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The Transcription Factor Fos-Related Antigen 1 Is Induced by Thiazolidinediones During Differentiation of 3T3-L1 Cells

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ABSTRACT

Thiazolidinediones (TZDs) are a new class of compounds that improve the insulin sensitivity in patients with non–insulin-dependent diabetes mellitus (NIDDM) as well as in rodent models of NIDDM. These compounds act as high-affinity ligands for a member of the nuclear hormone receptor superfamily PPARγ, which has been shown to play an important role in adipocyte differentiation. The strong correlation between the antidiabetic activity of TZDs and their ability to activate PPARγ has led to suggestions that PPARγ or downstream regulated genes mediate the effects of TZDs. To identify novel genes that potentially mediate the effects of TZDs, we have isolated genes that are differentially expressed during thiazolidinedione-stimulated differentiation of 3T3-L1 cells. Using mRNA differential display, we have compared 3T3-L1 cells treated to differentiate in the presence of BRL49653 with untreated 3T3-L1 cells and identified Fos-related antigen 1 (Fra-1), a member of the Fos protein family, as a novel molecular target for BRL49653 action in 3T3-L1 cells. Analysis of all members of the Fos-Jun family of transcription factors showed that Fra-1 was the only member that was specifically up-regulated by BRL49653. The only other member of the Fos-Jun family expressed in differentiated 3T3-L1 cells was JunD and a complex of Fra-1 and JunD was formed on a consensus AP-1 binding element in differentiated 3T3-L1 cells, suggesting that the complex of Fra-1 and JunD may play a role in the stimulation of the differentiation process of 3T3-L1 cells observed after treatment of the cells with insulin sensitizers.

Thiazolidinediones (TZDs) are oral antihyperglycemic agents that after chronic administration to patients with non–insulin-dependent diabetes mellitus (NIDDM) as well as animal models of NIDDM improve glycemic control. Several studies indicate that TZDs enhance insulin-mediated suppression of hepatic glucose output and increase insulin sensitivity in skeletal muscle and adipose tissue resulting in increased glucose uptake in these peripheral tissues (Reginato and Lazar, 1999).

In vitro, TZDs have been identified as potent and high-affinity ligands for peroxisome proliferator-activated receptor γ (PPARγ) (Lehmann et al., 1995), a member of the nuclear hormone receptor superfamily. Three related but distinct PPARs have been identified and are referred to as PPARα, PPARδ, and PPARγ (Issemann and Green, 1990; Kliewer et al., 1994). PPARs form heterodimers with RXRs and regulate gene expression by binding to PPAR response elements located in the promoter region of genes regulated by these transcription factors. To date, three isoforms of PPARγ (γ1, γ2, and γ3) derived from alternative promoter usage and splicing, have been identified. The three proteins are identical except for additional 30 amino acid residues at the N terminus of PPARγ2. PPARγ1 seems to be expressed at low levels in several tissues, whereas PPARγ2 is mainly found in adipose tissue and PPARγ3 is restricted to colon and adipose tissue (Tontonoz et al., 1994a; Fajas et al., 1997; Fajas et al., 1998). Interestingly, ligand-activation of PPARγ by TZDs regulates transcription of a variety of adipocyte-specific genes (Kletzien et al., 1992; Kelly et al., 1998; Motojima et al., 1998) and forced expression of PPARγ in fibroblasts has been shown to result in the development of an adipocyte phenotype, an event that is significantly enhanced by the presence of TZDs (Tontonoz et al., 1994b). Furthermore, a partial PPARγ antagonist was recently shown to prevent adipocyte differentiation in vitro (Oberfield et al., 1999); taken together, these results strongly indicate that ligand-activated/inactivated PPARγ plays an important role in adipocyte differentiation. These observations have led to the suggestion that the antidiabetic actions of the TZDs result from their ability to bind to and activate PPARγ in adipose tissue. This is supported by the finding of correlations between compound potencies as antidiabetic agents in animal models and binding-affinities to recombinant PPARγ (Berger et al., 1996; Willson et al., 1996) as well as with binding

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ABBREVIATIONS: NIDDM, non-insulin-dependent diabetes mellitus; PPAR, peroxisome proliferator-activated receptor; TZD, thiazolidinedione; RXR, retinoid X receptor; Fra-1, fos-related antigen 1; PCR, polymerase chain reaction; DTT, dithiothreitol; TOPO, pCR2.1 topoisomerase; AP-1, activating protein 1; BRL, BRL49653 [5-(4-[(*N*-methyl-*N*(2-pyridyl)amino)ethoxy]benzyl)thiazolidine-2–4-dione]; Dex, dexamethasone; Ins, insulin.

affinities to differentiated 3T3-L1 cells and rat and human adipocytes (Young et al., 1998). Besides, synthetic RXR agonists of the interaction partner for PPARγ increase insulin sensitivity in obese mice and work in combination with TZDs to enhance antidiabetic activity (Mukherjee et al., 1997).

The mechanism by which TZD activation of PPARγ improves glycemic control in NIDDM patients remains unclear. However, the ability of PPARγ to transcriptionally regulate a variety of genes in adipocytes suggests that the antidiabetic activity of TZDs is mediated by one or more PPARγ downstream regulated genes. To identify genes that are differentially regulated by TZDs during adipocyte differentiation, we have identified a set of genes in 3T3-L1 cells exhibiting altered expression after exposure to the potent TZD BRL49653. Among these genes we identified *fra*-1 (Fos-related antigen 1), a member of the Fos-Jun family of transcription factors, as a novel gene induced by BRL49653. BRL49653 and other TZDs, including troglitazone and pioglitazone, induced *fra*-1 expression in a dose-dependent manner corresponding to their binding to PPARγ, indicating that the induction of *fra*-1 occurs through PPARγ. Fra-1 was shown to form functional heterodimers with JunD in nuclear extracts from differentiated 3T3-L1 cells suggesting that the complex of Fra-1 and JunD plays a role in the differentiation process of 3T3-L1 cells caused by TZDs, and thus may be involved in the antidiabetic activity of these compounds.

Materials and Methods

Cell Culture. Reagents were obtained from Life Technologies (Taastrup, Denmark) unless otherwise noted. Murine 3T3-L1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were grown to confluence and induced to differentiate 2 days after confluence with media containing 1 μM dexamethasone (Sigma, St. Louis, MO), 10 μg/ml human insulin (Novo Nordisk A/S, Bagsvaerd, Denmark), and 1 μM BRL49653 (Novo Nordisk).

