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Lipoprotein lipase (LPL) is an important enzyme in adipocyte and lipid metabolism with complex cellular regulation. Previous studies demonstrated an inhibition of LPL activity and synthesis following depletion of protein kinase C (PKC) isoforms with long term treatment of 3T3-F442A adipocytes with 12-*O*-tetradecanoylphorbol-13-acetate. To identify the specific PKC isoforms involved, we treated cells with antisense oligonucleotides that block expression of specific PKC isoforms. An antisense oligonucleotide to PKCα inhibited LPL activity by 78 ± 8%, whereas antisense oligonucleotides directed against PKCδ or PKCϵ had no effect on LPL activity. The change in LPL activity was maximal at 72 h and was accompanied by a decrease in LPL protein and LPL synthetic rate but no change in LPL mRNA, suggesting regulation at the level of translation. However, PKC depletion resulted in no change in the polysome profile, indicating that translation initiation was not affected. However, the addition of cytoplasmic extracts from adipocytes treated with 12-*O*-tetradecanoylphorbol-13-acetate or PKCα antisense oligomers inhibited LPL translation *in vitro*. This inhibition of LPL translation *in vitro* was lost when the LPL mRNA transcript did not contain nucleotides 1599–3200, thus implicating the 3′-untranslated region of LPL in the regulation of translation by PKC depletion. Both LPL activity and Raf1 activity were decreased in parallel following depletion of either total PKC or specific inhibition of PKCα. An antisense oligonucleotide to RAF1, which inhibited RAF1 activity, also inhibited LPL activity by 48 ± 10%, and this decrease in LPL activity was not accompanied by a change in LPL mRNA. Cells were treated with U0126, a specific inhibitor of the ERK-activating kinases MEK1 and MEK2. Although U0126 inhibited ERK1 and ERK2 phosphorylation, U0126 had no effect on LPL activity, indicating that MEK/ERK pathways were not involved in this mechanism of LPL regulation. Together, these data indicate that PKCα and RAF1 are important in the translational regulation of LPL in adipocytes and that the mechanism of regulation is probably through an ERK-independent pathway.

Lipoprotein lipase (LPL)[1] is a central enzyme in lipid metabolism and is synthesized mainly in adipose tissue and muscle. This enzyme catalyzes the hydrolysis of triglyceride-rich lipoproteins into free fatty acids and glycerol (1, 2). The regulation of LPL is complex and may occur at the level of transcription, translation, or post-translational processing. We have previously described translational regulation of LPL in adipocytes in response to thyroid hormone and catacholamines (3, 4). This translational regulation is likely due to the presence of an RNA-binding protein that interacts with the 3′-UTR of the LPL mRNA (5). In addition, the induction of insulin-deficient diabetes in rats results in a translational down-regulation of LPL, also mediated by interactions of transacting factors with the 3′-UTR (6).

Among the possible mechanisms for diabetes-related changes are alterations in cellular protein kinase C (PKC) (7). High glucose levels stimulate the formation of cellular diacylglycerol (DAG), a natural activator of PKC (8–10). PKC activity can be attributed to at least 12 enzyme isotypes that have been grouped into three classes by their structures and cofactor requirements: (i) conventional PKCs (PKCα, PKCβI, PKCβII, and PKCγ) are DAG and calcium-dependent, (ii) novel PKCs (PKCδ, PKCϵ, PKCη, PKCθ, and PKCμ) are DAG-dependent but calcium-independent, and (iii) atypical PKCs (PKCζ, PKCι, and PKCλ) are not responsive to phorbol esters but can bind DAG. We have previously examined the regulation of LPL in adipocytes through modulation of PKC. Depletion of cellular PKC activity, either through prolonged treatment of cells with phorbol ester (11, 12) or with drugs that inhibit PKC, resulted in an inhibition of LPL activity through a decrease in translation (13). Thus, PKC is important in the regulation of LPL expression, although the precise mechanism for this effect is not known.

Antisense oligonucleotides inhibit the expression of the targeted proteins by binding to the sense mRNA or pre-mRNA, thus inhibiting the production of the protein product (14). Antisense oligonucleotides directed against PKC isoforms and RAF1 have been used to inhibit the expression of the targeted mRNA inside the cell (15–18). To identify the specific PKC isoforms involved in LPL regulation and to investigate the PKC-mediated pathways regulating LPL expression, we treated 3T3-F442A adipocytes with antisense oligonucleotides to PKC α, δ, or ϵ. Antisense oligonucleotides to PKCα inhibited both LPL activity and RAF1 activity. In addition, antisense

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[1] The abbreviations used are: LPL, lipoprotein lipase; UTR, untranslated region; PKC, protein kinase C; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; UTR, untranslated region.

oligonucleotides to RAF1 kinase inhibited LPL expression post-transcriptionally. These studies demonstrate the specific involvement of PKCα and Raf1 kinase in LPL translational regulation in adipocytes.

MATERIALS AND METHODS

Cell Culture and Differentiation—3T3-F442A cells were obtained from Dr. Howard Green (Harvard Medical School, Boston, MA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented to 10% with calf serum and containing penicillin 10 units/ml and streptomycin 10 μg/ml (Invitrogen). For the experiments, the cells were grown to confluence and stimulated to differentiate in DMEM containing 10% fetal calf serum and 100 nM insulin for 5–7 days.

U0126 (19) was purchased from Calbiochem (San Diego, CA). Stock solutions were made in Me_2SO. The 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was purchased from Sigma, and stock solutions were made in Me_2SO. The control cells received the same amount of Me_2SO (0.1% v/v).

