

# Synthesis and accumulation of a receptor regulatory protein associated with lipid droplet accumulation in 3T3-L1 cells

D. J. Orlicky,<sup>1,\*</sup>§ J. G. Lieber,\* C. L. Morin,<sup>†</sup> and R. M. Evans\*

Department of Pathology,\* Center for Human Nutrition,<sup>†</sup> and Colorado Cancer Center,<sup>§</sup> University of Colorado Health Sciences Center, 4200 East 9th Avenue, Denver, CO 80262

**Abstract** Synthesis and accumulation of the recently identified prostaglandin  $F_{2\alpha}$  receptor regulatory protein (FPRP) was found to correlate closely with lipid droplet accumulation by 3T3-L1 preadipose cells. FPRP, a transmembrane glycoprotein, has been shown to regulate the binding of ligand to certain seven-transmembrane receptors. Anti-FPRP immunohistochemistry, Western blotting, and metabolic labeling/immunoprecipitation experiments demonstrated that FPRP was not detectable in undifferentiated 3T3-L1 cells. Interestingly, low levels of FPRP mRNA were detected in the undifferentiated 3T3-L1 cells. After induction of adipose differentiation, FPRP mRNA increased ~3 fold whereas FPRP synthesis increased ~50 fold. Differentiation induction with either dexamethasone/insulin/isobutylmethylxanthine or the thiazolidinedione derivative ADD 4743 were both effective at inducing FPRP accumulation and accumulation of lipid droplets. By co-immunohistochemical and lipid staining, greater than 99% of the cells accumulating lipid droplets possessed FPRP. FPRP mRNA and protein are also found in rat adipose tissue. Treatment of 3T3-L1 cells with an FPRP anti-sense oligonucleotide during differentiation decreased FPRP accumulation and resulted in a decrease in lipid droplets without altering the level of induction of a late marker of adipocyte differentiation, glycerol-3-phosphate dehydrogenase activity. Transient expression of an FPRP cDNA in undifferentiated 3T3-L1 cells was insufficient to induce lipid droplet accumulation.—Orlicky, D. J., J. G. Lieber, C. L. Morin, and R. M. Evans. **Synthesis and accumulation of a receptor regulatory protein associated with lipid droplet accumulation in 3T3-L1 cells.** *J. Lipid Res.* 1998. 39: 1152–1161.

**Supplementary key words** 3T3-L1 adipocytes • adipogenesis • prostaglandin  $F_{2\alpha}$  • receptor regulatory protein • lipid accumulation • dexamethasone • thiazolidinedione

Prostaglandin  $F_{2\alpha}$  receptor regulatory protein (FPRP) is a glycosylated, type 1 integral membrane protein (1). It was originally identified and characterized due to its ability to associate with and inhibit [<sup>3</sup>H]prostaglandin  $F_{2\alpha}$  binding to the prostaglandin  $F_{2\alpha}$  receptor. The mecha-

nism by which FPRP inhibits ligand binding is not completely understood, but the observed inhibition in binding is due to a decrease in the number of receptors capable of binding ligand, rather than in receptor affinity (2). FPRP can also decrease ligand binding to certain other seven transmembrane receptors, including the  $\beta_1$ -adrenergic receptor (D. Orlicky, unpublished data). The predicted structure of FPRP, from conceptual translation of its open reading frame, includes a signal sequence, 6 glycosylated immunoglobulin loops, a single transmembrane domain, and a short, highly charged, intracellular carboxy tail. By sequence comparison, FPRP does not have any similarity either to other prostaglandin receptors or to the peroxisome proliferator-activated receptors (PPARs). A human homologue of FPRP has been partially cloned and mapped to chromosome 1 (3). BLASTP sequence data bank searches show FPRP to be significantly similar to only one other known protein, the human leukocyte protein V7 which functions to inhibit the T-cell receptor in activated T-cells (4, 5).

FPRP is expressed in a limited, cell-type specific manner including in adipocytes, cardiomyocytes, osteoclasts, granulosa lutein cells, Leydig cells, and a subset of mammary glandular cells (1, D. Orlicky, unpublished data). To further elucidate the function of FPRP, we chose to examine its expression in the 3T3-L1 preadipocyte cell line, a cell line representative of fat cell metabolism (6–8). 3T3-L1 preadipocytes differentiate into mature, lipid droplet-containing adipocytes when stimulated with an appropriate hormonal regimen. During the process of adipocyte conversion they lose their primitive mesenchymal character, assume a rounded morphology, and acquire many of the enzymatic and biochemical characteristics of adipocytes.

Abbreviations: PGF<sub>2 $\alpha$</sub> , prostaglandin  $F_{2\alpha}$ ; FPRP, prostaglandin  $F_{2\alpha}$  receptor regulatory protein; TAG, triacylglycerol; G3PDH, glycerol-3-phosphate dehydrogenase; ORO, oil red O; NR, Nile Red.

<sup>1</sup>To whom correspondence should be addressed.

Adipocytes play a key role in providing energy in times of demand and the storage of energy in the form of triacylglycerol (TAG)-rich lipid droplets. Understanding the factors and mechanisms involved in adipocyte lipid homeostasis is key to understanding conditions such as obesity, lipotrophies, and noninsulin-dependent diabetes as well as hypertension and coronary artery disease. Therefore, identification of proteins associated with lipid droplet accumulation is an important step in understanding the normal regulation of adipocytes or other lipogenic cells. The results of our studies indicate that adipocyte FPRP accumulation commenced during differentiation of 3T3-L1 cells, and that inhibition of FPRP accumulation decreases the accumulation of lipid droplets in these 3T3-L1 cells.

## MATERIALS AND METHODS

### Cells and cell culture

3T3-L1 cells were obtained from the American Type Culture Collection (ATCC CCL 92.1) and maintained in a 1:1 mixture of Ham's F-12:Dulbecco's MEM supplemented with 10% fetal bovine serum and 10  $\mu\text{g}/\text{ml}$  gentamycin (F12:DMEM:FBS). Adipocyte conversion was induced by treating 2 day post-confluent cultures with F12:DMEM:FBS containing 0.25  $\mu\text{M}$  dexamethasone, 0.5 mM isobutylmethylxanthine (IBMX), and  $10^{-7}$  M insulin for 48 h, then with F12:DMEM:FBS containing  $10^{-7}$  M insulin for the duration of the experiment (Diff. Media A and B, respectively). Diff. Media B was changed every 48 h as necessary. Alternatively, conversion was induced with F12:DMEM:FBS containing 2  $\mu\text{g}/\text{ml}$  ADD4743 (gift from Takeda Chemical Industries Ltd.) and  $10^{-7}$  M insulin for the entire experiment (with medium change every 48 h). Cells were harvested and analyzed between 0 and 5 days post differentiation induction.

### Evaluation of adipocyte conversion

Lipid accumulation in 3T3-L1 cells was identified by Oil Red O (ORO) staining and quantitated as described by Lieber and Evans (9). Cells were considered to be undergoing adipocyte conversion if they assumed a rounded morphology and contained ORO-positive lipid droplets equal to or greater than 3 microns in diameter. This operating definition of adipocyte conversion has been used because undifferentiated 3T3-L1 cells do not contain lipid droplets of this size (6, 7, 9). For quantitation, 400–800 cells in randomly chosen fields for each experimental group were evaluated independently by three individuals. These values were then averaged.