Plasmids. cDNA fragments from c-*fos*, *fosB*, *fra*-1, *fra*-2, c-*jun*, *junB*, and *junD* were cloned by reverse transcription PCR from RNA isolated from 3T3-L1 cells treated for 1 h with dexamethasone, insulin, and BRL49653 using the Superscript II kit (Life Technologies) after the manufacturer's instructions. The upstream and downstream primers were: 5′-TGAAACACACCAGGCTGTGGGCCTC-3′ and 5′-GACCACCTCGACAATGCATGATC for c-*fos*, 5′-GAGTCTGCTAACCCCACTTCCCGC-3′ and 5′-GCCTGAGCAAGAAGGCCTCCGGGA-3′ for *fosB*, 5′-GAGTTCATGAAGGTGGCTCACGGC-3′ and 5′-GTCCCCACTGCTACTGCTTCTGCG-3′ for *fra*-2, 5′-GCAACGGCTGCCAGTTGCTGCTAG-3′ and 5′-GACTTTAGACACGAAGTCGTGTTT-3′ for *junB*, 5′-CAGAGACAAACTTGAGAACTTGAC-3′ and 5′-CATACACAGTTGAGAGAGGCAGGC-3′ for c-*jun*, 5′-CAGGTCCCGGCGTACTGAGCCCG-3′ and 5′-GGAACAGGAATGTGGACTCGTAGC-3′ for *junD*, 5′-GCGGCCGCCATGTACCGAGACTACGGGGAACCG-3′ and 5′-GCGGCCGCTCACAAAGCCAGGAGTGTAGG-3′ for the coding region of *fra*-1, 5′-ATGGAAACGCCCTTCTATGGCGAG-3′ and 5′-GCGGCCGCGGGCTCAGTACGCCGGGACCTG-3′ for the coding region of *junD*, respectively. All fragments were cloned into TOPO 2.1 (Invitrogen, Leek, the Netherlands) and sequenced using an ABI 377 sequencer (Perkin-Elmer, Foster City, CA).

mRNA Differential Display Analysis. mRNA differential display was performed essentially as described previously (Liang and Pardee, 1992). Briefly, total cellular RNA was isolated from 3T3-L1 cells harvested 2 days after confluence as well as after 1 day or 7 days treatment with dexamethasone, insulin, and BRL49653, using RNAZol (BioSite, Täby, Sweden) according to the manufacturer's instructions. To remove traces of DNA, 50 μg of total RNA was treated with 3 U of RNase-free RQ1 DNase (Promega, Roedovre, Denmark) and purified on microcon 100 columns (Amicon, Beverly, MA) according to the manufacturer's recommended procedures. Subsequently, three individual reverse transcription reactions were performed on RNA from each time point, using one of three different 1-base-pair-anchored 3′-oligo(dT) primers with a *Hin*dIII recognition sequence (5′-AAGCTTT$_{11}$G-3′,5′-AAGCTTT$_{11}$A-3′,or 5′-AAGCTTT$_{11}$C-3′) in a reaction buffer containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 μM 1-base-pair–anchored 3′-oligo(dT) primer, 200 U Superscript II reverse transcriptase (Life Technologies), 20 μM dNTP, and 2 μg of DNase-treated RNA in a total volume of 20 μl for 60 min. Differential display PCR reactions were performed using the same 1-base-pair–anchored 3′-oligo(dT) primer together with one of a small collection of arbitrarily designed 5′-oligos of 13 base pairs in length including a *Hin*dIII recognition sequence (5′-AAGCTT-7 random nucleotides-3′). The sequence of the 5′-arbitrary oligonucleotide that gave a *fra*-1 PCR product was 5′-AAGCTTACACAGC-3′. The reverse transcribed cDNA (2 μl) was used for each PCR reaction containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 2 μM dNTP, 1 μCi [α-^{33}P]dATP (Amersham Pharmacia Biotech, Buckinghamshire, UK), 0.2 μM 5′-arbitrary oligo, 1 μM 1-base-pair–anchored 3′-oligo(dT) primer, 1 U *Taq*2000 (Stratagene, Aarhus, Denmark), and 0.22 μg of anti-*Taq* antibody (CLONTECH, Stockholm, Sweden) in a total volume of 20 μl. Parameters for PCR were 40 cycles of denaturing at 94°C for 15 s, annealing at 40°C for 2 min, and extension at 72°C for 30 s, finally followed by 5 min at 72°C. For PCR reactions, 0.6 μl was loaded onto a 6% cast-away sequencing gel (Stratagene), and PCR fragments were visualized by exposing the dried sequencing gel to X-ray film. Differentially amplified bands of interest were excised and the DNA was eluted by boiling the gel slice in 100 μl of water for 15 min. The eluted DNA fragments were reamplified using the same primer pair and PCR conditions as for the differential display PCR, and finally cloned into the TOPO TA cloning vector (Invitrogen, Leek, Netherlands). The sequence of the cloned DNA fragment was determined using an ABI 377 (Perkin-Elmer, Foster City, CA).

Northern Blots. Total RNA was isolated from 3T3-L1 cells using RNAZol after the instructions of the vendor. RNA (20 μg) were size-fractionated in a denaturing gel containing 1% agarose, 20 mM MOPS, 5 mM NaOAc, 6% formaldehyde, and 1 mM EDTA, transferred to a Hybond N^+ membrane (Amersham Pharmacia Biotech) by capillary blotting and immobilized by UV crosslinking. cDNAs from c-*fos*, *fosB*, *fra*-1, *fra*-2, c-*jun*, *junB*, and *junD* were labeled with the Prime It kit (Stratagene) using [α-^{32}P]dATP (3000 Ci/mmol; Amersham Pharmacia Biotech) and hybridized using Express Hyb (CLONTECH, Stockholm, Sweden) after the manufacturer's instructions and results were visualized by autoradiography.

Nuclear Run-on Transcription Assay. Nuclear run-on transcription assay was performed according to the protocol of Greenberg and Ziff (1984) with slight modifications. In brief, 5×10^7 3T3-L1 cells were washed twice with ice-cold PBS, harvested by scraping in PBS and pelleted by centrifugation. The cell pellet was resuspended in 4 ml lysis buffer (5 mM HEPES, pH 6.9, 3 mM $MgCl_2$, 0.32 M sucrose, 1% Nonidet P40, and 0.5 mM β-mercaptoethanol), vortexed briefly and incubated 5 min on ice before centrifugation at 500*g* for 5 min. The resulting pellet was resuspended in 4 ml of lysis buffer without Nonidet P40, incubated on ice for 5 min, and centrifuged for 5 min at 500*g*. The pellet containing the nuclei was resuspended in 200 μl of glycerol storage buffer [50 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 40% (v/v) glycerol, and 0.5 mM β-mercaptoethanol] and frozen in liquid N_2. Transcription reactions were initiated by adding 200 μl of 2× reaction buffer (10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 300 mM KCl, 5 mM DTT, 0.5 mM each ATP, GTP, and CTP) and 120 μCi [α-^{32}P]UTP (800 Ci/mmol) (Amersham Pharmacia Biotech) to the thawed nuclei suspension and was continued for 30 min at 30°C with agitation. RNase-free DNase was added to a final concentration

of 17.5 U/ml, incubation was continued for 10 min at 30°C, and the reaction was terminated by addition of 100 μg of yeast tRNA and 1.2 ml of RNAZol. RNA was isolated according to the manufacturer's instructions, resuspended in H_2O, and purified on Sephadex G-50 Nick columns (Amersham Pharmacia Biotech, Uppsala, Sweden).