Oligonucleotide Treatments—The oligonucleotides were synthesized as phosphorothioate-containing 2-methoxyethyl modifications at positions 1–5 and 15–20 (Isis Pharmaceuticals, Carlsbad, CA). The sequences were as follows: Isis 17251 antisense PKCα, CAGCCATGGTTCCCCCAAC; Isis 17250 scrambled PKCα, CCAGTCACTCGCACCATCGC; Isis 17256 antisense PKCδ, TCCAGGTCAACGCGGCATTC; Isis 17257 scrambled PKCδ, TCGGCCCTGCGGATCTCA; Isis 17260 antisense PKCϵ, GTCCATGCGATCTTGCGCCC; Isis 17261 scrambled PKCϵ, GCCAGCTCCGATCTTGCGCCC; Isis 15770 antisense Raf1, ATGCATTCTGCCCCCAAGGA; and Isis 103169 scrambled RAF1, AAGGATCCTCCCTCCATGGA. All of the oligonucleotides are synthesized using an Applied Biosystems 380B automated DNA synthesizer and purified as described previously (16). The cells were transfected 4–5 days after initiation of differentiation using serum-free DMEM containing 15 μg/ml Lipofectin (Invitrogen) and 1 μM PKC antisense or scrambled oligonucleotide for 6 h (17, 20) or 2.5 μM Raf1 antisense or scrambled oligomer for 6 h (18). The cultures were washed with serum-free DMEM three times to remove Lipofectin and incubated in differentiation medium for 72 h unless otherwise specified. The control cultures were treated with 15 μg/ml of Lipofectin in serum-free DMEM for 6 h.

Measurement of LPL Activity—Heparin-releasable and extractable LPL activities were determined as described previously (21). To measure heparin-releasable LPL, the cells were incubated in 1 ml of DMEM containing 10 units/ml heparin for 45 min at 37 °C. After collecting the heparin-released fraction, the remaining LPL was extracted in 50 mM Tris, pH 7.4, containing 0.2% Triton X-114. The extracts were diluted with three volumes of 50 mM Tris, pH 7.4, containing 10 units/ml heparin. LPL catalytic activity was measured as described previously using a substrate containing [^{3}H]triolein and fetal bovine serum as a source of apoC-II (22). LPL activity was expressed as nmol of free fatty acid released/h/mg of protein.

RNA Extraction and Northern Blotting—Northern blotting was performed as described previously (23). RNA was extracted from adipocytes (24), and equal amounts of total RNA (10 μg) from the various treatment groups were analyzed using 2.2 M formaldehyde, 1% agarose gels. Northern blots were probed using ^{32}P-labeled human LPL cDNA (25) and GAPDH cDNA probes followed by autoradiography.

LPL Synthetic Rate—The synthetic rate of LPL was measured in adipocytes using a 40-min pulse with [^{35}S]methionine (100 μCi/ml), as described previously (26). The unincorporated label was aspirated, and the total cellular proteins were extracted in cell lysis buffer containing 50 mM phosphate buffer, pH 7.4, 2% deoxycholate, 1% SDS, 20 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, and 2 mM EDTA. The extracts were immunoprecipitated using specific polyclonal antibodies as described previously (27). The immunoprecipitated samples were analyzed on 10% SDS-PAGE, followed by autoradiography.

Western Blot Analysis—The analysis of proteins LPL, PKCα, or ERK was performed essentially as described earlier (13). The cell monolayer was rinsed in ice-cold phosphate-buffered saline, and the total protein was extracted using the cell lysis buffer described above. The proteins (15 μg) were fractionated by 10% SDS-PAGE and transferred onto nitrocellulose membranes using 200 mA current for 2–3 h. The membranes were treated with 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.2% Tween 20, and 5% nonfat dry milk overnight at 4 °C. To identify the LPL protein, anti LPL polyclonal antibody (a generous gift from Dr. Ira Goldberg, Columbia University, New York, NY) was applied at 1:2000, followed by anti-rabbit horseradish peroxidase conjugate at 1:5000 (Sigma). The reaction product was visualized with chemiluminiscence reagents (Amersham Biosciences). To detect PKC, isoform-specific primary antibodies and horseradish peroxidase-conjugated secondary antibodies were purchased from Transduction Laboratories (Lexington, KY). The primary antibodies were applied at a dilution of 1:1000, and the secondary antibodies were applied at 1:5000. The membranes were washed, and the reaction product was visualized using chemiluminescence. To detect activated ERK1 and ERK2, antibodies that react with activation-specific phosphorylation sites on the ERK kinases were purchased from Promega Labs (Madison, WI). The primary antibody was applied at a dilution of 1:5000, and the alkaline phosphatase-conjugated secondary antibody was applied at 1:500. The reaction product was visualized using 5-bromo-4-chloro-3-indolylphosphate-nitro blue tetrazolium reagent (Sigma).

Measurement of Raf1 Kinase Activity—The cells were washed with serum-free medium and lysed in 50 mM Tris, pH 7.4, containing 1% Triton X-100 and 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 2 mM leupeptin, and 5 mM sodium vanadate. The cell lysates from control and TPA-treated cells were immunoprecipitated with Raf1 antibody and prebound to protein G-agarose beads. Raf1 activity was measured *in vitro* using a coupled assay (19). In this approach, the immunoprecipitated Raf1 is used to activate recombinant MEK in the presence of ATP (150 μM) and $MgCl_2$ (20 mM), which in turn activates recombinant ERK2. Activation of ERK2 is measured by detection of phosphorylation of myelin basic protein in the presence of [γ-^{32}P]ATP. The products of the reaction are spotted on phosphocellulose paper, unincorporated [γ-^{32}P]ATP is washed away with dilute phosphoric acid (0.85%), and the filters are counted by liquid scintillation. All of the substrates for the assay were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY).

Polysome Preparation and Reverse Transcription-PCR—The polysome profiles were obtained as described previously (3, 6, 26). In brief, postmitochondrial supernatants were prepared from control and TPA-treated 3T3-F442A adipocytes and reconstituted with 0.25 M sucrose and layered over a 10–50% sucrose gradient. The gradients were centrifuged for 3 h at 180,000 $\times$ *g* at 4 °C. A control extract treated with 5 mM EDTA was included to demonstrate the release of LPL mRNA from the polysomes into the free fraction. The gradients were fractionated into 750-μl fractions, and the UV absorption at A_{260} was recorded. The fractions were precipitated, and the RNA was extracted. Measurement of LPL mRNA in each fraction was done by reverse transcription-PCR, as described previously (3, 6). The primers derived from mouse LPL cDNA sequence encoded nucleotides 1158–1177 (upstream primer) and nucleotides 1369–1389 (downstream primer). Equal volumes of each fraction containing 0.1–1 ng of RNA was reverse-transcribed, followed by PCR for 20 cycles. The resulting ethidium bromide-stained gel was imaged and analyzed using the Eagle Sight™ 3.0 quantitation and analysis software (Stratagene, La Jolla, CA).