Alternatively, cells were plated and grown as indicated below for anti-FPRP immunohistochemistry, then at the time of harvest the coverslips were fixed in Bouin's fixative as previously described (2), and stained with Nile Red (NR) to detect lipid droplets as described by Greenspan, Mayer, and Fowler (10) using a final concentration of NR in phosphate-buffered saline of 1.7 ng/ml. When both FPRP and NR staining were to be performed on the same coverslip, the FPRP staining was performed first on coverslips fixed in Bouin's fixative. Due to the broad emission spectrum of NR, in co-stained samples both FPRP and lipid droplets were visualized at the same time.

### Oligonucleotides (Macromolecular Resources, Ft. Collins, CO)

Anti-sense oligonucleotides were chosen after examination of the predicted secondary RNA structure (1). The anti-sense oligonucleotide used here was 5' CCAGCAGCAGCGGCCTCG 3',

which corresponds to bp34–bp17 of the FPRP sequence. A sense oligonucleotide, 5' CGAGGCCGCTGCTGCTGG 3', which corresponds to bp17–bp34 of FPRP was used as a control. Oligonucleotides were not modified to inhibit their degradation. When used, oligonucleotides were added to both Diff. Media A and B.

### Anti-FPRP immunohistochemistry

Cells were plated on glass coverslips. At the time of harvest, the coverslips were washed twice in phosphate-buffered saline (PBS), fixed in acetone-methanol 70:30 (v/v) at  $-20^{\circ}\text{C}$  for 10 min, then rinsed twice in PBS and processed for anti-FPRP indirect immunofluorescence as described previously (11). The rabbit polyclonal anti-FPRP antibody was used at a concentration of 1:375 (2, 12), and a rhodamine-labeled goat anti-rabbit antibody was used at 1:250 (Boehinger Mannheim Corp., Indianapolis, IN).

### SDS gel electrophoresis and immunoblotting

Cells and tissue were harvested, solubilized, analyzed by polyacrylamide gel electrophoresis (PAGE), electrophoretically transferred, and immunoblotted all as previously described using 40 micrograms (determined using the Bradford reagent, 13) of whole cell lysate, primary antibody at a 1:300 dilution, secondary antibody at a 1:1,000 dilution, and the colorimetric substrate diaminobenzidine (Fig. 3) (2, 12). For quantitative evaluation of the relative content of FPRP by Western blot analysis (Fig. 7a, Table 2), only 8 micrograms of total cell protein was used per sample, the primary antibody was used at a dilution of 1:3000, the secondary at 1:12,000, and the Western blot was developed with an ECL reagent (New England Biolabs, Beverly, MA). The ECL film was scanned on a HP IICx/t flatbed scanner as a 256 grey scale image at  $300 \times 300$  dpi resolution. The scanned image was then densitometrically analyzed using SigmaGel gel analysis software (Jandel Scientific) to determine the pixel intensity of the immunoreactive bands.

### FPRP synthesis studies

Synthesis of FPRP was examined by incorporation of [ $^{35}\text{S}$ ]methionine and -cysteine into newly synthesized protein followed by immunoprecipitation, PAGE analysis of the precipitate, autoradiography and densitometric scanning of the exposed film using a modification of a previous method (14). In particular, treated cultures (60 mm dish) were first washed with PBS, then incubated in methionine and cysteine-free, serum-free medium (Dulbecco's modified Eagle's medium 1 $\times$ , high glucose, without l-methionine and l-cysteine, "deficient media") for 1 h, and labeled with 100  $\mu\text{Ci}$  [ $^{35}\text{S}$ ]methionine and -cysteine (translabel, Amersham Life Science, Arlington Heights, IL) in 1 ml deficient media for 1 h. The labeling media were removed and cultured in F12:DMEM:FBS for 1 h. Cells were rinsed twice with  $4^{\circ}\text{C}$  PBS, then solubilized on ice. The solubilization buffer (0.6 ml) consisted of PBS, 0.95% Triton X-100, 0.2% deoxycholic acid, plus the protease inhibitors phenylmethylsulfonyl fluoride (2 mM), aprotinin (250  $\mu\text{g}/\text{ml}$ ), leupeptin (250  $\mu\text{g}/\text{ml}$ ), antipain (25  $\mu\text{g}/\text{ml}$ ), and chymostatin (25  $\mu\text{g}/\text{ml}$ ). The solubilized culture plus buffer was scraped into an Eppendorf tube, placed on ice for 1 h, centrifuged at  $4^{\circ}\text{C}$  for 15 min, and the soluble fraction was removed to another tube. Polyclonal anti-FPRP antibody (0.4  $\mu\text{l}$ ) (2, 12) was added to the solubilized fraction, and the tube was tumbled overnight at  $4^{\circ}\text{C}$ . Protein A-Sepharose (Sigma, 20  $\mu\text{l}$  of a 1:1 slurry in PBS) was added, the tube was tumbled for 2 h, then centrifuged at the lowest setting in an Eppendorf microfuge for 15 sec. The supernatant was removed, and the pellet was washed 3 times with PBS containing 0.95% Triton X-100 plus the above protease inhibitors, followed by 3 times with PBS only (all at  $4^{\circ}\text{C}$ ). To the pellet was added 30  $\mu\text{l}$  urea sample buffer (0.14 M Tris, 12% sodium dodecyl sulfate, 8 M urea, 10% 2-mer-

captoethanol, 0.001% bromophenol blue, pH 6.8), it was heated to 65°C for 10 min. Thirty  $\mu$ l of regular sample buffer was added (0.14 M Tris, 12% sodium dodecyl sulfate, 10% 2-mercaptoethanol, 0.001% bromophenol blue, 22.3% glycerol, pH 6.8) and the soluble material was run on PAGE as described above. After electrophoresis, the gel was fixed for 1 h in 20% methanol plus 10% acetic acid, then washed in water for 1 h, impregnated with sodium salicylate (soaked in a 1 molar solution for 20 min), then dried on a gel drier at 60°C. The dried gel was exposed to Kodak X-OMAT AR film at -70°C for 3 days and developed.

### Evaluation of G3PDH activity

Glycerol-3-phosphate dehydrogenase (G3PDH) activity was assayed spectrophotometrically (15) as a late marker of adipose differentiation.

