11-8-2002

The Translational Regulation of Lipoprotein Lipase by Epinephrine Involves an RNA Binding Complex Including the Catalytic Subunit of Protein Kinase A

Gouri Ranganathan
University of Arkansas for Medical Sciences

Dan Phan
University of Arkansas for Medical Sciences

Irina D. Pokrovskaya
University of Arkansas for Medical Sciences

Joan E. McEwen
University of Arkansas for Medical Sciences

Chunling Li
University of Arkansas for Medical Sciences

The Translational Regulation of Lipoprotein Lipase by Epinephrine Involves an RNA Binding Complex Including the Catalytic Subunit of Protein Kinase A

Digital Object Identifier (DOI)
10.1074/jbc.M202560200

Authors
Gouri Ranganathan, Dan Phan, Irina D. Pokrovskaya, Joan E. McEwen, Chunling Li, and Philip A. Kern

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 277, No. 45, Issue of November 8, pp. 43281–43287, 2002
Printed in U.S.A.

The Translational Regulation of Lipoprotein Lipase by Epinephrine Involves an RNA Binding Complex Including the Catalytic Subunit of Protein Kinase A*

Received for publication, March 15, 2002, and in revised form, September 4, 2002
Published, JBC Papers in Press, September 5, 2002, DOI 10.1074/jbc.M202560200

Gouri Ranganathan, Dan Phan, Irina D. Pokrovskaya, Joan E. McEwen, Chunling Li, and Philip A. Kern‡

From the Central Arkansas Veterans HealthCare System, and Department of Medicine, Division of Endocrinology, and the Department of Geriatrics, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205

The balance of lipid flux in adipocytes is controlled by the opposing actions of lipolysis and lipogenesis, which are controlled primarily by hormone-sensitive lipase and lipoprotein lipase (LPL), respectively. Catecholamines stimulate adipocyte lipolysis through reversible phosphorylation of hormone-sensitive lipase, and simultaneously inhibit LPL activity. However, LPL regulation is complex and previous studies have described translational regulation of LPL in response to catecholamines because of an RNA-binding protein that interacts with the 3′-untranslated region of LPL mRNA. In this study, we identified several protein components of an LPL RNA binding complex. Using an LPL RNA affinity column, we identified two of the RNA-binding proteins as the catalytic (C) subunit of cAMP-dependent protein kinase (PKA), and A kinase anchoring protein (AKAP) 121/149, one of the PKA anchoring proteins, which has known RNA binding activity. To determine whether the C subunit was involved in LPL translation inhibition, the C subunit was depleted from the cytoplasmic extract of epinephrine-stimulated adipocytes by immunoprecipitation. This resulted in the loss of LPL translation inhibition activity of the extract, along with decreased RNA binding activity in a gel shift assay. To demonstrate the importance of the AKAPs, inhibition of PKA-AKAP binding with a peptide competitor (HT31) prevented epinephrine-mediated inhibition of LPL translation. C subunit kinase activity was necessary for LPL RNA binding and translation inhibition, suggesting that the phosphorylation of AKAP121/149 or other proteins was an important part of RNA binding complex formation. The hormonal activation of PKA results in the reversible phosphorylation of hormone-sensitive lipase, which is the primary mediator of adipocyte lipolysis. These studies demonstrate a dual role for PKA to simultaneously inhibit LPL-mediated lipogenesis through inhibition of LPL translation.

Lipoprotein lipase (LPL)[1] is a central enzyme in lipid metabolism and hydrolyzes the core of triglyceride-rich plasma lipoproteins into nonesterified fatty acids and monoacylglycerol (1). In adipose tissue and muscle, LPL is localized to the capillary endothelium, and contributes to the rapid removal of triglyceride-rich lipoproteins and their remnants.

Catecholamines are of considerable physiologic importance in the mobilization of adipose tissue lipid in response to fasting and exercise. Hormones that cause elevated cAMP (β-adrenergic agonists, ACTH, and glucagon) result in the activation of cAMP-dependent protein kinase A (PKA), which then activates hormone-sensitive lipase (HSL) (2, 3). HSL is the primary mediator of adipocyte lipolysis (4), and the release of nonesterified fatty acids from adipocytes play a central role in obesity and insulin resistance (5, 6). On the other hand, LPL hydrolyzes lipoproteins at the capillary endothelium generating nonesterified fatty acids for triglyceride storage. LPL and HSL serve opposing functions in adipose tissue, and they respond in an opposite fashion in response to hormonal regulation. In adipocytes, insulin and the fed state result in an increase in LPL activity along with a decrease in HSL activity, whereas hormones that are elevated during the fasting state, such as epinephrine and glucagon, inhibit LPL activity and stimulate HSL-mediated lipolysis (7–9).