Preparation of Cytoplasmic Extracts—A S-100 fraction was isolated from 6 $\times$ 10^6 adipocytes. The cells were homogenized using a Dounce homogenizer in 5 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 35 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, and 7 mM β-mercaptoethanol). The homogenates were centrifuged at 10,000 $\times$ *g* for 15 min at 4 °C, and the post-nuclear supernatants are centrifuged at 100,000 $\times$ *g* for 1 h. The proteins are fractionated using 60% ammonium sulfate precipitation at 4 °C for 30 min. The precipitated proteins are dissolved in 200 μl of buffer A (20 mM Tris-HCl, pH 7.4, 20 mM KCl, 7 mM β-mercaptoethanol, and 10% glycerol). Equal quantities of cytoplasmic extract proteins (0.5–1.0 μg) were used to analyze effects on *in vitro* translation of LPL transcripts using the rabbit reticulocyte lysate *in vitro* translation system (Promega).

In Vitro Translation and LPL Transcripts—RNA transcripts of LPL cDNA were prepared as described previously (6). RNA transcripts (0.1 μg) were translated for 60 min in the presence of [^{35}S]methionine using a rabbit reticulocyte lysate *in vitro* translation system. Translation products were analyzed on SDS-PAGE followed by autoradiography.

Statistics—All of the assays are done in triplicate, and the data are expressed as the means $\pm$ S.E. Changes in LPL activity were analyzed using Student's *t* test, with $p < 0.05$ taken as the level of significance.

RESULTS

PKC Isoforms Involved in LPL Regulation—In previous studies, we observed a decrease in LPL activity following depletion of PKC isoforms α, β, δ, and ϵ after overnight TPA treatment of adipocytes (13). To identify the individual isoforms of PKC involved in LPL regulation, we examined the effect of depleting specific PKC isoforms using antisense oligonucleotide technology. Differentiated adipocytes were treated with antisense ol-

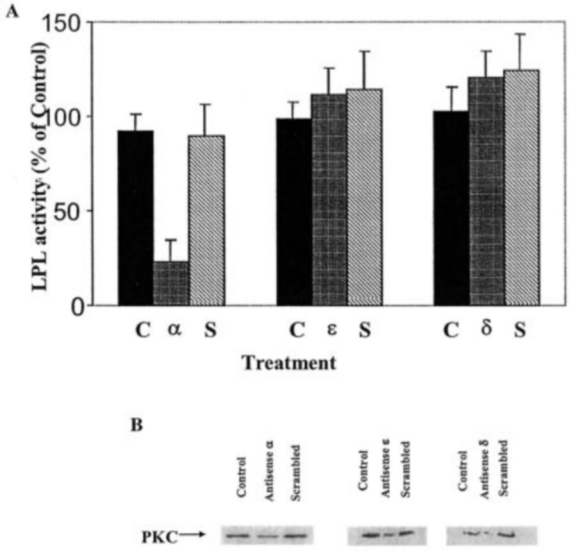

FIG. 1. **Effect of PKC antisense oligonucleotides on LPL activity.** Differentiated 3T3-F442A adipocytes were exposed to either antisense oligonucleotides or to an equal concentration of scrambled oligonucleotides. *A*, after 72 h, LPL activity was measured in cells that were treated with Lipofectin only (*C*) or treated with the antisense oligonucleotides PKCα, δ, or ϵ or the scrambled oligonucleotides (*S*). The data are expressed as percentages of control where the control activity was 1.2 ± 0.2 mmol of free fatty acid/h/mg of protein. The data represent the result of three experiments performed in triplicate and expressed as the means $\pm$ S.E. The decrease in LPL activity in the antisense PKCα-treated cells was statistically significant ($p < 0.05$). *B*, Western blot of cells treated as described in *A* and blotted with antibodies to PKC α, δ, and ϵ, respectively, and equal quantities of protein (15 μg) was loaded on SDS-PAGE gels. The data represent one of three independent experiments with similar results.

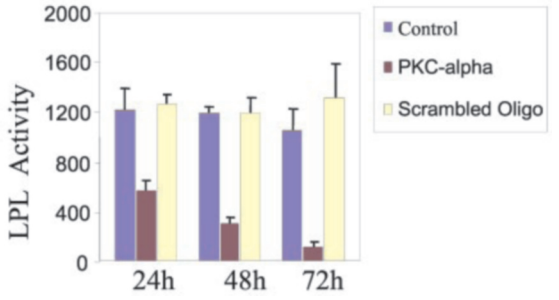

FIG. 2. **Time course of inhibition of LPL by antisense oligonucleotide to PKCα.** 3T3-F442A adipocytes were differentiated and then exposed to either antisense oligonucleotide to PKCα or scrambled oligonucleotide. Control cells were treated with Lipofectin alone. LPL was measured as the heparin-released activity, and the data are expressed as nmol of free fatty acid/min/mg of protein. The data represent the means of three similar experiments performed in triplicate. The changes in LPL activity in the antisense oligonucleotide-treated cells were statistically significant ($p < 0.05$).

igonucleotide to PKCα, δ, or ϵ or with an equal concentration of an oligonucleotide of scrambled sequence. LPL activity was measured 72 h after addition of the oligonucleotides. Antisense oligonucleotide to PKCα inhibited heparin-releasable LPL activity by $78 \pm 8\%$ when compared with control cells treated with Lipofectin alone (Fig. 1*A*). The scrambled oligonucleotide had no significant effect. Specificity was further demonstrated by the lack of effect observed with antisense oligonucleotide to PKCδ or PKCϵ (Fig. 1*A*). To confirm that the antisense oligonucleotides were effective in depleting specific PKC protein expression, we examined the levels of PKCα, δ, and ϵ proteins in cell lysates 72 h after oligonucleotide application. As detected by Western blot analysis, the antisense oligonucleotides effectively decreased the levels of the appropriate PKC isoforms when compared with control cells or to cells treated with scrambled oligonucleotides (Fig. 1*B*). Thus, the antisense oligonucleotides to PKCδ and ϵ were effective at reducing expression of those isoforms, but this resulted in no change in LPL activity.