### RT-PCR analysis

RNA was isolated from cells using Trizol (Gibco BRL, Gaithersburg, MD), a guanidine-containing solution. The RNA was purified using centrifugation and ethanol precipitation and treated with DNase 1. The following pairs of primers (obtained from Clontech, Palo Alto, CA or Gibco BRL, Gaithersburg, MD) were used: for both rat and mouse FPRP, upstream or 5' primer 5' AAGCCGAGATCCACTC 3' and downstream or 3' primer 5' GTGTAACACTCGGTTG 3'; for mouse  $\beta_2$ microglobulin, upstream primer 5' ATGGCTCGCTCGGTGACCCTAG 3' and downstream primer 5' TCATGATGCTTGATCACATGTCTCG 3'; and for rat  $\beta_2$ microglobulin, upstream primer 5' CTCCTCAAAT TCAAGTGTACTCTCG 3' and downstream primer 5' GAGTG ACGTGTTTAACTCTGCAAGC 3'. RT-PCR was performed as previously published (16). For every RNA sample a no-RT control was run to ensure no contamination was present. All no-RT reactions were negative in these studies indicating that all PCR products were of RNA origin.  $\beta_2$ microglobulin mRNA RT-PCR was performed as a control for RNA loading error and RT activity. Both the FPRP and  $\beta_2$ microglobulin PCR reactions were amplified at the same time (94°C, 30 sec; 60°C, 30 sec; 72°C, 1 min; Perkin-Elmer Corp 9600). To remain in the previously determined exponential range, reactions containing FPRP primers were amplified 40 cycles and those containing  $\beta_2$ microglobulin primers were amplified 25 cycles. These numbers of cycles had previously been determined to be within the respective exponential ranges for mRNA RT-PCR amplification (C. Morin, unpublished observations). PCR products were run on a 4% gel (NuSieve, FMC, Rockland, ME) and the gel was stained with ethidium bromide and photographed. Quantitation of the bands was performed densitometrically using the SigmaGel gel analysis software. The quantity of the FPRP product was then normalized to the  $\beta_2$ microglobulin product.

### Sequence analysis

After RT-PCR synthesis of the FPRP cDNA fragment indicated above, the 3T3-L1 culture FPRP RT-PCR product was subcloned into pCR-Script (Stratagene, La Jolla, CA). The subcloned cDNA was prepared for single-stranded sequencing and sequenced with the Sequenase 2.0 sequencing kit according to the manufacturer's instructions (Amersham Life Science, Arlington Heights, IL).

### Transient transfection

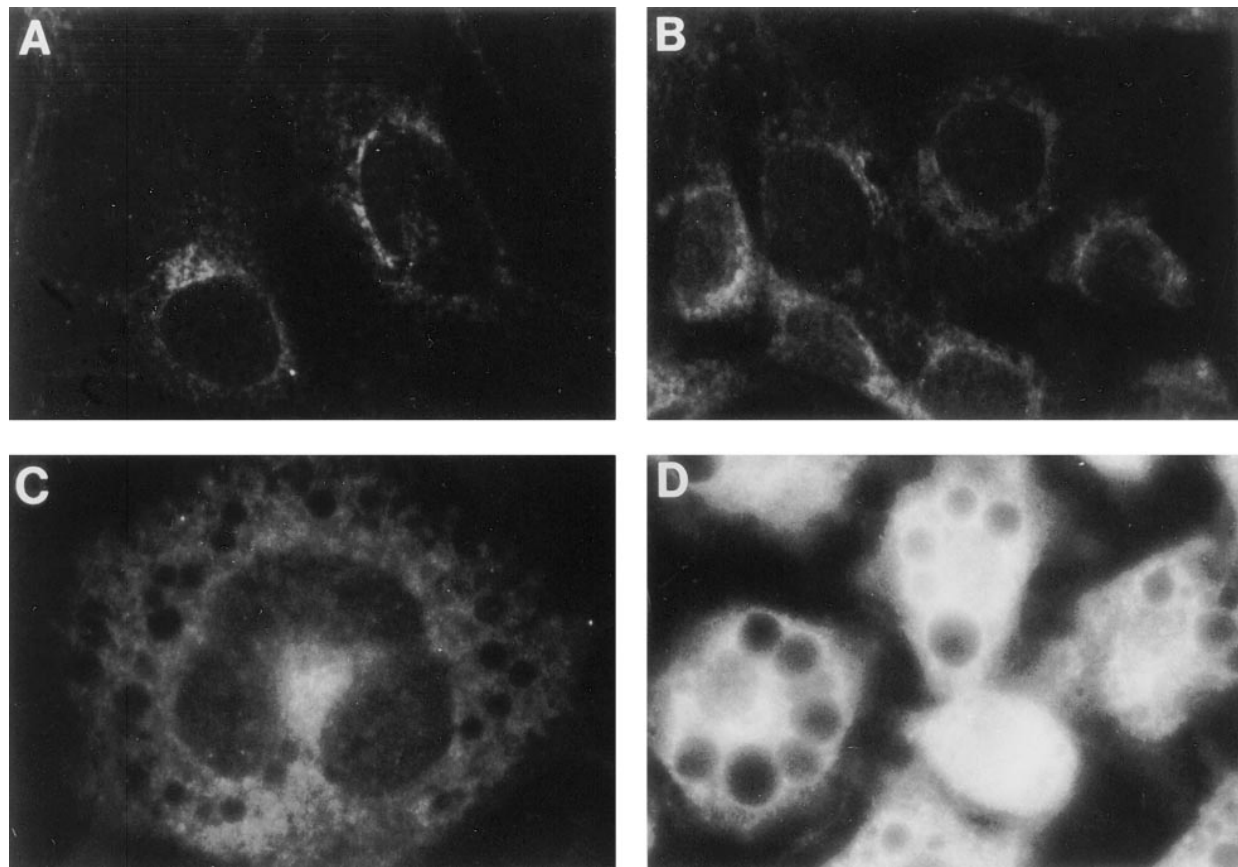
3T3-L1 cells were plated on cover slips as described above for immunohistochemical studies. At 2 days post confluence, cultures were transfected by a calcium phosphate method (17). Treatments included: no DNA, the pSG5 vector alone, or the FPRP cDNA (construct #232, 5) in the expression vector pSG5 (Stratagene, La Jolla, CA). Five micrograms of plasmid was transfected per 60 mm dish. Cells were harvested at 2, 3, 4, and 5 days

post transfection and analyzed using FPRP immunohistochemistry and ORO staining as described above.

## RESULTS

First, we demonstrated the presence of FPRP in adipocytes by immunohistochemistry. FPRP synthesis and accumulation is induced during the differentiation of 3T3-L1 cells. FPRP was not detectable in undifferentiated 3T3-L1 cells, however, by 1–2 days after induction, a small amount was detected (**Fig. 1**). There was some heterogeneity of content of FPRP on a per cell basis; this can be seen in Fig. 1 in which multiple cells are present in each portion of the figure. The amount of detectable FPRP then increased dramatically over the next 3 days. During differentiation, FPRP initially appeared in a pattern that resembled the trans-Golgi network or late endosome compartment, and then as lipid droplet formation proceeded, FPRP appeared to be distributed on membranes throughout the cytoplasm. The accumulation of FPRP was roughly coincident with, or slightly ahead of, the appearance and accumulation of lipid droplets during the adipocyte conversion of these cells (small amounts of FPRP are first seen at 1 and 2 days while lipid droplets are not visible until ~2–3 days). In these experiments, approximately 55–70% of the cells showed lipid accumulation after treatment with insulin, dexamethasone, and IBMX. By immunohistochemistry, there appeared to be concordance between the accumulation of lipid droplets and the expression of FPRP on a cell by cell basis. To test the hypothesis that all lipid-containing fat cells also contained FPRP, a method was devised using anti-FPRP immunofluorescence and Nile Red fluorescence together to visualize both FPRP and lipid droplets in individual cells (**Fig. 2**). Use of this method to examine 3T3-L1 cells 5 days post differentiation induction, followed by quantitation (in one of the two iterations of this experiment) produced the results shown in **Table 1**. In this particular experiment, 75% of the cells were FPRP<sup>+</sup>, 57.1% were NR<sup>+</sup>, and 99.2% of the cells that were NR<sup>+</sup> were also FPRP<sup>+</sup>. It should be noted that all 7 cells that were NR<sup>+</sup>/FPRP<sup>-</sup> had only small lipid droplets. Furthermore, by 5 days after induction of differentiation, expression of FPRP occurred in some cells without significant lipid droplet accumulation; however, greater than 99% of the cells that exhibited lipid droplet accumulation also exhibited FPRP accumulation.