Although the decrease in LPL activity by catecholamines has been described previously (7), the cellular mechanisms controlling LPL inhibition are complex. In rat adipocytes, we found that the LPL synthetic rate was inhibited more than 5-fold within 30 min of addition of epinephrine to the medium, with no change in LPL mRNA levels (10). Studies of 3T3 adipocytes demonstrated that the inhibition of LPL translation by epinephrine involved an RNA-binding protein that interacted with the proximal 3′-untranslated region (UTR) of the LPL mRNA (11). Subsequent studies found the first 24 nucleotides of the LPL 3′-UTR essential for translational regulation, and a 30-kDa RNA-binding protein was identified by cross-linking as an important component of LPL translational regulation (12).

This study was intended to identify the components involved in the translational regulation of LPL following cAMP elevation. As described below, we have identified the catalytic (C) subunit of PKA as the important 30-kDa protein involved in LPL translational regulation. However, the C subunit of PKA is likely part of an RNA binding complex, which also involves A kinase anchoring protein (AKAP) 121/149, which is involved in binding the PKA holoenzyme, and which contains a known RNA binding domain (13).

MATERIALS AND METHODS

Purification of the RNA-binding Protein—Approximately 100 T-75 flasks of 3T3-F442A adipocytes, representing ~10^9 cells, were induced

* This work was supported by a Career Development Award from the American Diabetes Association (to G. R.), Grant DK 39176 from the National Institute of Health, and a Merit Review Grant from the Veterans Administration. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Research, 151 LR, Central Arkansas Veterans Healthcare System, 4300 W. 7th St., Little Rock, AR 72205. Tel.: 501-257-4816; Fax: 501-257-4821; E-mail: kernphilipa@uams.edu.

[1] The abbreviations used are: LPL, lipoprotein lipase; PKA, protein kinase A; HSL, hormone-sensitive lipase; UTR, untranslated region; AKAP, A kinase anchoring protein; ACTH, adrenocorticotropic hormone.

to differentiate with insulin, and after 8 days of differentiation they were treated with epinephrine (10^{-5} M). LPL translation was inhibited in these cells, and a cytoplasmic extract was prepared as described previously (11). In brief, adipocytes were scraped from the plate and the cell pellet was resuspended in 10× the pellet volume of lysis buffer (50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 35 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 7 mM β-mercaptoethanol), and homogenized with 10 strokes of a glass homogenizer. Homogenates were centrifuged at 10,000 × *g* for 15 min at 4 °C. The postnuclear extract was used to prepare a high speed supernatant fraction (S-100) by centrifugation at 100,000 × *g* for 2 h at 4 °C. Solid ammonium sulfate was added to the cytosolic fraction to 60% saturation and precipitated for ½ h on ice. Precipitated proteins were collected by centrifugation at 6,000 × *g* for 10 min at 0 °C, redissolved and dialyzed against Buffer A (20 mM Tris-HCl, pH 7.4, 20 mM KCl, 7 mM β-mercaptoethanol, 0.1 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride). The sample was then diluted to 25 ml and passed through a DEAE-cellulose column equilibrated in 20 mM Tris-HCl, pH 7.4. After washing the column with the same buffer, proteins were eluted with 5 ml of buffer containing 400 mM KCl. The DEAE fraction that passed unbound through the column demonstrated LPL RNA binding properties, as demonstrated by a gel shift experiment, whereas the 400 mM KCl eluted fraction had no RNA binding activity. Therefore, the flow-through was dialyzed against the initial column buffer, and fractionated on a LPL 3′ UTR-oligo(dT)-Sepharose column.

To prepare the RNA binding column, poly(A) RNA transcripts were generated containing the C-terminal 50 nucleotides of coding sequence, and the first 100 nucleotides of the LPL 3′-UTR (nucleotides 1512 to 1635). A tracer amount of [^{32}P]UTP was also added during transcription, to follow the binding and quality of the RNA. The poly(A) RNA was incubated with presoaked poly(T)-Sepharose beads for 60 min, and packed into a column. The column was washed with low salt buffer (20 mM Tris-HCl, pH 7.4, 20 mM KCl) to remove the unbound excess RNA.