The time course of the effect of PKCα antisense oligonucleotide on LPL activity was examined. Following differentiation, adipocytes were exposed to the PKCα antisense oligonucleotide, and heparin-releasable LPL activity was measured at 24, 48, and 72 h after treatment. A decrease in LPL activity was evident 24 h after treatment of cells with oligonucleotide, and LPL activity decreased further at 48 and 72 h after treatment (Fig. 2). There was no corresponding change in LPL activity in the cells treated with scrambled oligonucleotide. Extractable LPL activity was also measured in the cells after 72 h of oligonucleotide treatment when the decrease in heparin-releasable LPL activity was maximum. Extractable LPL activity was also inhibited by $30 \pm 4\%$ ($p < 0.05$) in the antisense PKCα-treated cells and not in the scrambled or control cells (data not shown). There was no change in extractable LPL activity in the cells treated with antisense PKCδ and ϵ.

Mechanism of LPL Regulation—To study the cellular mechanism involved in the regulation of LPL activity by PKCα, the steady state of LPL mRNA and protein levels were measured, as well as the LPL synthetic rate. Northern blot analysis of LPL mRNA was performed on adipocytes treated with antisense or scrambled PKCα oligonucleotide, along with mock-treated control cells. There was no change in LPL mRNA between the control cultures or those treated with PKCα antisense or scrambled oligonucleotide (Fig. 3), indicating that the decrease in activity in the antisense-treated cells did not result from a decrease in LPL mRNA. Western blot analysis of LPL protein indicated a decrease in LPL protein expression in antisense-treated cells as compared with control cells, indicating that the decrease in LPL activity resulted from a decrease in steady-state levels of LPL protein (Fig. 4*A*). To study LPL synthesis, cells treated with PKCα antisense or scrambled oligonucleotide were pulse-labeled with [^{35}S]methionine followed by immunoprecipitation. The LPL synthetic rate was inhibited in cells exposed to PKCα antisense oligonucleotide but not in those exposed to scrambled oligonucleotide nor in control cells (Fig. 4*B*).

Translational regulation involving inhibition of translational initiation has been demonstrated previously (28). To determine whether translational initiation was inhibited by depletion of PKC using TPA, we studied the distribution of LPL mRNA on the polysomes in control and TPA-treated 3T3-F442A adipocytes. As shown in Fig. 5 there was no change in the distribution of LPL mRNA associated with the polysomes between control and TPA-treated adipocytes, indicating that translation initiation was not inhibited. As a control, EDTA was added to the cell lysate to dissociate the mRNA from the polysomes. As shown in Fig. 5, EDTA released the mRNA from the polysomes and yielded a shift of the LPL mRNA to the less dense fractions of the gradient.

The translational regulation of LPL both by catacholamines and diabetes (3, 6) is mediated by transacting factors and involves the 3′-UTR of LPL. To determine whether a similar mechanism was involved in the regulation by PKCα and to identify the region on LPL mRNA involved in LPL regulation, we studied the *in vitro* translation of LPL mRNA in the presence of cytoplasmic extracts from PKCα or scrambled oligomer treated cells. The cytoplasmic extracts were prepared as described in methods 72 h after transfection of PKCα or a scrambled oligomer when LPL inhibition was maximum. Equal quantities of cell protein from the cytoplasmic extracts were added

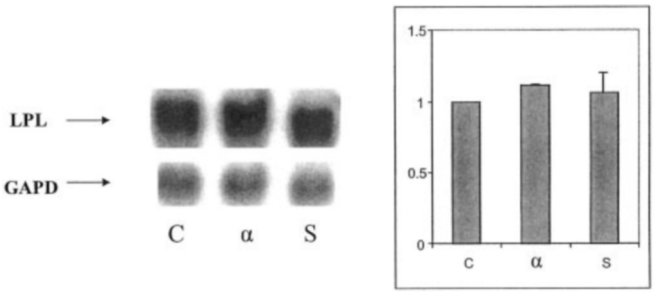

FIG. 3. **Effect of antisense PKCα on LPL mRNA.** *A*, Northern blot using the cDNAs for LPL and GAPDH. The data shown represent one of three experiments with similar results. *C*, control cells; *α*, cells treated with PKC*α* antisense oligonucleotide; *S*, cells treated with scrambled oligonucleotide. The *bar graph* represents the densitometric analysis of the Northern blot; the results are expressed as arbitrary units ± S.E.

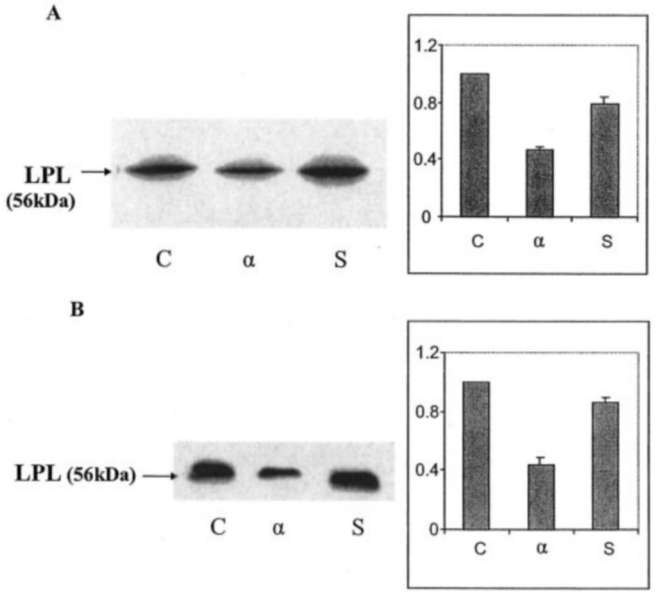

FIG. 4. **Effect of PKCα on LPL protein expression.** All of the gels are representative of at least three experiments with similar results. *A*, Western blot for LPL. *B*, LPL synthetic rate following a 40-min pulse with [^{35}S]methionine and immunoprecipitation. *C*, control cells; *α*, cells treated with PKC*α* antisense oligonucleotide; *S*, cells treated with scrambled oligonucleotide. The *bar graphs* represent the densitometric analysis of the autoradiograph; the results are expressed as arbitrary units ± S.E.

