. 6, panels D-H), making it likely that TNFα accelerated TIARP synthesis and trafficking to the plasma membrane at this early step of cell differentiation.

Our previous experiments were performed on differentiating or fully differentiated 3T3-L1 adipocytes. Thus, it was of importance to investigate whether TNFα responsiveness also existed in undifferentiated cells. As shown in Fig. 1A, there was a strong induction in TIARP mRNA expression after a 6-h exposure of differentiating or differentiated 3T3-L1 cells to TNFα. By contrast, the response to TNFα was virtually absent in undifferentiated 3T3-L1 cells (1 day before cell confluence). Since TIARP mRNA was also clearly present in liver (Fig. 4), we also tested its modulation in primary cultures of rat hepatocytes. Likewise, TIARP gene expression was present at moderate levels in the basal state, and was strongly induced (15-fold) in TNFα-treated hepatocytes (Fig. 10A). We also examined whether the determination toward a cell lineage influenced the regulation of TIARP expression by TNFα. For this purpose, we measured TIARP mRNA levels in three undetermined models, the NIH-3T3 and 3T3-C2 fibroblastic cell lines, and the mesodermic mutipotent cell line C3H10T1/2. Fig. 10B shows that in the absence of TNFα, TIARP mRNA expression was undetectable in C3H10T1/2 and 3T3-C2 cells, and clearly present in NIH-3T3 cells. Whatever the considered fibroblastic cell line and its basal expression in TIARP mRNA, no significant response to the cytokine was detectable. Taken together, these results demonstrate that determination toward a specific cell lineage is required but not sufficient to observe TIARP induction in the presence of TNFα, and that commitment into a differentiation process is also a prerequisite for the modulation of TIARP mRNA levels by the cytokine. This suggests that suppression of an inhibitory mechanism or emergence of a stimulatory mechanism occurs during the adipocyte differentiation process, and allows the cells to acquire TIARP responsiveness to TNFα.

**DISCUSSION**

Since TNFα represents a major effector of adipose tissue development and metabolism, our objective was to identify novel genes and proteins that are markedly regulated by this cytokine in differentiating preadipocytes or in mature adipose...
Fig. 9. Effect of TNFα on TIARP protein expression. Day 2 post-confluent 3T3-L1 cells were exposed (+) or not (−) to 0.5 nM TNFα for 24 h, then processed as described in the legend to Fig. 6. A, untreated cells at day 3, showing the accumulation of punctuate staining throughout the cytoplasm (compare with untreated cells at day 2 in Fig. 6, panel B); B-E, changing distribution to plasma membrane and Golgi when cells were exposed to TNFα from day 2 to day 3; C and D, views of a single cell when the focal plane was adjusted at 7 or 0.5 μm distance from the substratum-attached surface of the cell, respectively, showing intense staining of the plasma membrane and a dense vesicular pattern similar to that observed on untreated cells at day 10 (compare with Fig. 6, panels E-G). E, cut-off showing staining of the plasma membrane especially at contacts with neighboring cells. These views are representative of observations of several preparations from four independent series of cell cultures. Control assays were systematically performed using preimmune serum or omitting the primary antibody and gave negative results. Bars, 30 μm (A and B) or 10 μm (C-E).

Fig. 10. Differentiation dependence of TIARP mRNA responsiveness to TNFα. A, total RNA was prepared from growing (1 day before confluence) or day 2 (differentiating) or day 8 (mature) post-confluent 3T3-L1 cells exposed (+) or not (−) for 6 h to 1 μM TNFα. B, total RNA was extracted from day 2 post-confluent C3H10T1/2, NIH-3T3, or 3T3-C2 cells treated (+) or not (−) for 6 h by 1 μM TNFα. Northern blot analysis was then performed as mentioned in the legend to Fig. 1B.
is noteworthy that besides the strong induction of TIARP by TNFα that led to its identification, TIARP mRNA levels and the related protein also spontaneously and markedly emerge during the adipocyte differentiation process. This supports the view that TIARP might be implicated not only in TNFα effects on the fat cell during pathological states associated with local or systemic TNFα overproduction, but also in the normal progress of adipocyte development and metabolism.

Immunocytochemical and Western blot analyses indicate that TIARP is mainly associated with plasma membranes of 3T3-L1 cells. In agreement with the pattern of TIARP mRNA expression during 3T3-L1 adipose conversion, the protein is virtually absent in preadipocytes and its level progressively increases when cells become mature adipocytes. Apart from this strong pericellular distribution, other aspects of subcellular location deserve to be emphasized. First, the signal appears to be the strongest at the cell-cell junctions, suggesting that TIARP could be involved in intercellular communications. Second, the intense staining that is also present on plasma membrane invaginations raises the possibility that the protein could play a role in extracellular trafficking. Protein sequence analysis indicates that TIARP is a membrane-associated protein including six hydrophobic helices with the topology of six or five probable transmembrane domains, and a long putative intracytosolic NH2-terminal chain lacking a signal sequence. The transmembrane portion of TIARP presents characteristics of a ion-transport protein and shares a strong homology with two recently discovered proteins, STEAP and pHyde. STEAP is a protein with unknown functions and with a tissue distribution quite different from that of TIARP, since it is mainly expressed in prostate tissue (15). Nevertheless, at the subcellular level this protein is also preferentially expressed at the plasma membrane. pHyde has also been identified from prostate cancer cells, and seems to inhibit the growth of tumor cells and to promote their apoptosis (16). Structure prediction analysis reveals that STEAP and pHyde display six putative transmembrane domains.