For the initial binding reaction, the epinephrine-treated 3T3-F442A adipocyte extracts were added in low salt (20 mM KCl) buffer containing heparin and yeast tRNA to prevent degradation and inhibit nonspecific binding. After washing extensively, bound proteins were eluted with a salt gradient varying from 0.1 to 0.5 M KCl in 20 mM Hepes, pH 7.5. Fractions were dialyzed against 40 mM KCl buffer, and analyzed by SDS-PAGE with colloidal blue staining (Novex), as described under "Results."

Peptide Sequencing—To obtain sequence information, pooled column fractions from the LPL 3′-UTR column were run on a preparatory 10% polyacrylamide gel. Parallel lanes were stained for identification, and a discrete band at 30 kDa was cut from a wet gel and sent to the Harvard Microchemistry facility (Cambridge, MA) for sequencing. The sequence analysis was performed on tryptic fragments by microcapillary reverse-phase high performance liquid chromatography nanoelectrospray tandem mass spectrometry on a Finnigan LCQ quadrupole ion trap mass spectrometer.

In Vitro Translation—In vitro translation of RNA transcripts was performed as described previously (11). RNA transcripts were made from an LPL cDNA construct (LPL 35 of Wion *et al.* (14)). Equal quantities of RNA transcripts (0.1 μg) were translated in a rabbit reticulocyte lysate system (Promega) in the presence of [^{35}S]methionine, and the translation products were analyzed by SDS-PAGE and autoradiography. The intensity of the images was quantitated with the Eagle Sight™ 3.0 image capture and analysis software (Stratagene 2, La Jolla, CA). We previously demonstrated that cell extracts from epinephrine-treated cells inhibited LPL translation *in vitro* (11, 12). The cell extracts (S-100 fractions) from control and epinephrine-treated adipocytes were prepared as described above followed by ammonium sulfate precipitation and dialysis against buffer A. Protein concentration in the cell extract was determined with a Bio-Rad protein assay, with bovine serum albumin as a standard. Equal quantities of the cell extract (0.1 μg) were used in the rabbit reticulocyte lysate reaction and the reaction was carried out for 60 min. To assess the role of PKA C subunit, a specific antibody to the Cα subunit (polyclonal antibody to the C terminus, Santa Cruz Biotechnology) was added to the cell extract 30 min prior to addition to the *in vitro* translation reaction. As a control, extracts were treated with antibodies to β-actin (Calbiochem).

RNA Gel Shift—To assess the binding of the epinephrine cell extract to LPL RNA sequences, a [^{32}P]RNA sequence corresponding to the proximal 3′-UTR of LPL (nucleotides 1512 to 1635) was synthesized. This ^{32}P-labeled transcript (50,000 cpm) was incubated for 20 min in buffer A containing 10 μg/ml yeast tRNA, 10 units/ml heparin sulfate, along with 5 μg of cytoplasmic extract from control or epinephrine-treated 3T3-F442A adipocytes, and the products were analyzed on a 5% nondenaturing polyacrylamide gel. In some experiments, PKA C subunit was removed from the cell extract prior to incubation with the [^{32}P]RNA. To eliminate PKA Cα from the extracts, 0.1 μg of anti-Cα antibody was incubated with the cell extract followed by 5 μl of 1:1 diluted protein A-agarose beads. The extract was centrifuged at 1500 × *g*, and the Cα-depleted supernatant was then added to the ^{32}P-transcript and gel shift analysis was performed as described above. The effect of the anti-Cα antibodies was compared with irrelevant antibodies (β-actin). In additional experiments, PKA Cα subunit (0.5 Units, Calbiochem) was added back after immunoprecipitation of Cα. To inhibit PKA activity, cells were pretreated for 15 min with H89 (10 mM, Sigma), which is a specific inhibitor of the Cα ATP binding site (15), followed by epinephrine treatment as described above. The gel shift was then performed as described above.