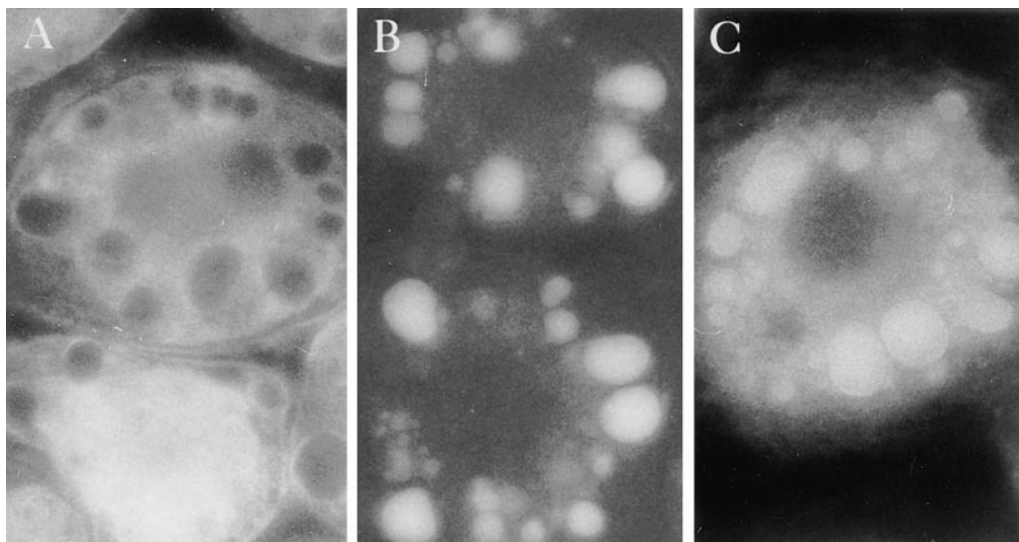
Mature FPRP in tissue has been identified as a 130 kD glycoprotein (2). Consistent with the immunohistochemistry, anti-FPRP Western blotting of 3T3-L1 cell lysates showed that although no 130 kD FPRP protein was detectable in undifferentiated cells, a 130 kD immunoreactive protein was easily detected in cells 5 days after induction of adipocyte conversion (**Fig. 3**, lane D). This protein appears to become an abundant component during differentiation. Comparison of the Western blots to the cell lysate SDS-PAGE Coomassie blue protein-staining pattern allowed identification of a protein band that comigrated with the 130 kD FPRP immunoreactive protein and that



**Fig. 1.** FPRP accumulation after 3T3-L1 cell differentiation induction. Immunohistochemical detection of FPRP is shown after: A) 1 day, B) 2 days, C) 3 days, or D) 5 days of induction. At time zero, 2-day post-confluent cultures of 3T3-L1 cells were induced to differentiate. At the indicated times, cells were fixed and immunohistochemically stained for FPRP. This experiment was performed twice, each in triplicate. Results from one experiment are shown.

was not detectable in uninduced cell lysates but became a prominent protein during adipocyte conversion (Fig. 3, lane B). Due to the very abundant anti-FPRP immunohis-

tochemical staining previously observed, the presence of a 130 kD band on the Coomassie-stained gel was not unexpected. Rat epididymal fat pad was also analyzed (Fig. 3,



**Fig. 2.** Correlation of FPRP accumulation and lipid droplet accumulation. Immunohistochemical detection of FPRP (A), Nile red detection of lipid droplets (B), or staining for both FPRP and lipid droplets (C) is shown from a culture 5 days post differentiation induction. This experiment was performed twice, each in quadruplicate. Results from one experiment are shown.

TABLE 1. Accumulation of lipid droplets and FPRP in 3T3-L1 cells determined by simultaneous Nile Red and anti-FPRP immunofluorescence microscopy

Nile Red/FPRP Fluorescence <sup>a</sup>	Number of Cells <sup>b</sup>	% of Total
NR <sup>-</sup> FPRP <sup>-</sup>	381	24.6
NR <sup>-</sup> FPRP <sup>+</sup>	284	18.3
NR <sup>+</sup> FPRP <sup>-</sup>	7 <sup>c</sup>	0.4
NR <sup>+</sup> FPRP <sup>+</sup>	877	56.7

<sup>a</sup>The presence (+ or absence (-) of 3 micron lipid droplets (NR) and FPRP was determined in individual 3T3-L1 cells 5 days post differentiation induction by simultaneous Nile Red fluorescence and anti-FPRP immunofluorescence microscopy as shown in Fig. 2.

<sup>b</sup>Total of 1549 cells were examined in random fields.

<sup>c</sup>All 7 cells in this grouping had only very small lipid droplets.

lane E) and the 130 kD immunoreactive protein was clearly present in this tissue as well, indicating that FPRP expression is not simply a property of cultured preadipocyte cells. The results using epididymal fat pad were confirmed on Bouin's fixed tissue by anti-FPRP immunohistochemistry (data not shown).

After establishing the presence of FPRP in adipocytes, we examined the levels of FPRP mRNA during 3T3-L1 cell differentiation. As shown in Fig. 4, after use of RT-PCR, FPRP mRNA was detected in subconfluent, uninduced cells. By this method, there was approximately a 3-fold increase of mRNA in cells 5 days after the induction of differentiation. FPRP mRNA was also easily detected in rat epididymal fat pad. To ensure that the RT-PCR product obtained from the differentiated 3T3-L1 cells was FPRP cDNA, it was subcloned and sequenced. The nucleic acid sequence of the amplified 3T3-L1 fragment displayed 98% identity in the coding region (4/199 base pairs were non-identical, two of these are silent, two are not) and 79% identity in the non-coding region to rat FPRP. The amino acid identity is 64/66 = 97% (one of the non-identities is a conservative substitution, L to V; one is a non-

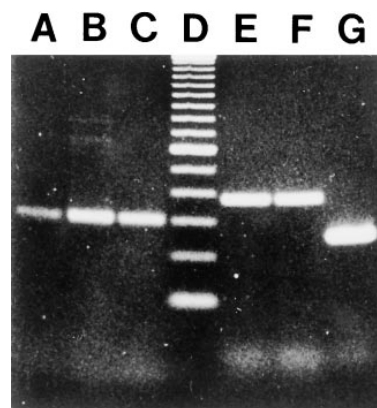


Fig. 4. FPRP mRNA RT-PCR analysis. Non-confluent, uninduced 3T3-L1 cells (lanes A, E), 3T3-L1 cells after 5 days of differentiation induction (lanes B, F), and Wistar rat epididymal fat pad (lanes C, G) were analyzed for FPRP mRNA (lanes A-C) or  $\beta_2$ microglobulin mRNA (lanes E-F) by RT-PCR. The expected size of the rat FPRP cDNA fragment is 304 bp, mouse  $\beta_2$ microglobulin cDNA fragment is 373 bp, and the rat  $\beta_2$ microglobulin cDNA fragment is 249 bp. Lane D contains size marker DNA (100 bp ladder, Gibco BRL, Gaithersburg, MD). This experiment was performed three times, each in duplicate. Results from one experiment are shown.