The 800-ng PCR product of the selected cDNAs were denatured for 15 min at room temperature in 0.2 N NaOH in a total volume of 200 μl and loaded onto Hybond N^+ membranes using a Schleicher & Schuell (Keene, NH) slot blot apparatus. The membranes were prehybridized at 65°C for 3 h in 5 ml of hybridization buffer [5× SSC, 1% SDS, 10 mM EDTA, 10× Denhardt's solution, 150 μg/ml poly(A) (Sigma, St. Louis, MO), and 50 μg/ml yeast RNA (Sigma, St. Louis, MO)] followed by hybridization with 2×10^7 cpm of radio labeled RNA for 72 h. After washing the membranes for 1 h with 2× SSC and 0.1% SDS, followed by 30 min washing with 0.2× SSC and 0.1% SDS, hybridizations were visualized by autoradiography and by exposing the membranes to a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA).

mRNA Stability Assay. Cells 2 days after confluence and cells treated for 7 days with dexamethasone, insulin, and BRL49653 were treated with ActinomycinD (25 μg/ml) (Sigma, St. Louis, MO) for 1 h to inhibit transcription. RNA was isolated at time points thereafter, treated with DNase, and reverse transcribed using oligo(dT) priming and Superscript II reverse transcriptase following the manufacturer's instructions. mRNAs for *fra*-1 and 36B4 were quantified by PCR using real-time fluorescent detection (Roche, Mannheim, Germany). The following primer combinations 5′-GAGCTGGCCTATCATAATTTGC-3′ and 5′-GTTCTAGGCTAGTCAAAGGGCAC-3′ for *fra*-1 and 5′-TAAAGACTGGAGACAAGGTGGGAG-3′ and 5′-AGAAAGCGAGAGT-GCAGGGC-3′ for 36B4 were used. The half-life of mRNA was calculated by "linear estimation" from the best fit of each line.

Western Blot Analysis. Cells were washed twice with PBS, harvested by scraping in PBS, and resuspended in 10 mM HEPES, pH 8.0, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride, corresponding to two thirds of the volume of packed cells. Cells were lysed by being pressed 10 times through a 25-gauge syringe, and the lysate was centrifuged for 40 s at 12,000*g*. The pellet consisting of nuclei was resuspended in 20 mM HEPES, pH 8.0, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, pH 8.0, 26% glycerol, 1 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride, corresponding to two thirds of the volume of packed nuclei and incubated on ice for 30 min. The lysate was centrifuged for 5 min. at 12,000*g* and the supernatant containing nuclear proteins was frozen at −80°C until use. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) according to the vendor's instructions. Proteins were separated by SDS-PAGE using 4 to 12% NuPAGE gels (Novex, San Diego, CA) following the manufacturer's instructions and transferred to a nitrocellulose membrane, using a semidry blotter from Kem-En-Tec (Copenhagen, Denmark). The membrane was subsequently blocked in PBS buffer containing 5% nonfat dry milk, and 0.05% Tween 20, incubated for 3 h with rabbit anti-Fra-1 antiserum (sc-605) or goat anti-JunD antiserum (sc-74-G) (Santa Cruz Biochemicals, Santa Cruz, CA) diluted 1:1000 in the blocking buffer mentioned above, washed four times in blocking buffer, and finally incubated with horseradish peroxidase (HRP) conjugated swine anti-rabbit IgG antibody or rabbit anti-goat IgG antibody (DAKOPATTS A/S, Glostrup, Denmark) diluted 1:2000 in blocking buffer. Bound HRP was detected with enhanced chemiluminescence Western blotting detection reagents according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Electrophoretic Mobility Shift Assay. Plasmids encoding the murine Fra-1 and JunD were in vitro transcribed and translated using the TNT coupled system (Promega, Roedovre, Denmark) following the manufacturer's protocol. Nuclear cell extracts from 3T3-L1 cells were prepared as described above. The pairs of corresponding oligonucleotides used in the experiments included: AP-1 binding site containing oligonucleotides: nucleotides A, 5′-CGCTTGATGACTCAGCCGGAA-3′ and 5′-TTCCGGCTGAGTCATCAAGCG-3; nucleotides B, 5′-AGCTGTGTCTGACTCATGCT-3′ and 5′-AGCATGAGTCAGACACAGCT-3′; and NF-κB binding site containing oligonucleotides, 5′-TCTCAGAGGGGACTTT-3′ and 5′-AAAGTCCCCTCTGAGA-3′. These annealed oligonucleotides (10 pmol) were end-labeled with [γ-^{32}P]ATP (5000 Ci/mmol; Amersham Pharmacia Biotech, Buckinghamshire, UK) and T4 polynucleotide kinase (New England Biolabs, Hellerup, Denmark) and purified on Nick columns. For electrophoretic mobility shift assays 2 μl of in vitro transcribed and translated Fra-1, JunD, or 3.5 μg of nuclear cell extracts were incubated at 4°C for 30 min with 2 μg of poly(dI-dC) (Amersham Pharmacia Biotech, Uppsala, Sweden) in binding buffer containing 20 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 50 mM KCl, and 15% glycerol. The ^{32}P-labeled probe (approximately 200 fmol) was then added to a final volume of 20 μl and the incubation continued for 30 min. The resulting DNA-protein complexes were resolved from the free probes by electrophoresis on a 6% nondenaturing polyacrylamide gel for 4 h at 4°C at 200 V in 0.25× Tris-borate-EDTA buffer and visualized by autoradiography. For supershift experiments, proteins were preincubated for 1 h at 4°C with 2 μg of antibodies before the addition of the labeled oligonucleotides. The antibodies used were rabbit anti-Fra-1 (sc-605-X), rabbit anti-JunD (sc-74-X) or rabbit anti-RXR (sc-774-X) (Santa Cruz Biochemicals). When binding competitions were performed, a 10- or 100-fold excess of unlabeled oligonucleotides were premixed with the ^{32}P-labeled probe before addition to the protein mixtures.