Northern, Western, and Ligand Blotting—RNA was extracted from adipocytes (16), and equal amounts of total RNA were resolved by electrophoresis in 2.2 M formaldehyde, 1% agarose gels. Northern blots were probed using [^{32}P]dCTP-labeled cDNA probes to AKAP149 and glyceraldehyde-3-phosphate dehydrogenase, which have been reported previously (17, 18). Antibodies to AKAP149 (Santa Cruz Biotechnology) were directed against the C-terminal region of the molecule, which is homologous with AKAP121, but not with other AKAPs (13, 19). Antiphosphoserine antibodies were obtained from Zymed Laboratories, San Francisco, CA. Western blotting was performed as described previously (20). Samples containing 15 μg of total protein were fractionated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. 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. Secondary antibodies were antispecies-specific peroxidase-labeled IgG (Sigma). Ligand blotting with [^{32}P]PKA RII subunit was performed as described previously (21).

To determine whether the PKA C subunit was associated with AKAP149/121, co-precipitation experiments were performed as described previously (22). Epinephrine-treated cells were lysed in phosphate-buffered saline containing 0.1% SDS, 0.5% sodium deoxycholate, and protease inhibitors and immunoprecipitated with either anti-PKA Cα antibodies or anti-AKAP149/121 antibodies. These immunoprecipitated products were analyzed on SDS-PAGE, followed by ligand blotting with the [^{32}P]PKA RII subunit, as described above.

LPL Synthetic Rate—The LPL synthetic rate was measured in cultured 3T3-F442A cells as described previously (23). Cells were incubated in methionine-free medium for 2 h prior to the addition of 50 μCi of [^{35}S]methionine for 30 min. The cells were lysed and immunoprecipitated with anti-LPL antibodies (24), followed by analysis of the samples on a 10% polyacrylamide-SDS gel, followed by autoradiography. Within each experiment, an aliquot of cell lysate was precipitated with trichloroacetic acid and counted, and the amount of lysate taken for immunoprecipitation was adjusted to give equal trichloroacetic acid counts. To study the effects of AKAP-PKA disruption (25), 10 μg of myristylated HT31 (Promega) was added to the cells for 15 min prior to epinephrine treatment.

RESULTS

When adipocytes were treated with epinephrine, LPL translation was inhibited (12), and the epinephrine-treated cell extract caused a gel shift when added to a [^{32}P]RNA fragment corresponding to LPL mRNA nucleotides 1512 to 1635 (Fig. 1*A*). To purify the RNA-binding protein, our methodology was designed to take advantage of the affinity of the RNA-binding protein for the 3′-UTR of LPL. When the epinephrine-treated cell extract was applied to a DEAE column, as described under "Materials and Methods," the RNA binding properties of the extract were predominantly found in the unbound fraction from the column. This material was then dialyzed against a low salt buffer (see "Materials and Methods"), and applied to a poly(U)-Sepharose column containing the relevant binding region of the 3′-UTR of the LPL mRNA. After washing the column extensively with the initial column buffer, increasing salt concentrations were applied, and the gradual elution of proteins was monitored by SDS-PAGE and colloidal blue staining.

Fig. 1 shows the stained gel of the column elution fractions from the 3′-UTR column. With progressive salt elution, we observed the appearance of a predominant protein that migrated at 30–35 kDa. Other less prominent bands were also