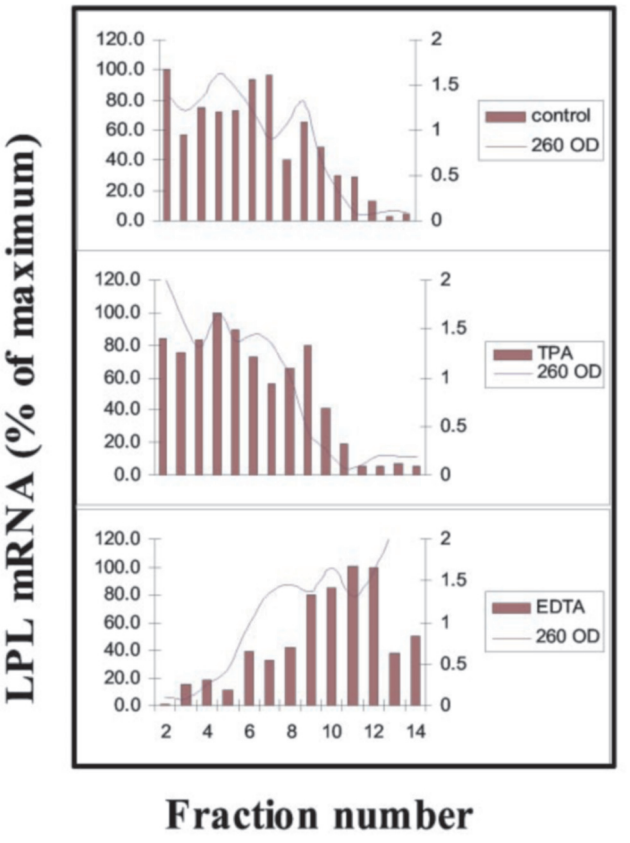

FIG. 5. **Effect of TPA treatment on the distribution of LPL mRNA in the polysomes.** Total RNA was extracted from control, TPA, or EDTA treated fractions, following sucrose density gradient fractionation. The fractions are numbered 1–15 from the *bottom* to the *top* of the gradient. The A_{260} of each fractions was recorded. Fractions 1–12 contain both 18 and 28 S ribosome subunits. LPL mRNA is quantitated in equal volumes of fractions by reverse transcription-PCR and expressed as a percentage of the maximum LPL mRNA. The data shown represent one of two experiments with similar results.

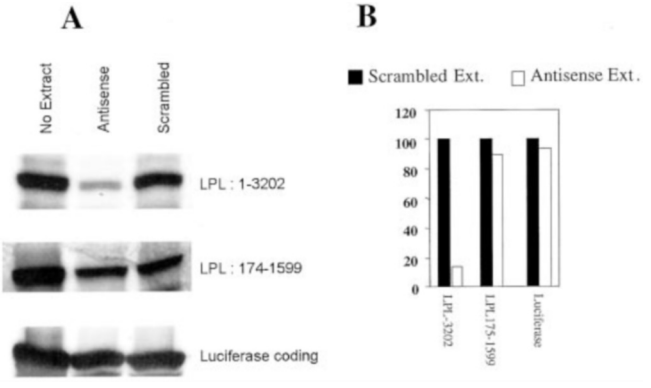

FIG. 6. *A*, effect of the cytoplasmic extracts on LPL *in vitro* translation. Cytoplasmic extracts from PKC*α* antisense oligomer or scrambled oligomer-treated cells were added to reticulocyte lysate in the presence of specific transcripts. The *autoradiogram* represents one of three independent experiments with similar results. *B*, the *bar graph* for each construct represents the mean of the three experiments. The data are expressed as the percentage of control.

to a rabbit reticulocyte *in vitro* translation system containing LPL transcripts or control transcript. If transacting factors interacting with regions of LPL mRNA were present in the PKC*α* antisense-treated cells, we would detect a decrease in LPL translation in the *in vitro* translation assay. In this assay, the full-length LPL transcript (3.2 kb) and the LPL transcript lacking the 5′- and 3′-UTR were studied along with a luciferase control transcript (3). As shown in Fig. 6, the LPL transcript containing the full-length 3′-UTR was inhibited by 80 ± 5% in the presence of the S-100 extract by the PKC*α* antisense-treated cell but not by the scrambled oligomer treated extract. There was no significant decrease in translation with the addition of the PKC*α* antisense-treated extract to the truncated LPL transcript or the control luciferase transcript.

Role of Other Kinases in LPL Regulation—PKC*α* can interact with and activate Raf1 (29). To determine whether Raf1 kinase is involved in the PKC-mediated regulation of LPL, we measured Raf1 activity and LPL activity in adipocytes where PKC isoforms had been depleted by long term TPA treatment. As demonstrated previously (13), TPA treatment inhibited LPL activity by 70 ± 6%. As shown in Fig. 7, there was a parallel decrease in Raf1 activity in the TPA-treated cells. Similar data were obtained when only PKC*α* was inhibited using the anti-