Results

Fra-1 Is Induced by BRL49653 During Differentiation of 3T3-L1 Cells. To identify novel genes regulated by thiazolidinediones in developing adipocytes, we treated 3T3-L1 cells with dexamethasone (Dex), insulin (Ins), and BRL49653 (BRL) and used mRNA differential display analysis to isolate differentially regulated cDNAs. Total RNA isolated from 3T3-L1 cells at three different time points were used: from cells 2 days after confluence (referred to as day 0), from cells treated for 1 day, and from cells treated for 7 days. cDNAs derived from day 0, day 1, and day 7 were then used as templates for the differential display analysis (see *Materials and Methods*). As reported elsewhere, a number of differentially expressed bands were isolated and sequenced (T. Albrektsen, K. S. Frederiksen, W. E. Holmes, E. Boel, K. Taylor, J. Fleckner, in preparation). Here we report on the characterization of a 251-base-pair fragment that was specifically up-regulated after 7 days of differentiation (Fig. 1A). This fragment was in GenBank found to be identical to sequences of the murine *fra*-1 (Fos-related antigen 1). The induction of *fra*-1 was confirmed by Northern blot analysis using full-length cDNA as probe (Fig. 1B). Furthermore, the use of two different differentiation protocols, one using Dex and Ins and another using Dex, Ins, and BRL, revealed that the induction of *fra*-1 was dependent on the presence of BRL (Fig. 1B). We next examined whether BRL alone or BRL in combination with Dex or Ins was sufficient to induce the expression of *fra*-1 but as shown in Fig. 1C all three components of the differentiation mix was required for the induction of *fra*-1. We also tested the concentration dependent induction of *fra*-1 by BRL and other PPAR ligands, including troglitazone and pioglitazone (Fig. 1D). Induction of *fra*-1 was observed at concentrations of 100 nM BRL, 1 μM pioglitazone, and 10 μM troglitazone, which is well in agreement with the rank order of potency of these compounds to bind to recombinantly expressed hPPARγ, as well as to rat and hu-

man adipocytes (Young et al., 1998), suggesting that the induction of *fra*-1 is mediated by PPARγ.

Up-Regulation of Fra-1 Is Caused by Increased Transcription of the Fra-1 Gene and Stabilization of Fra-1 mRNA. To elucidate the mechanism by which Dex, Ins, and BRL induce the expression of *fra*-1, we first analyzed whether *fra*-1 transcription was increased. Using nuclear run-on transcription analysis, nuclei isolated from cells 2 days after confluence (day 0) and nuclei isolated from cells treated for 7 days with the differentiation mixture (day +7) were compared. As seen in Fig. 2A, the *fra*-1 gene transcription was enhanced after 7 days of treatment with Dex, Ins, and BRL, suggesting that the rise of *fra*-1 mRNA is caused, at least in part, by increased de novo synthesis of *fra*-1 mRNA. Densitometric scanning of the filter showed that *fra*-1 transcription was elevated 2.5-fold. aP2 and PPARγ genes were used as positive controls because they are known to be regulated at the transcriptional level. In our experiment, transcription of PPARγ and aP2 were elevated 6.4- and 16-fold, respectively. The 36B4 gene was used as an internal control and the PCR product from the TOPO 2.1 plasmid, into which all cDNAs were cloned using T7 and m13 reverse primers, was used as negative control. We next examined whether post-transcriptional regulation could also account for part of the increase of the *fra*-1 mRNA. To address this,

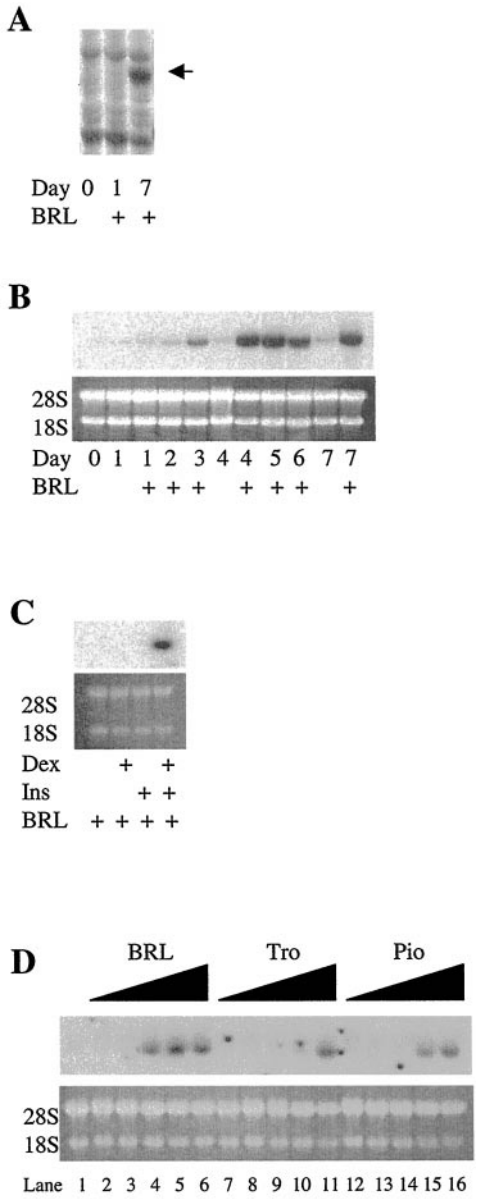

Fig. 1. Induced expression of *fra*-1 by BRL during differentiation of 3T3-L1 cells. A, differential display analysis of 3T3-L1 cells induced to differentiate with Dex, Ins, and BRL. B, Northern blot analysis of *fra*-1 expression in 3T3-L1 cells induced to differentiate with Dex and Ins, or Dex, Ins, and BRL. C, Northern blot analysis of *fra*-1 expression in 3T3-L1 cells differentiated for 7 days with the indicated combinations of compounds. D, Northern blot analysis of *fra*-1 expression in 3T3-L1 cells differentiated for 7 days with Dex and Ins alone (lane 1) or Dex, Ins and increasing concentrations [1 nM (lanes 2, 7, and 12), 10 nM (lanes 3, 8, and 13), 100 nM (lanes 4, 9, and 14), 1 μM (lanes 5, 10, and 15), 10 μM (lanes 6, 11, and 16)] of BRL, troglitazone, or pioglitazone.