conservative substitution, G to E). The 3T3-L1 product (accession #AF006201) corresponds with nucleotides 2426-2729 of the rat FPRP sequence (accession #26595). This particular region was chosen for RT-PCR analysis because it contains the active domain of FPRP and was expected to be highly conserved (2). These results indicate that although FPRP expression is not detectable by immunohistochemistry or Western blotting in uninduced 3T3-L1 cells, there is a small but detectable level of FPRP mRNA present. Moreover, while the level of FPRP mRNA did increase in cells during differentiation, the apparent fold-induction was less than the fold-induction in content of FPRP.

To directly examine the induction of FPRP synthesis during differentiation, the incorporation of radiolabeled amino acids into FPRP was analyzed by immunoprecipitation and PAGE/autoradiography. Synthesis of the 130 kD FPRP protein was not detectable in uninduced cells (not shown) and barely detectable in the cells 1 day after induction of differentiation (Fig. 5). However, over the next 3 days there was a significant increase in the FPRP synthetic rate of the cultures as demonstrated by an increased amount of radiolabeled FPRP that was immunoprecipitated. Quantitation of the apparent incorporation of radiolabel into the 130 kD protein band indicated that the rate of FPRP synthesis increased approximately 50-fold during the first 4 days of differentiation (Fig. 5). These results implied that the induction of FPRP accumulation observed in differentiating 3T3-L1 cells by immunohistochemistry and Western blotting was the result of newly synthesized FPRP.

As shown above, FPRP accumulation could be induced in 3T3-L1 cells by treatment with insulin, dexamethasone, and IBMX, and was also detected in adipose tissue, sug-

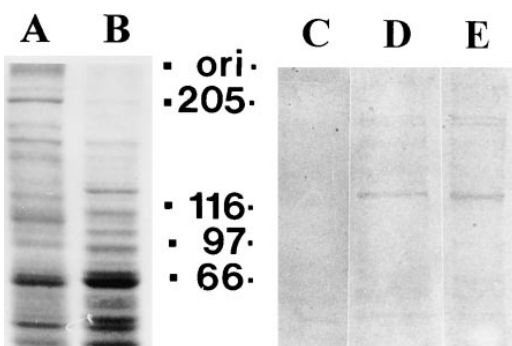
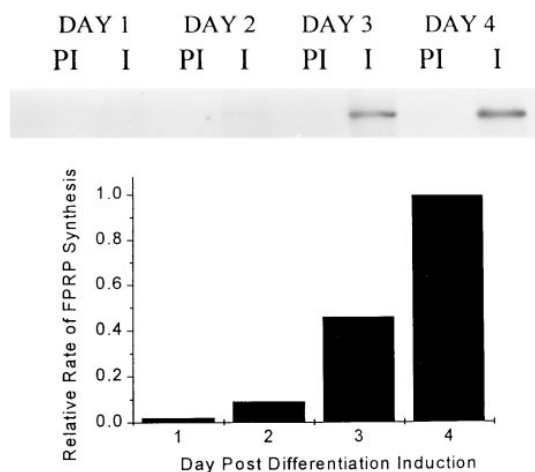


Fig. 3. Western analysis of 3T3-L1 Cell FPRP. Uninduced (A, C) or differentiated (B, D) 3T3-L1 cell cultures, and rat epididymal fat pad (E) were analyzed for immunoreactive FPRP after 8% PAGE and electrophoretic transfer (lanes C, D, E). Lanes A and B show Coomassie-stained proteins from a gel run in parallel to the transfer gel. Molecular weight markers are indicated, ori indicates origin of the gel, df indicates the dye front of the gel. The arrow head marks the expected size of FPRP. This experiment was performed three times, each in duplicate. Results from one experiment are shown.

gesting that expression of this protein is associated with the differentiated adipose cell phenotype. To further analyze whether expression of this protein was a response to one of the inducing agents and could occur independently of adipose differentiation in these 3T3-L1 cells, the effect of each of the differentiation-inducing components (insulin, IBMX, and glucocorticoid) on the induction of FPRP accumulation and adipocyte conversion was determined. Neither insulin nor IBMX, when administered separately or in combination, could induce expression of immunohistochemically detectable FPRP (Fig. 6) or significant adipocyte conversion (not shown) in confluent cultures of 3T3-L1 cells. However, treatment of cells with glucocorticoid either alone or in the presence of the other components was effective in inducing FPRP accumulation (Fig. 6) as well as adipocyte conversion. Approximately 30–40% of the cells underwent differentiation in response to glucocorticoid alone whereas approximately 70% of the cells differentiated when treated with insulin, IBMX, and glucocorticoid. Again, in these experiments it appeared that virtually all cells possessing lipid droplets also accumulated FPRP as noted above. Treatment of 3T3-L1 cells with the thiazolidinedione derivative ADD4743, which induces adipocyte differentiation by a glucocorticoid independent mechanism, also induced FPRP accumulation in those cells that accumulated lipid (Fig. 6). These results indicated first, that the induction of FPRP by glucocorticoid may not be direct and second, that lipid accumulation was not separable from the induction of FPRP.

To examine whether FPRP expression was required for some aspect of 3T3-L1 cell differentiation or lipid accu-