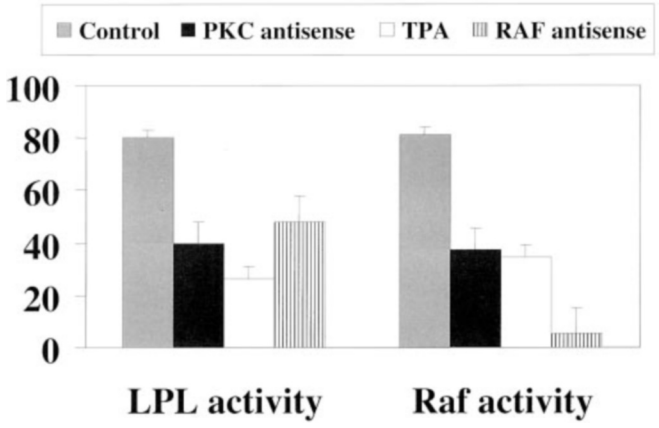

FIG. 7. **Effect of PKCα and RAF1 inhibition on Raf1 activity.** 3T3-F442A adipocytes were treated with TPA and were exposed to PKCα-antisense, Raf1 antisense, or scrambled oligonucleotide. Heparin-releasable LPL activity and Raf1 activity was measured as described under "Materials and Methods." The data are expressed as percentages of control activity, with *Control* representing untreated cells. The data represent the means ± S.E. of three similar experiments performed in duplicate. The decreases in both LPL and Raf1 activity in TPA- and antisense-treated cells were statistically significant ($p < 0.05$).

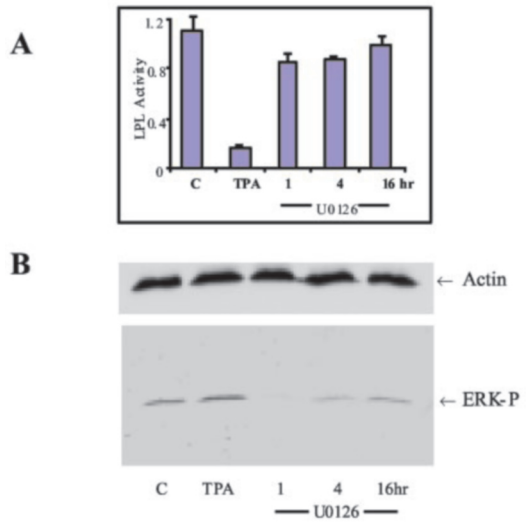

FIG. 8. **Lack of a relationship between changes in ERK1/2 and LPL activity.** *A*, cells were treated with U0126 or TPA, and heparin-releasable LPL activity was measured. LPL activity is expressed as nmol of free fatty acid/h/mg of protein. The data represent the means ± S.E. of three identical experiments done in duplicate. *B*, Western blot of cells treated with U0126 or TPA probed with antibodies to activated ERK1/2 or β actin. Equal quantities (15 μg) of protein were loaded on SDS-PAGE gels. The data represent one of three identical experiments with similar results.

sense oligonucleotide. As shown in Fig. 7, the depletion of PKCα after treating cells with PKCα antisense oligonucleotide inhibited Raf1 activity and LPL activity by 55 ± 8%, whereas scrambled oligonucleotide had no significant effect on either LPL or Raf1 activity. Thus, decreases in Raf1 activity were accompanied by a significant decrease in LPL activity (Fig. 7). Antisense oligonucleotides to RAF1 were effective and inhibited Raf1 activity by 90 ± 8%. This inhibition of Raf1 activity was accompanied by a 48 ± 10% inhibition of LPL activity, which was not accompanied by a decrease in LPL mRNA (data not shown).

Activated Raf1 can phosphorylate MAPK and ERK kinase (MEK), leading to phosphorylation-dependent activation of ERK1 and ERK2 (30). To determine whether the regulation of LPL activity by PKCα involved the activation of ERK1/2, we studied the effect of the MEK inhibitor U0126 (19). Differentiated 3T3-F442A adipocytes were treated with U0126 for 1, 4, or 16 h or with TPA for 16 h. LPL activity was not inhibited in the U0126-treated adipocytes (Fig. 8*A*), whereas prolonged TPA treatment (16 h) inhibited LPL activity as observed earlier (13). ERK1/2 activity was assayed by Western blot analysis using an antibody that specifically detects activation-associated phosphorylation sites in ERK1/2. U0126-treated cells demonstrated a decrease in levels of active ERK1/2 following treatment as compared with the untreated control, but 16 h TPA-treated cells demonstrated no such change in ERK1/2 (Fig. 8*B*).

DISCUSSION

The regulation of LPL expression is complex and occurs at transcriptional and post-transcriptional levels of processing (1). Under a specific condition, LPL translation in adipocytes is inhibited because of the activity of an RNA-binding protein that binds to the 3′-UTR of LPL mRNA (4, 5). This translational inhibition is activated by treating cells with epinephrine with the subsequent stimulation of cAMP-dependent protein kinase. We have also described translational inhibition of LPL in the adipose tissues of diabetic rats (6). Adipocytes from diabetic rats also demonstrate evidence for an RNA-binding protein; however, the RNA-binding motif on the 3′-UTR is further downstream from the motif for the epinephrine-induced RNA binding protein, suggesting that a different mechanism is in place for diabetes-induced translational regulation.

LPL translational regulation is dependent on PKC-controlled mechanisms. In previous studies, we examined the effect of phorbol ester (TPA), which can mimic in part the effect of DAG, the natural ligand for PKC (31). Phorbol esters activate conventional and novel PKCs immediately (32, 33), but long term treatment with TPA results in a decrease in PKC in several cell types (34), mainly because of an increased rate of degradation of PKC protein (35, 36). Because activation of PKC did not stimulate LPL, our data suggested that PKC plays a constitutive role in maintaining LPL synthesis.