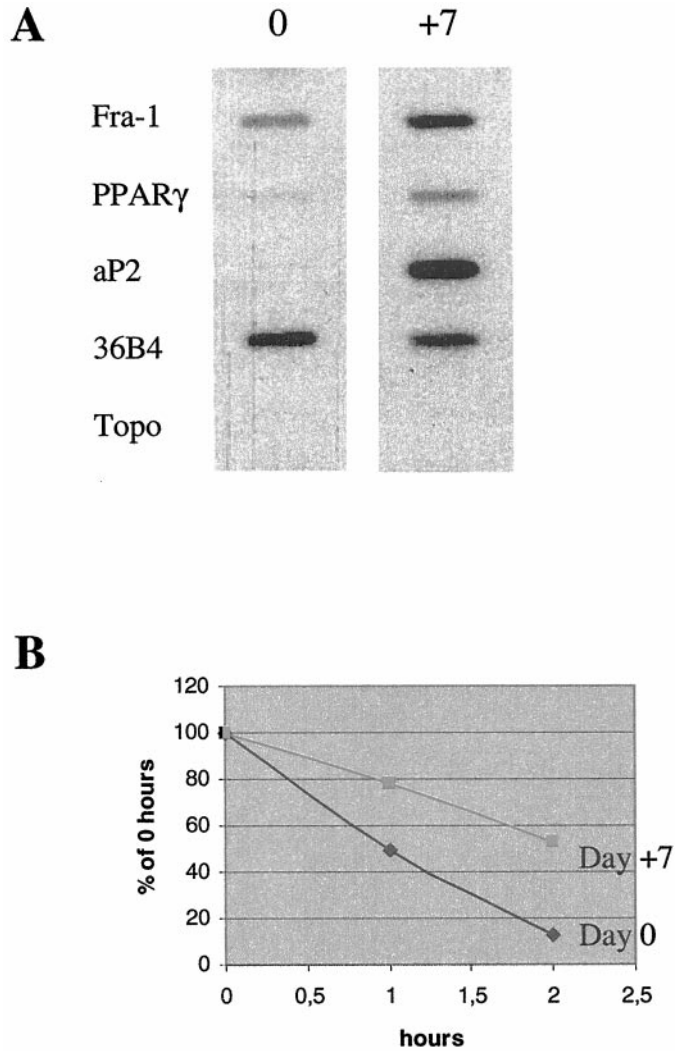

Fig. 2. Transcriptional regulation of *fra*-1 gene and stabilization of *fra*-1 mRNA. A, nuclear run-on transcripts were isolated from 3T3-L1 cells 2 days after confluence and from 3T3-L1 cells treated for 7 days with Dex, Ins, and BRL and equal amounts of ^{32}P-labeled transcripts were hybridized to slot-blotted *fra*-1, PPARγ, aP2, and 36B4 cDNAs, and flanking regions of PCR-products using T7 and m13 reverse primers from TOPO 2.1 plasmid. B, two days later, confluent 3T3-L1 cells and 3T3-L1 cells treated for 7 days with Dex, Ins, and BRL were treated with actinomycin D for 1 h and total RNA was isolated at the indicated time points thereafter. Degradation of *fra*-1 mRNA was analyzed by quantitative PCR using real-time fluorescent detection and normalized to mRNA levels of 36B4. Data points are means of duplicate measurements of *fra*-1 and 36B4.

we analyzed the stability of *fra*-1 mRNA in cells in which ongoing RNA synthesis had been blocked by actinomycin D. Because only a very small amount of *fra*-1 mRNA could be detected by Northern blotting in cells before differentiation, we decided to detect the degradation of *fra*-1 mRNA after transcriptional inhibition by quantitative PCR using real-time fluorescent detection (Fig. 2B). mRNA levels of *fra*-1 was normalized to mRNA levels of 36B4, which was not degraded within the time of the measurements. Inhibition of transcription by actinomycin D resulted in a rapid decay of *fra*-1 mRNA in nondifferentiated confluent cells with a half-life of 60 min. In contrast, the half-life of *fra*-1 mRNA in cells treated for 7 days with Dex, Ins, and BRL was 130 min. corresponding to a 2.2 fold increase in stability of *fra*-1 mRNA in differentiated 3T3-L1 cells compared with nondifferentiated cells. Therefore, these data imply that the increase in *fra*-1 mRNA in 3T3-L1 cells treated to differentiate with Dex, Ins, and BRL results from both enhanced transcription rate of the *fra*-1 gene and an increase in the stability of *fra*-1 mRNA.

Fra-1 and JunD Are the Only Members of the AP1 Family that Are Expressed in Differentiated 3T3-L1 Cells. As Fra-1 is a member of the AP-1 family of transcription factors, comprising c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB, and JunD this raised the issue of whether Fra-1 was the only member of this family to be specifically induced by BRL or whether this was a general phenomenon among these proteins. As shown in Fig. 3A, none of the AP-1 genes, except *junD*, were expressed in cells before induction of differentiation while all, except *junD*, were up-regulated after 1 h of treatment with the differentiation cocktail. Longer exposure of the cells to the differentiation mixture showed decreased expression of all induced genes and only *fra*-1 expression increased again after 3 days of treatment while none of the other members were affected.

Members of the AP-1 family are described as cellular immediate-early response genes and previous analyses have demonstrated that selected members of the AP-1 family are induced by treatment of 3T3-L1 cells with Dex, Ins, and methylisobutylxanthine (Stephens et al., 1993). We therefore also analyzed the immediate-early response of these genes to the differentiation mixture consisting of Dex and Ins, or Dex, Ins, and BRL (Fig. 3A). Importantly, the immediate-early responses were independent of the presence of BRL because treatment of the cells with Dex and Ins was sufficient to induce the expression of the genes. Further analyses showed that treatment of the cells with Dex, Ins, or BRL alone, and even the change of media, resulted in an increased expression of *fra*-1 (Fig. 3B). A temporary weak induction of *fra*-1, corresponding to the induction seen with change of media, was also seen throughout the course of differentiation with the combination of Dex and Ins each time media were changed (data not shown). To ensure that the observed increased expression of *fra*-1 was not a result of media change, we therefore always changed media 24 h before harvesting RNA.

Taken together, these data illustrate that *fra*-1 is the only member of the AP-1 transcription family that is induced after longer exposure of 3T3-L1 cells with a combination of Dex, Ins, and BRL. Furthermore, Northern blot analysis of the adipocyte marker AdipoQ shows that the cells do differentiate by treatment of the cells with a combination of Dex and Ins alone, but that the induction of *fra*-1 depends on the presence of the high-affinity PPARγ ligand BRL49653.