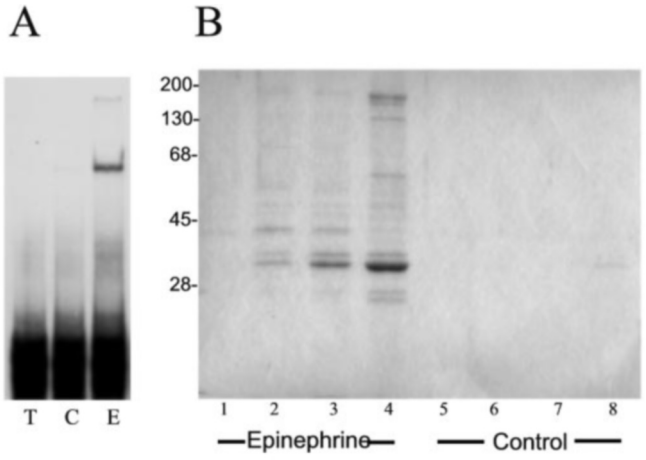

FIG. 1. **Proteins that bind to the 3′-UTR of the LPL mRNA.** *A*, gel shift assay. As described under "Materials and Methods," a cell extract was prepared from control (*C*), and epinephrine (*E*)-treated cells and added to the [^{32}P]RNA corresponding to the proximal LPL 3′-UTR, followed by analysis by nondenaturing polyacrylamide gel. *T*, transcript alone; *C*, transcript plus control cell extract; *E*, transcript plus epinephrine-treated cell extract. This figure is representative of eight similar experiments. *B*, proteins eluted from the LPL 3′-UTR column. As described under "Materials and Methods," the cell extract was precipitated with ammonium sulfate, passed through a DEAE-cellulose column, and then passed through a column containing nucleotides 1512 to 1635 of LPL mRNA. Fractions were then eluted off with progressively higher salt concentrations, analyzed by SDS-PAGE, and the gel was stained with colloidal blue. *Lanes 1*, *2*, *5*, and *6*, 100 mM KCl; *lanes 3* and *7*, 250 mM KCl; *lanes 4* and *8*, 500 mM KCl. This figure is representative of four experiments.

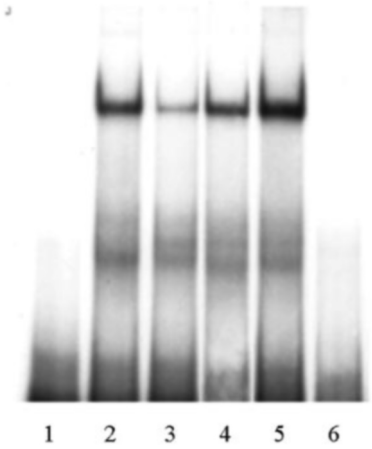

FIG. 2. **Effect of PKA C subunit on LPL RNA binding.** A 123-nucleotide [^{32}P]RNA fragment corresponding to the proximal 3′-UTR of LPL was incubated in the presence of either cell extract, followed by the addition of RNase. *Lane 1*, the transcript was incubated with control cell extract. *Lane 2*, epinephrine-treated cell extract. *Lane 3*, epinephrine-treated cell extract from which the C subunit was immunoprecipitated prior to incubation with the [^{32}P]RNA by the addition of 0.5 μg of anti-Cα antibody followed by the addition of protein A. *Lane 4*, same as *lane 3*, except for the addition of only 0.05 μg of anti-Cα antibody. *Lane 5*, same as *lane 3*, except for the addition of 5 units of activated PKA Cα following the immunoprecipitation. *Lane 6*, activated PKA Cα (5 units) was added to the [^{32}P]RNA transcript in the absence of cell extract. This figure is representative of three similar experiments.

apparent, mostly at higher molecular weights. A cytoplasmic extract from control cells, which had been through the same column procedures, demonstrated essentially no proteins eluting from the RNA binding column except for a faint 30–35-kDa band (Fig. 1, *lanes 5–8*). In addition, no proteins were eluted when an epinephrine-treated cell extract was passed through a column containing irrelevant RNA (data not shown). Because of our previous identification of a cross-linked band at about 30–35 kDa (12), the prominent 35-kDa band was cut from the wet gels, and subjected to sequence analysis of proteolytic peptides from this band. The results demonstrated the presence of several proteolytic fragments belonging to the C subunit of PKA. Peptides to aldolase and cyclophilin were also identified inconsistently. Because these are abundant cellular proteins and do not fit a known mechanism for LPL regulation, further studies were not pursued. There were no unassigned peptides.

Role of PKA Cα in Translation Inhibition—As a result of the elution of PKA C subunit from the LPL RNA affinity column, we sought additional evidence that this subunit was involved in the inhibition of LPL translation. To further characterize this interaction, and to obtain direct evidence for PKA C binding to the LPL mRNA, we performed a gel shift assay. A ^{32}P-labeled transcript corresponding to nucleotides 1512 to 1635 of the LPL mRNA was incubated with the control and epinephrine-treated cell extracts. As shown in Fig. 2, the cell extract from epinephrine-treated cells resulted in a gel shift (*lane 2*) when compared with the control extract (*lane 1*). To confirm the role of PKA C subunit, we added anti-PKA Cα antibody to the epinephrine-treated cell extract, followed by protein A-agarose, to immunoprecipitate the PKA Cα subunit. This PKA Cα-depleted extract was then added to the [^{32}P]RNA transcript in a gel shift reaction. As shown in Fig. 2 (*lane 3*), there was a greatly reduced intensity of the shifted band, and the addition of less anti-PKA Cα antibody resulted in a greater intensity of the gel-shifted band (*lane 4*). The addition of irrelevant antibodies did not reduce the intensity of the shifted band (data not shown). To determine whether we could then restore RNA binding, we added active PKA Cα (0.5 units) back to the PKA Cα-depleted cell extract. Addition of Cα subunit after the immunoprecipitation restored and augmented the mobility gel shift (Fig. 2, *lane 5*). However, the addition of the active PKA Cα protein to the [^{32}P]RNA, in the absence of cell extract, did not cause a gel shift (*lane 6*), suggesting that the PKA Cα subunit is part of a binding complex that involves other proteins.