The current studies were performed to determine the specific isoforms of PKC involved in LPL regulation and to gain better knowledge of the signal transduction pathways involved in PKC-mediated regulation of LPL. We treated adipocytes with antisense oligonucleotides to the α, δ, and ϵ isoforms of PKC. Antisense oligonucleotide inhibitors have been used to block PKC isoform expression previously (37). The Phosphorothioate-modified oligonucleotides include sulfur in the phosphate backbone of the DNA, which improves stability (38), and these modified antisense oligonucleotides have been used previously to inhibit the expression of specific isoforms of PKC (16, 17, 20). We confirmed that each specific antisense oligonucleotide inhibited the expression of the corresponding PKC isoform, whereas the scrambled oligomers had no such effect. Only the depletion of PKCα was accompanied by inhibition of LPL activity. In the previous study using TPA, where many PKC isoforms were inhibited (13), the decrease in LPL activity was accompanied by an inhibition of LPL protein and LPL synthesis with no change in LPL mRNA levels, suggesting translational regulation. In this study we focused on the PKC isoforms that were responsive to phorbol esters. The data indicate that PKCα, a member of the "conventional" class of PKCs, is a critical component in the translational regulation of LPL in adipocytes.

The regulation of gene expression at the level of translation occurs with numerous proteins. One of the best characterized systems involving translational regulation of gene expression is the inhibition of ferritin mRNA translation initiation in

response to iron limitation (28). The 3′-UTR is important for mRNA stability but can also be involved in translational efficiency and initiation (5, 39–41). To examine whether the inhibition of translation resulting from the depletion of PKC following TPA treatment caused an inhibition of translational initiation, we studied the polysome profiles of control and PKC-depleted adipocytes. We found that there was no change in the distribution of LPL mRNA on the polysomes, indicating that the inhibition was not at the level of translational initiation. In previous studies by us, LPL translational regulation has been identified in response to catecholamines, thyroid hormone, and diabetes (4–6), and in each instance, the 3′-UTR has been involved. In the present study, cytoplasmic extracts from adipocytes treated with antisense PKCα were added to the *in vitro* translation assay in the presence of full-length LPL transcript. The extract inhibited translation of LPL transcript containing the full-length UTR; however, the same extract did not inhibit the transcript of LPL lacking the 3′-UTR and did not inhibit the translation of an irrelevant mRNA construct. These data suggest that sequences involved in the translational regulation of LPL by PKCα depletion were located on the 3′-UTR of LPL mRNA.

Activation by PKCα phosphorylates Raf1, and this triggers a protein kinase cascade, which has the sequence: PKCα → Raf1 → MEK1/2 → ERK1/2. ERK1/2 comprise one set of three groups of MAPK have been identified in mammalian cells: (i) ERKs, also referred to as p42/44 MAPK; (ii) Jun N-terminal kinase or stress-activated protein kinase; and (iii) p38 MAPK. Although there is some cross-talk, these groups appear to be activated by distinct upstream kinases: ERK1/2 are activated primarily by MEK1/2; p38 responds to MKK3, MKK4, and MKK6; and Jun N-terminal kinase/stress-activated protein kinase to MKK4 and MKK7 (42, 43). ERK1/2 kinase and p38 MAPK have been identified in adipocytes. The activity of these enzymes is higher during the earlier stages of adipocyte differentiation than in the completely differentiated adipocyte (44). The stress-activated kinases Jun N-terminal kinase/stress-activated protein kinase have been studied extensively in fibroblasts, but the roles of any of these kinases in lipase regulation in the mature adipocyte have not been studied. We examined the potential role of MAPK in the regulation of LPL in adipocytes using U0126 and found no effect. Because U0126 is a selective inhibitor of MEK1/2 and has little or no effect on other kinases (19), these data suggest that the connection between PKCα to LPL regulation does not involve MEK1/2 → ERK1/2.

PKCα is an activator of Raf1 in NIH 3T3 fibroblasts (29). To determine whether the PKCα signal transduction pathway involved Raf1, we measured Raf1 activity in cells that were subjected to depletion of PKCα. Raf1 activity in the cells paralleled the changes in LPL activity. The activities of Raf1 and LPL were decreased by long term TPA treatment, which decreased all PKC isoforms (13), and by the specific inhibition by the antisense PKCα oligonucleotides. These data indicated a possible role of Raf1 kinase in LPL regulation. We therefore treated cells with an antisense oligonucleotide to Raf1 kinase, which inhibited LPL activity significantly. However, the specific inhibition of PKCα had a greater inhibitory effect on LPL activity than the inhibition of RAF1 using RAF1 antisense oligomers. Thus, these studies indicate that PKCα and RAF1 are both involved in LPL regulation, but PKCα could also be inhibiting LPL by some RAF1-independent mechanism.

Because inhibition of ERK1/2 did not inhibit LPL activity, Raf1 is likely not mediating the effect on LPL through ERK1/2. However, Raf1 may function through other pathways. For example, Raf1 can activate p97 MAPK (45) and also can facilitate ERK5 activation in an allosteric manner.

These studies yield greater insight into the cellular regulation of LPL expression. The specific inhibition of PKCα with an antisense oligonucleotide resulted in an inhibition of LPL translation. The parallel inhibition of Raf1 activity and the inhibition of LPL expression by antisense oligonucleotides to RAF1 kinase confirm an important role for both PKCα and Raf1 in the signal transduction pathway involved in the translational regulation of LPL in adipocytes.