Fra-1 and JunD Form Functional Heterodimers in Nuclear Extracts from Differentiated 3T3-L1. As shown in Fig. 3A, *fra*-1 and *junD* are the only members of the AP-1 family that are expressed at the mRNA level in differentiated 3T3-L1 cells. The expression of the corresponding proteins was verified by Western blotting as seen in Fig. 4. The expression profile of the proteins corresponded very well with that of the mRNAs. JunD protein was ubiquitously expressed throughout the course of differentiation of the cells, whereas Fra-1 protein was expressed in the immediate early phase as well as after longer exposure in the presence of Dex, Ins, and BRL.

Jun family proteins are able to bind to the consensus AP-1 binding site sequence as homodimers, whereas Fos family proteins form only heterodimers with Jun proteins, complexes that are more stable and have greater *trans*-activation capacities than Jun homodimers. To analyze whether Fra-1 and JunD were able to form heterodimers that bind to DNA,

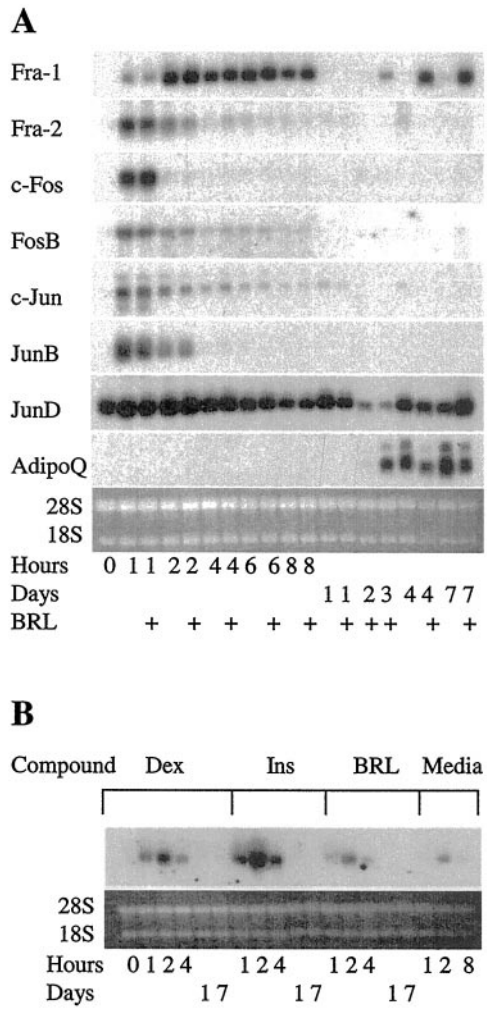

Fig. 3. Immediate early and late regulations of AP-1 transcription factors during differentiation of 3T3-L1 cells. A, total RNA was isolated at different time points (hours and days) after initiation of differentiation with Dex and Ins, or Dex, Ins, and BRL. Northern blot analysis of the expression of the members of the *fos-jun* family as well as AdipoQ was performed. B, Northern blot analysis of *fra*-1 expression at the indicated time points after treatment of 3T3-L1 cells with Dex, Ins, BRL, or media change alone.

we first analyzed the ability of in vitro transcribed and translated Fra-1 and JunD proteins to bind to a consensus AP-1 motif. Unlabeled Fra-1 and JunD proteins were synthesized in vitro in rabbit reticulocyte lysates and the proteins were either mixed together or used alone in electrophoretic mobility shift assays employing a ^{32}P-labeled oligonucleotide containing an AP-1 consensus binding sequence. In agreement with results reported previously (Ryseck and Bravo, 1991) and as illustrated in Fig. 5A, only JunD could form homodimers that bound to the AP-1 motif (Fig. 5A, lane 3), whereas a combination of Fra-1 and JunD formed a complex migrating slightly faster than the JunD homodimer (Fig. 5A, lane 4). No Fra-1 homodimer formation could be detected (Fig. 5A, lane 2). Antibodies against Fra-1 were shown to supershift the Fra-1-JunD heterodimer (Fig. 5A, lane 5), whereas antibodies against JunD inhibited the binding of the Fra-1-JunD heterodimer but had only minor effects on the JunD homodimer (Fig. 5A, lane 6). Control rabbit sera raised against RXR had no effect on the Fra-1-JunD heterodimer binding to the AP-1 motif (Fig. 5A, lane 7). We next performed experiments to analyze the ability of Fra-1 and JunD proteins expressed in differentiated 3T3-L1 cells to form heterodimers that bind to an AP-1 consensus sequence. Nuclear protein extracts from 3T3-L1 cells differentiated for 7 days with Dex, Ins, and BRL contained an AP-1 binding complex migrating as the Fra-1-JunD heterodimer (Fig. 5A, lane 8). Preincubation of the extracts with antibodies raised against Fra-1 showed a reduced binding of the complex (Fig. 5A, lane 9) and longer exposure of the gel showed a supershifted band (Fig. 5A, lane 13). Antisera that recognize JunD also reduced the complex formation (Fig. 5A, lane 10), whereas RXR antisera had no effect on the complex formation (Fig. 5A, lane 11). The specificity of the Fra-1-JunD complex, formed by in vitro synthesized proteins (Fig. 5B, lanes 2–6) and nuclear extracts from 3T3-L1 cells differentiated for 7 days with Dex, Ins, and BRL (Fig. 5B, lanes 7–11) was assessed by its complete disappearance in the presence of 100-fold excess of unlabeled AP-1 probe (Fig. 5B, lanes 4 and 9), whereas incubation in the presence of an unrelated competitor (NFκB) did not modify the retarded band (Fig. 5B, lanes 6 and 11). To analyze whether the AP-1 complex was induced by BRL-stimulated differentiation of the cells, the AP-1 binding capability of nuclear extracts from 3T3-L1 cells at confluence (day 0) and from cells treated for 7 days with Dex and Ins alone (day −7) were compared with the AP-1 binding capability of nuclear extracts from cells treated for 7 days with all components (Dex, Ins, and BRL) (day +7). As shown in Fig. 5C, a complex bound to the AP-1 consensus sequence independent of the BRL treatment. However, only cells treated with BRL contained Fra-1 (Fig. 5C, lane 3). Thus, these data indicate that 3T3-L1 fibroblasts, as well as differentiated 3T3-L1 cells, contain AP-1 binding complexes, most likely formed by JunD homodimers. However, only when differentiated in the presence of BRL do these complexes contain Fra-1. Together, these data suggest that BRL-stimulated differentiation of 3T3-L1 cells results in cells expressing

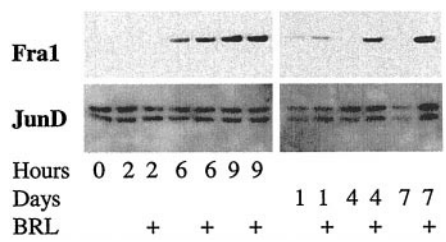

Fig. 4. Expression of Fra-1 and JunD proteins during differentiation of 3T3-L1 cells. Nuclear cell extracts were isolated from 3T3-L1 cells treated with Dex and Ins, or Dex, Ins, and BRL at the indicated time points and nuclear proteins were subjected to SDS-PAGE followed by Western blotting using antibodies against Fra-1 and JunD.