The above experiments demonstrate the involvement of PKA Cα in RNA binding, but do not necessarily imply inhibition of translation. As described previously, the cell extract from control adipocytes inhibited LPL translation *in vitro* when compared with the addition of no extract, and epinephrine-treated cell extract yielded a much greater inhibition of LPL translation (11, 12). To determine whether the PKA Cα subunit is involved in translation inhibition, antibodies to PKA Cα were added to the *in vitro* translation reaction containing the LPL mRNA and cell extracts. As shown in Fig. 3, the cytoplasmic extract from epinephrine-treated cells inhibited LPL translation *in vitro* (*lane 2*). However, when the PKA Cα subunit was immunoprecipitated from the epinephrine-treated cell extract, the inhibition of LPL translation was abolished (*lane 4*). Indeed, the *in vitro* translation of LPL was increased in both the control and epinephrine-treated cell extracts after depletion of PKA Cα, which likely reflected the constitutive presence of the PKA Cα subunit. Addition of irrelevant antibody had no effect on the proportional change in LPL translation because of control and epinephrine extracts, although there was a small nonspecific decrease in translation in both *lanes 5* and *6* (Fig. 3). Thus, these data suggested that PKA Cα subunit was involved in LPL translation inhibition.

Other Components of the RNA Binding Complex—These data indicated that the Cα subunit was not by itself sufficient to cause a gel shift or inhibit translation, and suggested the presence of other proteins as part of a complex. PKA regulatory subunit was not detected by Western blotting of the column

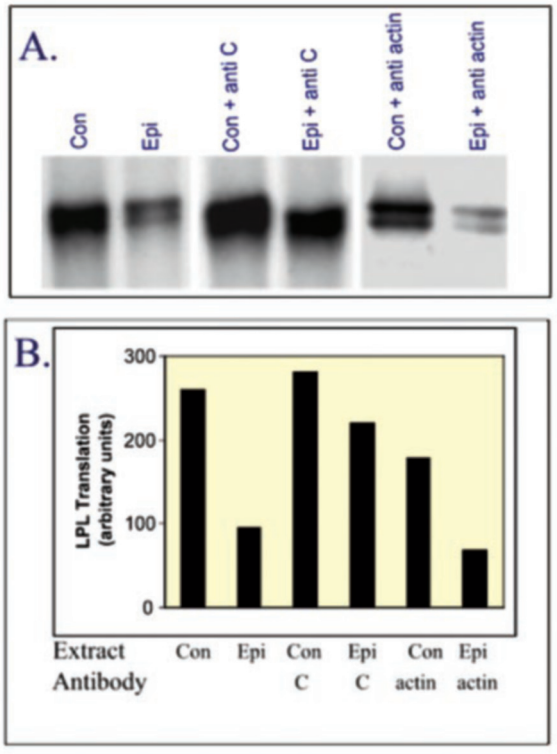

FIG. 3. **Effect of antibody to PKA C subunit on LPL translation *in vitro*.** Cytoplasmic extracts were prepared, as described under "Materials and Methods," and added to the *in vitro* translation reaction in the presence of LPL mRNA (nucleotides 1–2435). *Con* and *Epi* refers to the addition to the *in vitro* translation reaction of the cytoplasmic extracts from control or epinephrine-treated 3T3-F442A adipocytes. *Anti-C* and *anti-actin* refers to the addition of antibody to either PKA Cα or actin (irrelevant antibody). *A*, SDS gels of the *in vitro* translation reactions. This figure is representative of three experiments. *B*, *bar graph* demonstrating the relative density of each reaction.