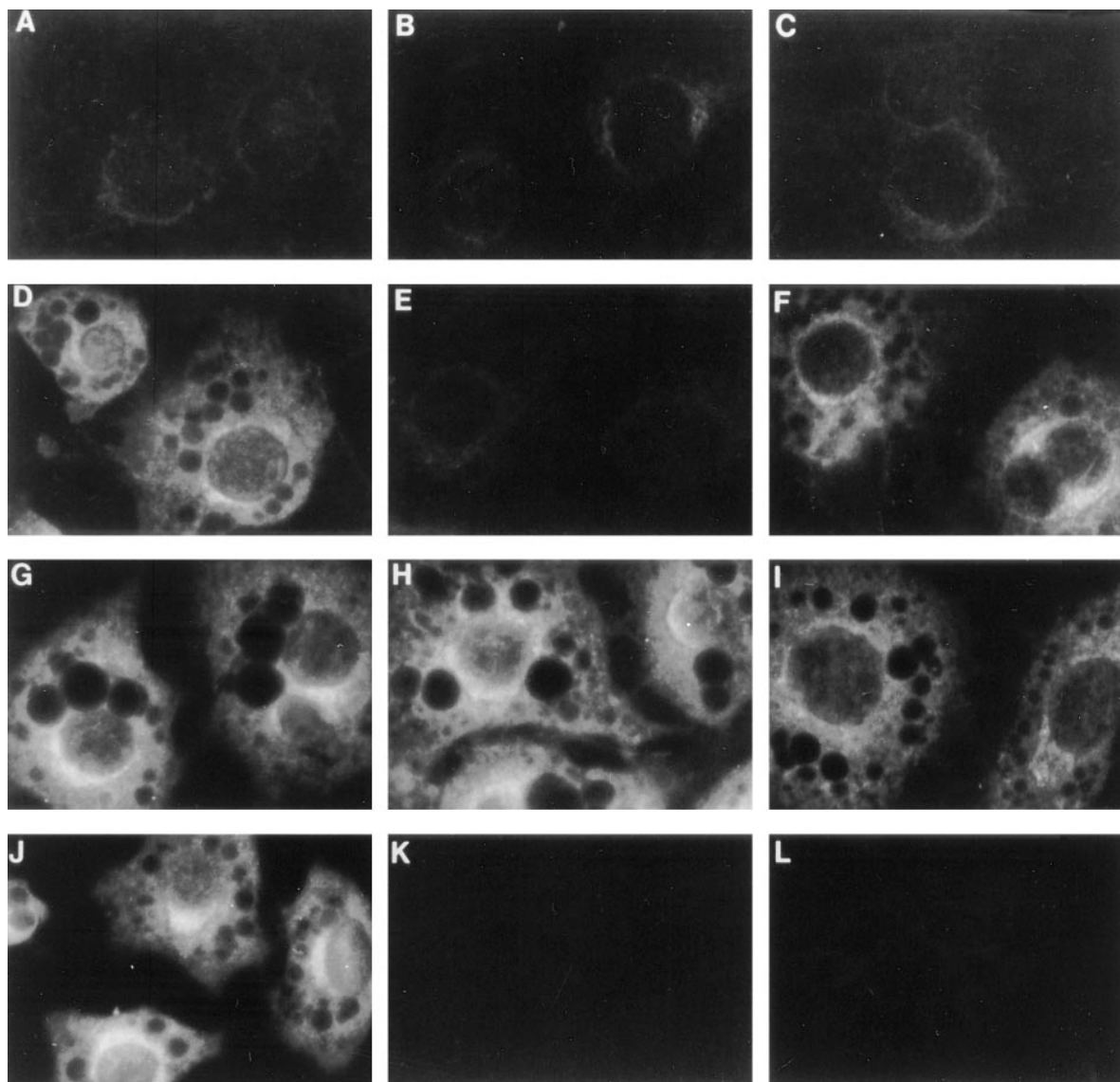


**Fig. 5.** FPRP synthesis after induction of 3T3-L1 cell differentiation. The relative FPRP synthetic rate was quantitated by [ $^{35}$ S]met and -cys metabolic labeling of 3T3-L1 cultures at the indicated day post induction of differentiation and then by immunoprecipitation, PAGE, and autoradiography. (Top) The portion of the autoradiogram shown corresponds to the area around and including the 130 kD band. PI indicates preimmune serum; I indicates anti-FPRP immune serum. (Bottom) Data shown in the top portion of Fig. 5 were densitometrically scanned and quantitated. Graphed is the relative rate of FPRP synthesis (band density) of the 130 kD band versus the day of induction. This experiment was performed twice, each in duplicate. Results from one experiment are shown.

mulum, experiments were carried out to determine the effect of inhibition of FPRP synthesis using anti-sense oligonucleotides. The anti-sense oligonucleotide used in these studies was an 18 mer directed against a region of the mRNA near the AUG start codon which is hypothesized to be looped out (single-stranded in a secondary structure model of this mRNA) and not involved in the self-annealing structure prevalent in the FPRP mRNA 5' end (1). For these experiments, differentiation was induced in cells, and simultaneously the cells were treated with anti-sense oligonucleotide, sense oligonucleotide, or without any addition. After 4 days the effect of the anti-sense oligonucleotide on FPRP expression was examined. Anti-FPRP immunohistochemistry suggested that there was a small but observable decrease in the FPRP content in cells exposed to the anti-sense oligonucleotide during differentiation (data not shown). Quantitation of the FPRP content after anti-FPRP Western blotting (Fig. 7, bottom left) indicated that, in three micromolar anti-sense oligonucleotide-treated cultures, the level of FPRP was reduced to  $58 \pm 5\%$  that of the untreated differentiated cultures (arbitrarily designated 100%, Table 2). FPRP content in sense oligonucleotide-treated cultures was not significantly different from untreated differentiated cultures.

The effect of the anti-sense oligonucleotide on the level of adipocyte conversion was next accessed by Oil Red O (ORO) staining. As shown in Fig. 7b, a smaller percentage of cells accumulated ORO-stained lipid droplets in the anti-sense oligonucleotide-treated cultures than in the untreated cultures or cultures treated with a sense oligonucleotide. While increasing concentrations of the sense oligonucleotide between 1–25  $\mu$ M had no measurable effect on the percentage of cells that accumulated ORO<sup>+</sup> lipid droplets, treatment of cells with increasing concentrations of the anti-sense oligonucleotide resulted in a decrease in the percentage of cells that contained visible ORO<sup>+</sup> lipid droplets (Fig. 7, bottom left). In addition to this reduction in the percentage of cells that contained ORO-positive vesicles, many of the cells in the anti-sense oligonucleotide-treated cultures contained visibly fewer and smaller vesicles; however, this response varied somewhat from cell to cell. One concentration of anti-sense oligonucleotide was studied in more detail: 3  $\mu$ M anti-sense oligonucleotide caused both a 33% decrease in the percentage of cells accumulating lipid droplets and a 42% decrease in FPRP protein accumulation. Treatment with the anti-sense oligonucleotide did not appear to interfere with the formation of bi-nucleate cells, a phenomenon associated with the differentiation of 3T3-L1 cells (9, Fig. 7, top).

Because the effect of anti-sense oligonucleotides on the accumulation of both lipid droplets and FPRP could have been due to a general inhibition of adipose differentiation, the effect of the anti-sense oligonucleotides was examined on the expression of G3PDH activity, a late marker of adipocyte conversion. No significant difference in the induction of G3PDH activity between non-treated and either sense or anti-sense oligonucleotide-treated cultures was observed (Table 2). In all cases, G3PDH activity was increased approximately 30-fold, 4 days after induction of



**Fig. 6.** FPRP accumulation after various treatments of undifferentiated 3T3-L1 Cells. Two-day post-confluent cultures of 3T3-L1 cells were cultured with the indicated treatments for 5 days, then fixed and stained for anti-FPRP immunohistochemistry. These treatments included: A) no hormone; B) isobutylmethylxanthine (IBMX); C) insulin (Ins); D) dexamethasone (Dex); E) IBMX + Ins; F) IBMX + Dex; G) Ins + Dex; H) and I) IBMX + Ins + Dex; J) ADD4743 + Ins. After treatment of A–H for 48 h, cultures were switched to control medium. After treatment of I for 48 h, the culture was switched to Ins. Culture J was cultured continuously in ADD4743 + Ins (medium changed every 48 h). Photomicrographs K and L show staining with preimmune sera after: K) no hormone; and L) IBMX + Ins + Dex. This experiment was performed twice, each in duplicate. Results from one experiment are shown.