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REFERENCES

1. Enerbäck, S., and Gimble, J. M. (1993) *Biochim. Biophys. Acta* **1169,** 107–125
2. Olivecrona, G., and Olivecrona, T. (1995) *Curr. Opin. Lipidol.* **6,** 291–305
3. Yukht, A., Davis, R. C., Ong, J. M., Ranganathan, G., and Kern, P. A. (1995) *J. Clin. Invest.* **96,** 2438–2444
4. Kern, P. A., Ranganathan, G., Yukht, A., Ong, J. M., and Davis, R. (1996) *J. Lipid Res.* **37,** 2332–2340
5. Ranganathan, G., Vu, D., and Kern, P. A. (1997) *J. Biol. Chem.* **272,** 2515–2519
6. Ranganathan, G., Li, C., and Kern, P. A. (2000) *J. Biol. Chem.* **275,** 40986–40991
7. Kellerer, M., and Haering, H. U. (1995) *Diabetes Res. Clin. Practice* **28,** S173–S177
8. Craven, P. A., Davidson, C. M., and DeRubertis, F. R. (1990) *Diabetes* **39,** 667–674
9. Farese, R., Standaert, M., Arnold, T., Yamada, K., Musunuru, K., Hernandez, H., Mischak, H., and Cooper, D. (1994) *J. Clin. Invest.* **93,** 1894–1899
10. Draznin, B., Leitner, J. W., Sussman, K. E., and Sherman, N. A. (1988) *Biochem. Biophys. Res. Commun.* **156,** 570–575
11. Ballester, R., and Rosen, O. M. (1995) *J. Biol. Chem.* **260,** 15194–15199
12. Borner, C., Eppenberger, U., Wyss, R., and Fabbro, D. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 2110–2114
13. Ranganathan, G., Kaakaji, R., and Kern, P. A. (1999) *J. Biol. Chem.* **274,** 9122–9127
14. Maier, J. A., and Ragnotti, G. (1993) *Exp. Cell Res.* **205,** 52–58
15. Dean, N. M., and Griffey, R. H. (2000) *Antisense Nucleic Acid Drug Development* **7,** 229–233
16. Dean, N. M., McKay, R., Miraglia, L., Geiger, T., Muller, M., Fabbro, D., and Bennett, C. F. (1996) *Biochem. Soc. Trans.* **24,** 623–629
17. Dean, N. M., McKay, R., Condon, T., and Bennet, F. C. (1994) *J. Biol. Chem.* **269,** 16416–16424
18. O'Dwyer, P. J., Stevenson, J. P., Gallagher, M., Cassella, A., Vasilevskaya, I., Monia, B. P., Holmlund, J., Dorr, F. A., and Yao, K. S. (1999) *Clin. Cancer Res.* **5,** 3977–3982
19. Favata, M. F., Horiuch, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Feeser, W. S., Van Dyk, D. W., Pitts, W. J., Earl, R. A., Hobbs, F., Copeland, R. A., Magolda, R. L., Scherle, P. A., and Trzaskos, J. M. (1998) *J. Biol. Chem.* **273,** 18623–18632
20. Marcusson, E. G., Bhat, B., Manoharan, M., Bennett, C. F., and Dean, N. M. (1998) *Nucleic Acids Res.* **26,** 2016–2023
21. Ranganathan, S., and Kern, P. A. (1998) *J. Biol. Chem.* **273,** 26117–26122
22. Nilsson-Ehle, P., and Schotz, M. C. (1976) *J. Lipid Res.* **17,** 536–541
23. Ranganathan, G., and Kern, P. A. (1999) in *Lipase and Phospholipase Protocols* (Doolittle, M. H., and Reue, K., eds) pp. 329–336, Humana Press, Totowa, NJ
24. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
25. Holm, C., Kirchgessner, T. G., Svenson, K. L., Fredrikson, G., Nilsson, S., Miller, C. G., Shively, J. E., Heinzmann, C., Sparkes, R. S., Mohandas, T., Lusis, A. J., Belfrage, P., and Schotz, M. C. (1988) *Science* **241,** 1503–1506
26. Ong, J. M., Kirchgessner, T. G., Schotz, M. C., and Kern, P. A. (1988) *J. Biol. Chem.* **263,** 12933–12938
27. Goers, J. F., Petersen, M. E., Kern, P. A., Ong, J., and Schotz, M. C. (1987) *Anal. Biochem.* **166,** 27–35
28. Klausner, R. D., and Harford, J. B. (1989) *Science* **246,** 870–872
29. Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., and Marme, D. R. U. (1993) *Nature* **364,** 249–252
30. English, J. M., Pearson, G., Hockenberry, T., Shivakumar, L., White, M. A., and Cobb, M. H. (2000) *J. Biol. Chem.* **274,** 31588–31592
31. Liu, J. P. (1996) *Mol. Cell. Endocrinol.* **116,** 1–29
32. Ishizuka, T., Hoffman, J., Cooper, D. R., Watson, J. E., Pushkin, D. B., and Farese, R. V. (1989) *FEBS Lett.* **249,** 234–238
33. Farese, R. V., Standaert, M. L., Francois, A. J., Ways, K., Arnold, T. P., Hernandez, H., and Cooper, D. R. (1992) *Biochem. J.* **288,** 319–323
34. Adams, J., and Gullick, J. (1989) *Biochem. J.* **257,** 905–911
35. Junco, M., Webster, C., Crawford, C., Bosca L., and Parker, P. J. (1994) *Eur. J. Biochem.* **223,** 259–263
36. Eto, A., Akita, Y., Saido, T. C., Suzuki, K., and Kawashima, S. (1995) *J. Biol. Chem.* **270,** 25115–25120
37. Farese, R. V., Standaert, M. L., Ishizuka, T., Yu, B., Hernandez, H., Waldron, C., Watson, J., Farese, J. P., Cooper, D. R., and Wickstrom, E. (1991) *Antisense Res. Dev.* **1,** 35–42
38. Woolf, T. M., Jennings, C. G., Rebagliati, M., and Melton, D. A. (1990) *Nucleic Acids Res.* **18,** 1763–1769

39. Ch'ng, J. L., Shoemaker, D. L., Schimmel, P., and Holmes, E. W. (1990) *Science* **245,** 1003–1006
40. Ch'ng, J. L., Mulligan, R. C., Schimmel, P., and Holmes, E. W. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 10006–10010
41. Ostareck-Lederer, A., Ostareck, D. H., Standart, N., and Thiele, B. J. (1994) *EMBO J.* **13,** 1476–1481
42. Han, J., Lee, J. D., Jiang, Y., Li, Z., Feng, L., and Ulevitch, R. J. (1996) *J. Biol. Chem.* **271,** 2886–2891
43. Moriguchi, T., Toyoshima, F., Masuyama, N., Hanafusa, H., Gotoh, Y., and Nishida, E. (1997) *EMBO J.* **16,** 7045–7053
44. Engelman, J. A., Lisanti, M. P., and Scherer, P. E. (1998) *J. Biol. Chem.* **273,** 32111–32120
45. Janulis, M., Trakul, N., Greene, G., Schaefer, E. M., Lee, J. D., and Rosner, M. R. (2001) *Mol. Cell. Biol.* **21,** 2235–2247