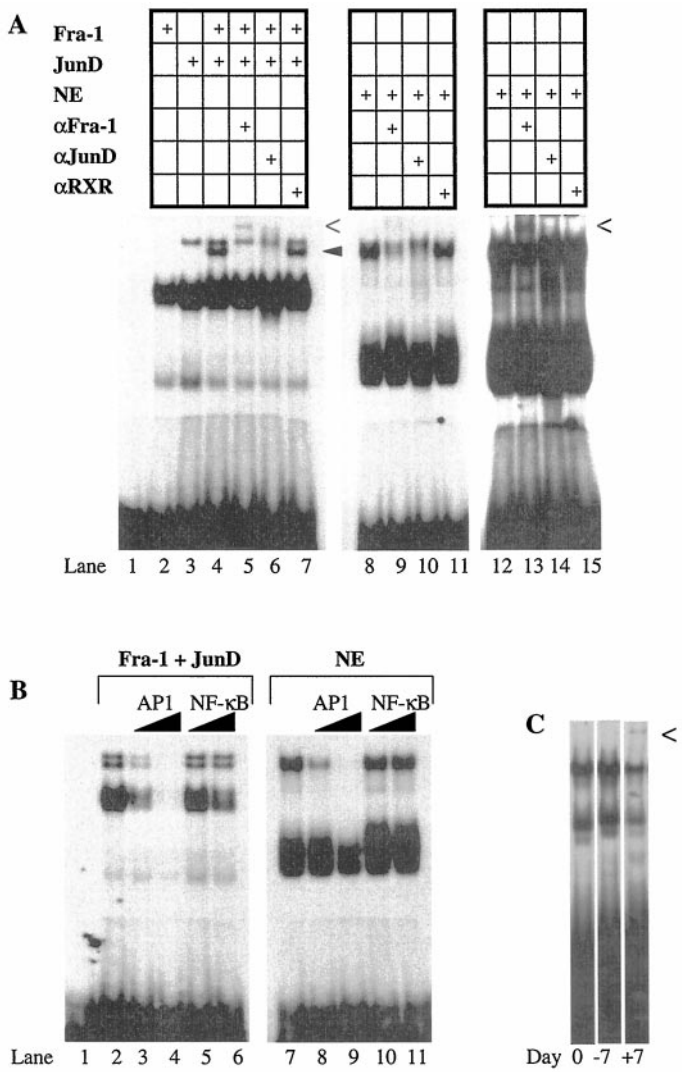

Fig. 5. In vitro binding activity of 3T3-L1 adipocyte nuclear proteins to an AP-1 consensus sequence. A, in vitro transcribed and translated Fra-1 and JunD (lanes 2–7) as well as nuclear extracts (NE) from 3T3-L1 cells differentiated for 7 days with Dex, Ins, and BRL (lanes 8–15) were incubated with ^{32}P-labeled double stranded oligonucleotides containing the AP-1 consensus binding site (nucleotides A) and complexes were separated on a 6% nondenaturing acylamide gel as described under *Materials and Methods*. ^{32}P-labeled double-stranded oligonucleotide alone is shown in lane 1. Immunological detection of Fra-1 and JunD in the complexes was performed by preincubation of protein complexes with polyclonal antibodies before addition of ^{32}P-labeled double-stranded oligonucleotides. Fra-1-JunD heterodimers are indicated by closed arrow and Fra-1 supershifts are indicated by open arrow. B, competition experiments were performed by addition of 10- or 100-fold excess of nonradioactive oligonucleotides containing AP-1 or NFκB consensus response elements to the labeled probe. C, immunological detection of Fra-1 in the AP-1 complexes created in nuclear extracts from 3T3-L1 cells 2 days after confluence (day 0), cells treated for 7 days with Dex and Ins alone (day −7) as well as cells treated for 7 days with Dex, Ins, and BRL (day +7) using nucleotides B. Fra-1 supershift is indicated by open arrow.

functional Fra-1 and JunD proteins that form heterodimers, which bind to a consensus AP-1 binding site.

Discussion

The treatment of NIDDM has recently been improved by the identification of TZDs as novel agents with the ability to improve the sensitivity to insulin and thereby lower serum glucose levels. Furthermore, TZDs have been shown to efficiently lower the levels of free fatty acids in the plasma. The finding of TZDs as high-affinity ligands for PPARγ and the observed correlation between the antidiabetic potency of selected TZDs and their abilities to activate PPARγ suggest that PPARγ through downstream-regulated genes mediates the effect of TZDs.

To delineate molecular targets, potentially responsible for the antidiabetic effects of TZDs, we have identified genes in differentiating adipocytes exhibiting altered expression after exposure to the potent TZD, BRL49653. The model system used for these studies was 3T3-L1 mouse embryo fibroblasts, which by treatment with dexamethasone, insulin, and TZD can efficiently differentiate into adipocytes. Using mRNA differential display analysis, a variety of genes inducible during this process was identified and cloned, including fatty acid transporter, FSP27, and inorganic pyrophosphatase (T. Albrektsen, K. S. Frederiksen, W. E. Holmes, E. Boel, K. Taylor, J. Fleckner, in preparation). Here we have characterized details of the regulation of a novel molecular target of TZDs, the transcription factor Fra-1.

Fra-1 is a component of the AP-1 transcription factor complex, consisting of dimer combinations formed between proteins of the Jun and Fos families. To date, three Jun proteins (c-Jun, JunB, and JunD) and four Fos family members (c-Fos, FosB, Fra-1, and Fra-2) have been identified (reviewed by Angel & Karin, 1991). The members of the two families share a high degree of sequence homology and they all contain a "leucine-zipper" domain and a basic region that mediate protein-protein interactions and DNA binding, respectively. Jun proteins are able to homodimerize, whereas Fos proteins function only upon heterodimerization with members of the Jun family proteins. All homodimers and heterodimers recognize a consensus AP-1 site (TGAC/GTCA), which is found in a number of cellular promoters. AP-1 complexes in response to a variety of extracellular stimuli have been implicated in a range of very different processes in different cell types, including proliferation, growth arrest, differentiation, and apoptosis. Therefore, the biological function of AP-1 proteins is highly dependent on the cell type, its differentiation state, and the particular environment.