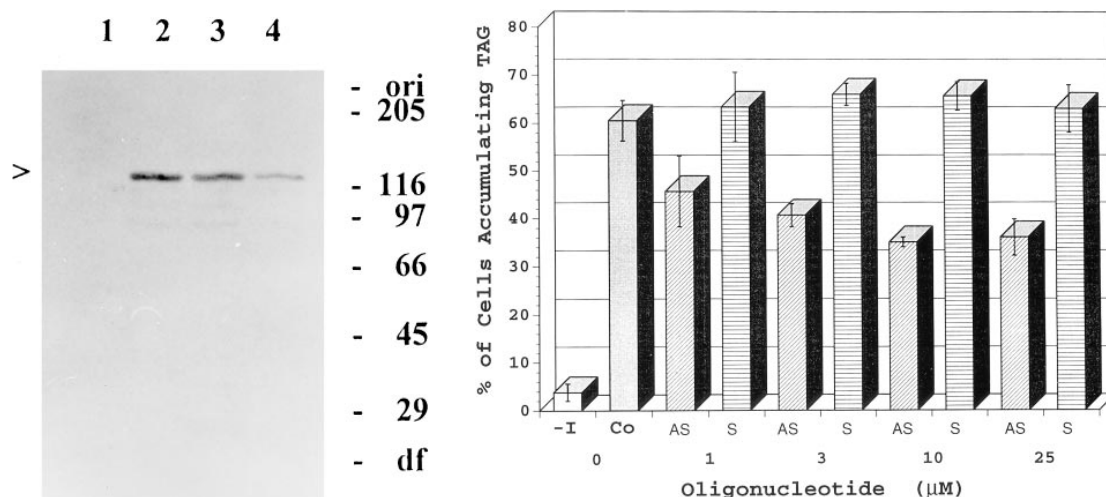
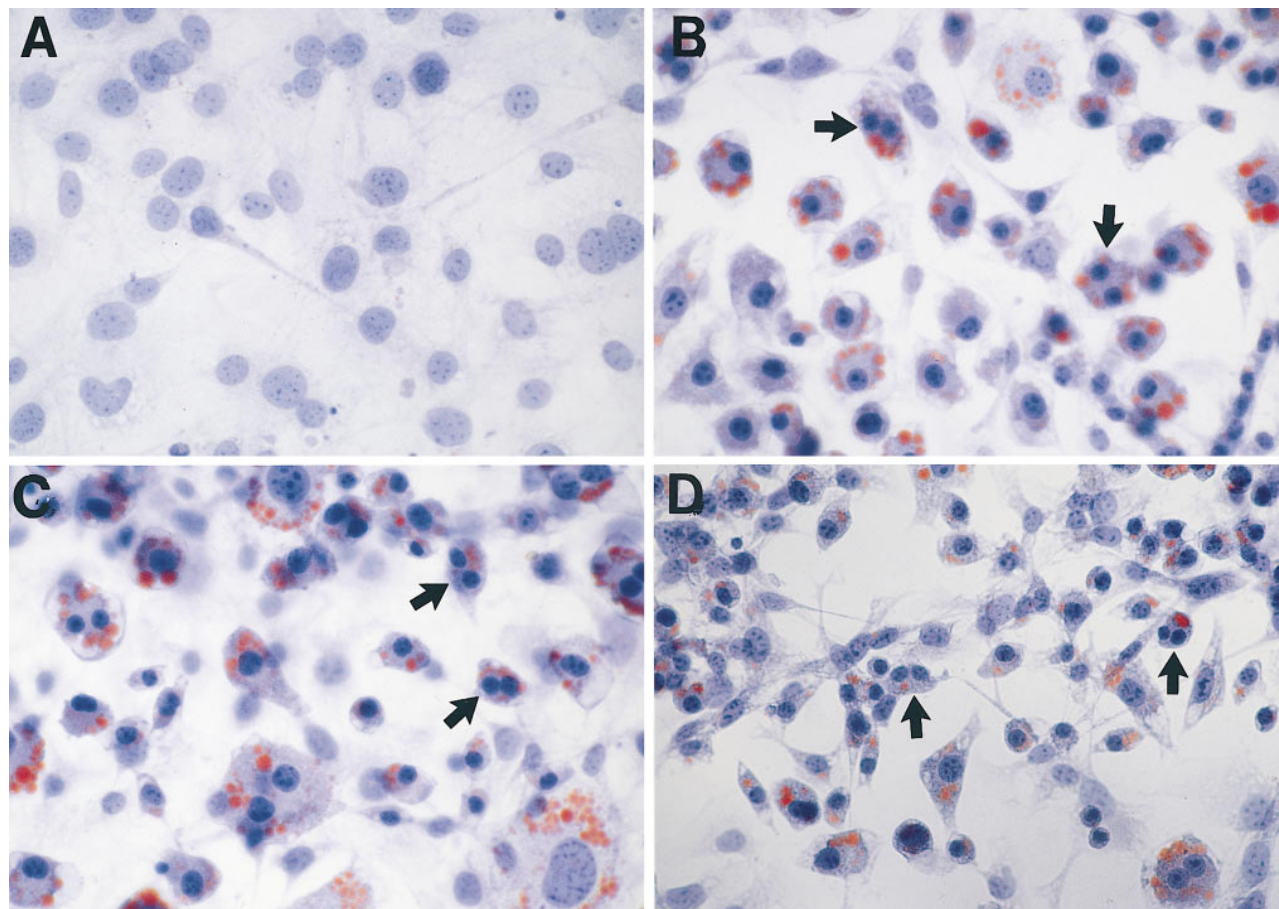
adipocyte conversion. The level of G3PDH activity observed in these experiments was consistent with previous reports (6), and indicated that anti-sense oligonucleotide treatment did not affect the expression of this late marker of differentiation.

Last, the effect of FPRP expression on uninduced 3T3-L1 cells was examined. Confluent 3T3-L1 cells were transiently transfected with an expression plasmid encoding the full length FPRP open reading frame. Anti-FPRP immunohistochemistry detected FPRP transgene expression in these cells; however, its intracellular localization was distinctly different from that seen in differentiating preadipocytes and mature adipocytes, and no increase in lipid

droplet accumulation was detected by ORO staining (data not shown). This result indicates that FPRP expression by itself is not sufficient to cause accumulation of ORO-positive lipid droplets.