Interestingly, cell-surface molecules containing six transmembrane domains include water and ion transport channels (41–44). Electrophysiological studies of ionic fluxes in adipocyte and of the protein structures responsible for the transport of ions are limited. However, several works suggest that channels could be involved in adipocyte development or metabolism (45–49). For instance, voltage-gated potassium channels seem necessary for the normal proliferation and differentiation of brown fat cells in culture (50, 51). Recently, a novel member of the aquaporin family, the adipose-specific AQPap has been isolated (47, 49). Several lines of evidence support the view that AQPap is the physiological glycerol channel specific to adipocytes (49). In 3T3-L1 adipocytes, a short-time exposure of epinephrine translocates AQPap from perinuclear cytoplasm to the plasma membrane (49). Likewise, we have found that TIARP was translocated from the cytoplasm to the cell periphery in response to a short-time isoproterenol exposure (results not shown). Thus, a cAMP-dependent mechanism might induce a translocation of these two transmembrane proteins at the cell surface, suggesting coordinated and rapid cellular fluxes changes of ions, water, or metabolites.

Several considerations favor the hypothesis that TIARP could work as a potassium channel. Potassium channels are classically described as containing six transmembrane domains, five being hydrophobic and the other positively charged and localized within a cluster formed by the other helices. It is postulated that it may constitute the voltage sensor region, moving outward on depolarization, and causing a conformational change. Concerning the putative transmembrane domains present in the TIARP protein, it must be recalled here that sequence predictions were ambiguous. The controversy concerned the penultimate helical domain (TM5 at positions 384–404) predicted as either a true or a probable transmembrane segment which could hypothetically behave as a moving region responsible for conformational changes. A possible function as a potassium channel for TIARP is strengthened by the presence in the NH2-terminal sequence of a NAD-binding domain which is found in various transporters, namely in proteins that regulate potassium fluxes described in bacteria or eukaryotes and are believed to play a role in the defense against osmotic shock (30, 31, 52, 53). Interestingly, pyridine nucleotides are known to control potassium channels opening state (54).

Another remarkable feature of TIARP is the glycine-rich region of the NH2-terminal domain suggestive of an involvement in oxidoreductase activity. In line with this characteristic is the significant homologies of the complete NH2-terminal sequence with several NAD(P)/NAD(P)H-dependent oxidoreductases (29–31) supporting the idea that TIARP might be involved in electron transport and energy metabolism. A role for TIARP in oxidoreductase activity could accommodate the results of previous studies that showed the presence of a NADPH-dependent H2O2-generating system in human and rodent adipocyte plasma membranes (55, 56). This enzyme is linked to multiple cell-surface receptors. β-Adrenergic agonists and growth factors such as fibroblast growth factor and platelet-derived growth factor inhibit (57–60), while insulin and TNFα stimulate (44–58, 61) this H2O2-producing system. Further molecular and functional studies are required to establish a potential relationship between TIARP and this particular NADPH-dependent oxidase only characterized at biochemical and pharmacological levels.

The relationship between the putative channel properties of TIARP and its potent TNFα-induced expression also remains an unresolved question. TIARP may represent an important mediator of the physiological or pathological effects of TNFα on several aspects of adipocyte biology, such as differentiation, lipogenesis, lipolysis, insulin sensitivity, or apoptosis. Interestingly, it has been recently suggested that in addition with an increase in mitochondrial membrane permeability, potassium and chloride plasma membrane channels participate at early steps in pathways leading to TNFα-mediated cell death (62). The recent identification of a protein displaying strong sequence homology with TIARP, pHyde, that seems to act as a tumor suppressor by inhibition of the growth of prostate cancer cells at least in part through apoptosis (16), could support an implication of TIARP in the balance between physiological events linked to cell differentiation and TNFα effects. Thus, it is conceivable that in adipocytes TIARP may mediate some biological adaptive effects in response to the cytokine or cellular stresses. In the present work, the stimulation of synthesis and trafficking of TIARP-associated vesicles in response to TNFα is consistent with an adaptive function of TIARP.

An interesting observation is that TIARP induction in response to TNFα not only depends on cell determination toward a specific lineage, but is also influenced by commitment of the preadipocyte into the differentiation process per se. Indeed, while TIARP transcript abundance was strongly induced by TNFα in differentiating or fully mature 3T3-L1 cells, undifferentiated 3T3-L1 preadipocytes were unresponsive to the cytokine. Likewise, several target cell- or differentiation-specific actions of TNFα have been reported, including effects on preadipocyte and adipocyte (63–67). This illustrates the requirement of unknown differentiation-dependent mechanisms to activate a permissive response to TNFα.

In conclusion, we identified by a differential display ap-
proach a novel protein, TIARP, that appears remarkable by several features. The expression of TIARP is strongly induced during TNFα exposure and adipose conversion, and is essentially localized at the plasma membrane. The predictive structural analysis of the protein suggests that it may have channel and oxidoreductase properties. Future studies addressing the biochemical and functional properties of TIARP will provide insights into its role in adipocyte biology.

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