Several different compounds as well as cellular manipulations have previously been shown to result in differential expression of Fra-1 in a variety of cell lines. First, *fra*-1 has been shown to be transcriptionally induced by oleate in a dose-dependent manner in another preadipocyte cell line, 3T3-F442A (Distel et al., 1992). In this respect, it is tempting to speculate that the induction by oleate was mediated by PPARγ, because long-chain fatty acids in high concentrations have been shown to activate this receptor. However, only a very small amount of PPARγ can be detected in preadipocytes, suggesting that additional mechanisms are at play to explain the induced expression of *fra*-1 by oleate. Distel et al. did not, however, analyze the effect of fatty acids on *fra*-1 expression in differentiated adipocytes. Second, *fra*-1 has been demonstrated to be transcriptionally activated by AP-1 through a consensus AP-1 site found in the first intron of the *fra*-1 gene (Bergers et al., 1995) and recent experiments suggest that *fra*-1 is a transcriptional target of c-Fos during osteoclast differentiation (Matsuo et al., 2000). Third, the testis-determining factor Sry (Cohen et al., 1994) and the transcription factor Tax-1 of the human T-cell leukemia virus type I (Tsuchiya et al., 1993) have been shown to transcriptionally stimulate *fra*-1 gene expression. Fourth, *fra*-1 has been reported to be a target of the high-mobility group I-C chromatin component (Vallone et al., 1997). Finally, overexpression of β-catenin in colorectal cell lines results in transcriptional induction of *fra*-1 expression (Mann et al., 1999). In summary, previous experience shows that *fra*-1 expression can be regulated by several transcription factors; our results add PPARγ to the list of factors controlling Fra-1.

A search through publicly available DNA databanks retrieved a 931-base-pair sequence upstream of the structural mouse *fra*-1 gene. Inspection of this sequence revealed the presence of one stretch of sequence with homology (10 of 13 bases in a DR-1 element) to a consensus PPRE (AGGTCAnAGGTCA). However, no PPARγ responsive enhancer effect of the entire 931 base pair fragment could be detected in transient cotransfection experiments of CV-1 and HEK 293 cells (data not shown). This suggests either that other potential PPREs are to be found further up-stream in the *fra*-1 promoter or that transcriptional activation of *fra*-1 by PPARγ is mediated via an additional induced protein.

In addition to the transcriptional induction of *fra*-1 expression, *fra*-1 was recently illustrated to be up-regulated by the retinoid acid receptor ligands ATRA and 13-*cis*-RA in a post-transcriptional manner (Kaiser et al., 1999). This post-transcriptional regulation of *fra*-1 is well in accordance with the stabilization of *fra*-1 mRNA observed in our experiments. The mechanism by which TZDs increase the stability of *fra*-1 mRNA remains to be established. However, previous studies of another member of the Fos family, c-Fos, have demonstrated that the extreme instability of the mRNA of this protein is partly caused by the presence of multiple AU-rich destabilizing elements in its 3′-UTR (Fort et al., 1987). Different hypotheses have been proposed for the mechanisms explaining the increased mRNA stability, including down-regulation of auto-repressing proteins, allowing continued maintenance of the mRNA available for translation (Pontecorvi et al., 1988).

Analysis of the expression pattern of all members of the Fos-Jun family of transcription factors during the course of differentiation of 3T3-L1 cells revealed that *fra*-1 was the only member that was specifically induced after prolonged exposure of 3T3-L1 cells with the differentiation cocktail containing Dex, Ins, and TZD. The only other member of the family expressed in differentiated 3T3-L1 cells was *junD*. Functional heterodimers between and Fra-1 and JunD was found in these cells, and taken together these data suggest that Fra-1 is the TZD-sensitive component of the AP-1 complex found in 3T3-L1 adipocytes. Fra-1 and JunD have previously been shown to form heterodimers in vitro (Ryseck and Bravo, 1991) and complexes of Fra-1 and JunD and/or FosB have been demonstrated to bind to AP-1 sequences in the promoter regions of and transcriptionally activate interleukin-2 in activated T-cells (Boise et al., 1993) and involu-

crin in keratinocytes (Welter et al., 1995). However, no target genes for Fra-1/JunD heterodimers have yet been found in adipocytes. It is therefore tempting to speculate that the Fra-1/JunD complex regulates genes that play an important role for the effect of TZDs. Treatment of 3T3-L1 cells with TZDs in combination with Dex and Ins results in stimulation of differentiation of the cells. This is well in accordance with previous in vivo reports demonstrating that TZDs increases the number of small size adipocytes and stimulates the expression of genes involved in lipid metabolism of adipocytes in obese Zucker *fa/fa* rats (Hallakou et al., 1997). It is therefore possible that the induced Fra-1 expression and the resulting Fra-1/JunD complex binds to promoter regions of genes important for the development and/or maintenance of the adipocyte phenotype. In this respect, it is important to note that Fra-1 lacks a *trans*-activation domain (Bergers et al., 1995). It is therefore possible that Fra-1 limits the activity of other potent *trans*-activators, such as c-Fos and FosB or other unknown factors interacting with AP-1 proteins, by competing for Jun-proteins as dimerization partners, sequestering them into DNA-bound AP-1 factors with low transcriptional activity. Thus, Fra-1 could act as a repressive factor, controlling genes specific for a preadipocyte phenotype. Previous studies of a related adipocyte cell line, 3T3-F442A, has revealed that c-fos binds to a specific site in the promoter region of the differentiation-sensitive adipocyte gene, aP2, and acts as a negative regulator in preadipocytes (Distel et al., 1987). However, this specific site does not play a negative role in adipocytes, and it could be speculated that Fra-1 substitutes for c-Fos for binding to this element suppressing the repressive function of c-fos in this cell line. Alternative roles of the induced expression of Fra-1 could involve the regulation of proteins secreted by the adipocytes, which affect the glucose uptake by the skeletal muscle or hepatic glucose output from the liver. Finally, it cannot be ruled out that Fra-1 interacts with proteins other than JunD. Previous studies have demonstrated that several other proteins, distinct from Jun and Fos, can dimerize directly with AP-1 proteins that affect the activity of the transcription factor complexes. These proteins include activating transcription factor/cAMP response element-binding protein (Hai and Curran, 1991), Maf (Kataoka et al., 1994), and nuclear factor of activated T cells (Jain et al., 1993). However, further studies must be performed to answer these questions.

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