## DISCUSSION

Lipid accumulation by developing adipocytes is a complex process that involves many changes in gene expression. These changes result in storage of energy rather than its immediate use. Preadipocyte cell lines such as 3T3-L1 have been an important tool in characterizing this



**Fig. 7.** Bottom, left: FPRP anti-sense oligonucleotide-inhibition of FPRP accumulation. Two-day post confluent 3T3-L1 cultures were treated with Diff. Media A and then B containing either no oligonucleotide, anti-sense, or sense oligonucleotide. At 4 days post differentiation induction, cultures were harvested and their relative content of FPRP was determined by Western analysis using the ECL method. Shown is a sample blot from this analysis. Lane 1) non-differentiated, 2) differentiated, no oligonucleotides, 3) differentiated, sense oligonucleotide (3 micromolar), and 4) differentiated, anti-sense oligonucleotide (3 micromolar). Molecular weight standards are indicated to the right of the blot. To the left of the blot is an arrow indicating where FPRP is expected to migrate. This experiment was performed three times, each in duplicate. Results from one experiment are shown. Quantitation of results from these experiments is shown in Table 2. Top: Oil-Red-O staining of sense and anti-sense oligonucleotide-treated 3T3-L1 cell cultures. Parallel cultures to those described in Fig. 7, bottom left, were formalin-fixed, stained with Oil-Red-O for neutral lipids, and counterstained with hematoxylin. (A) non-differentiated, (B) differentiated, no oligonucleotides, (C) differentiated, sense oligonucleotide (3 micromolar), and (D) differentiated, anti-sense oligonucleotide (3 micromolar). Bottom, right: Quantitation of the FPRP anti-sense oligonucleotide inhibition of lipid droplet accumulation. Figure 7, bottom right, is a quantitation of the experiment presented in Fig. 7, top. The percentage of cells accumulating lipid droplets larger than 3 microns when cultured in the presence of anti-sense (AS) and sense (S) oligonucleotides, as well as for uninduced cells (-I) and induced in the absence of any oligonucleotide (Co) are shown.



TABLE 2. FPRP content and G3PDH activity in oligonucleotide-treated 3T3-L1 cell cultures

Treatment	FPRP	G3PDH activity
Non-differentiated, no oligonucleotides	N.D.	9 ± 3
Differentiated, no oligonucleotides	100	288 ± 9
Differentiated, sense oligonucleotide	121 ± 14	311 ± 18
Differentiated, anti-sense oligonucleotide	58 ± 5	319 ± 10

Quantitated levels of FPRP after the Western analysis shown in Fig. 7a are compared with levels of G3PDH activity from parallel cultures. FPRP content is indicated relative to the differentiated, no oligonucleotide-treated group (arbitrarily designated 100%). N.D., not detected. The quantity of FPRP in the anti-sense oligonucleotide (3 micromolar)-treated group was significantly different from the sense oligonucleotide (3 micromolar)-treated group (by *t*-test at the 0.01 level). G3PDH activity is expressed as nanomoles of reduced NADH formed per milligram of culture lysate protein per minute. All three differentiated groups were significantly different from the non-differentiated group; however, none are significantly different from each other (paired *t*-test at the 0.05 level). Means and standard error of the mean are indicated, *n* = 3.

aspect of adipogenesis (6, 7). Our studies show that a prominent feature of this process is the induction of synthesis and accumulation of FPRP. FPRP was undetectable in undifferentiated 3T3-L1 cells, and both the level of mRNA and protein were found to increase during differentiation. It is of note that both FPRP mRNA and the 130 kD FPRP were also found to be present in adipose tissue. The presence of FPRP is highly tissue specific, it is also found in other cell types where lipid metabolism plays a prominent role including cardiomyocytes and certain reproductive cells.

A rat FPRP cDNA and part of a human FPRP gene have been cloned; however, the nature of the promoter and other regulatory sequences has not yet been characterized (1, 3). Therefore, the factors that may regulate FPRP transcription during adipogenesis are not known. Based on the induction of FPRP during adipocyte conversion, it will now be useful to determine whether glucocorticoids and thiazolidinediones act directly to regulate the expression of this gene in adipose and other tissues. Certainly other adipocyte-related genes appear to be under the control of these two classes of inducing agents. Included in this group are the genes for 422/aP2, the insulin-sensitive glucose transporter (GLUT4), and stearoyl-CoA desaturase 1 (SCD1, for a current review, see ref. 7).

Although the FPRP protein was not detectable in undifferentiated 3T3-L1 cells by immunohistochemistry, metabolic labeling and immunoprecipitation, or Western blotting, FPRP mRNA was detected in these cells by RT-PCR. This apparent difference between mRNA and protein expression could have been due to the greater sensitivity of RT-PCR in detecting FPRP mRNA than immunodetection techniques for FPRP detection. However, the subsequent level of induction of FPRP synthesis was considerably greater than the apparent level of induction of FPRP mRNA, suggesting that FPRP expression may also involve post-transcriptional regulation. The importance of post-transcriptional regulation of gene expression in differentiation is not clear. While numerous studies have focused on the role of transcriptional regulation of gene expres-

sion, there is some evidence that post-transcriptional regulation may also be important in this process. A protein able to stabilize mRNA and accelerate formation of translation initiation complexes has been reported to accelerate 3T3-L1 cell differentiation and lipid accumulation (18). Additional studies are clearly required, but the induction of FPRP during adipocyte conversion may represent an example of this type of regulation.

Our results suggest that FPRP expression was involved in some aspect of lipid droplet accumulation. There was a correspondence between the accumulation of FPRP and the appearance of lipid droplets on a cell by cell basis. Moreover, treatment of cells with anti-sense oligonucleotides reduced the accumulation of FPRP during differentiation and resulted in a measurable reduction in lipid droplet accumulation. This inhibition of FPRP expression appeared to specifically affect lipid droplet accumulation as the anti-sense oligonucleotide treatment neither affected the induction of G3PDH, a late marker of differentiation, nor did it affect the morphological changes in the cells that are characteristic of adipocyte conversion such as the appearance of bi-nucleate cells. Previously, others have shown that in the 3T3-L1 cell line lipid accumulation can be decreased by inhibition or antagonism of certain transcription factors (19, 20), nutritional (vitamin) deficiency from the culture media (15), overexpression of a lipolytic enzyme (21), overexpression of a key signal transduction enzyme (22), treatment with known inhibitory cytokines (23–26), disruption of the vimentin intermediate filament system (9), treatment with PGF<sub>2α</sub> (27), or inhibition of poly(ADP-ribose) polymerase (28). However, only the vitamin (biotin) deficiency and disruption of the vimentin filament organization were reported to decrease lipid accumulation without also inhibiting the induction of late markers of adipocyte conversion.

FPRP was originally identified as a transmembrane glycoprotein that inhibited the capacity of the prostaglandin F<sub>2α</sub> receptor to bind ligand. Subsequent studies have shown that expression of FPRP in COS cells can regulate the binding of ligand to other seven-transmembrane receptors, including the β1 beta adrenergic receptor. Both the prostaglandin F<sub>2α</sub> and β1 beta adrenergic receptors have been shown to influence lipolysis (8, 27, 29). The coincident expression of FPRP in cells that accumulate lipid droplets raises the possibility that its role in this process may involve effects on hormone receptors that regulate lipid accumulation. Alternatively, the true role of FPRP in adipocytes may yet be discovered.

It is estimated that a third of all Americans are clinically obese (30). Furthermore, obesity is a major cause of cardiovascular pathologies as well as hypertension and diabetes. Presently, a large percentage of the research directed at biochemical control of obesity attempts to do so at the level of satiety control. Interference with the activity of a protein like FPRP may provide a novel mechanism for control of obesity at the level of the adipocyte.

In summary, we have shown that the accumulation of FPRP is associated with the accumulation of lipid in 3T3-L1 adipocytes. Decreasing the accumulation of this pro-

tein decreases the accumulation of lipid droplets in these cells. The presence of FPRP in adipocytes and cardiomyocytes as well as osteoclasts, Leydig cells and granulosa lutein cells may indicate that it provides a function common to cells involved in lipid metabolism in the adipose, cardiovascular and reproductive systems. ■■

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