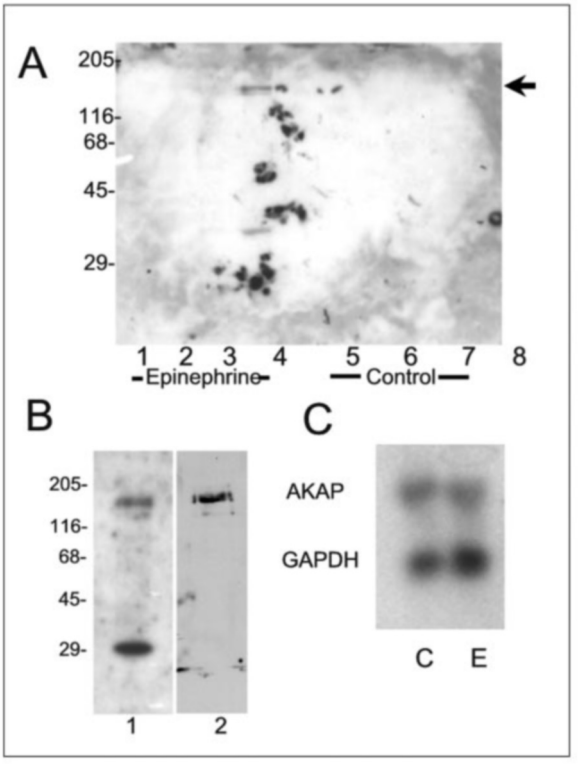

FIG. 4. **Identification of AKAP121/149 as an LPL RNA binding protein.** *A*, from both control and epinephrine-treated cells, a cell extract was prepared as described for Fig. 1, and then passed through a column containing nucleotides 1512 to 1635 of LPL mRNA. Fractions were then eluted off the LPL RNA column with progressively higher salt concentrations, analyzed by SDS-PAGE, and then blotted with specific antibodies to AKAP121/149. The lanes are the same as in Fig. 1: *lanes 1*, *2*, *5*, and *6*, 100 mM KCl; *lanes 3* and *7*, 250 mM KCl; *lanes 4* and *8*, 500 mM KCl. *Arrow* marks the migration of the AKAP121/149 band. *B*, a cell extract was prepared from epinephrine-treated cells as described above, and eluted off the LPL 3′-UTR column with 500 mM KCl, dialyzed, and subjected to SDS-PAGE. *Lane 1*, this fraction was blotted with the ^{32}P-labeled PKA RII subunit as a ligand blot. *Lane 2*, this fraction was Western blotted with anti-phosphoserine antibodies. *C*, Northern blot from control (*C*) and epinephrine (*E*)-treated adipocytes.

eluate from the epinephrine-treated cell extract (data not shown). Indeed, the R subunit was tightly bound to the DEAE column, and was not present in either the flow-through or the high salt wash. However, one class of proteins that is known to anchor PKA through the regulatory subunit is the AKAPs. To determine whether AKAPs were involved in the RNA binding complex, we used the ^{32}P-labeled PKA regulatory subunit to perform ligand blotting of the elute from the LPL RNA affinity column described in Fig. 1. As shown in Fig. 4*B* (*lane 1*), a ligand blot of the proteins eluted off the column at 500 mM KCl demonstrated two bands: the expected band at 30 kDa, which represented the C subunit, and a band that migrated with a molecular mass between the markers at 116 and 205 kDa.

AKAP121/149 is a member of the AKAP family of PKA-binding proteins that are notable for a consensus KH domain (26, 27), which is found in many known RNA-binding proteins (28). To determine whether the slower migrating band from the ligand blot was AKAP121/149, we performed Western blotting with specific antibodies to AKAP121/149. As shown in Fig. 4*A*, the anti-AKAP121/149 antibodies identified the same protein that was identified in the ligand blot. In addition, the anti-AKAP antibodies did not detect any AKAP121/149 in the 3′-UTR column eluate from the control cell extract fraction, or from the lower salt eluate from the epinephrine-treated cells. A negative result was obtained when the blot was probed with antibodies to AKAP150, which has no C-terminal homology to AKAP121/149, but which has a similar migration (data not shown). Hence, AKAP121/149 and the PKA Cα subunit co-eluted from the 3′-UTR LPL mRNA column. The PKA kinase activity has numerous targets, and we performed Western blots with antiphosphoserine antibodies to determine whether any proteins from the 3′-UTR LPL mRNA column were phosphorylated. As shown in Fig. 4*B*, the same band identified as AKAP121/149 was also identified by the antiphosphoserine antibodies, suggesting that AKAP121/149 is phosphorylated. Antiphosphoserine antibodies identified no other proteins from the 3′-UTR column, and identified no proteins from the control cell extract column (data not shown). The Cα subunit was present, as described above, but was not detected with antiphosphoserine antibodies, suggesting that it became dephosphorylated during the purification.

PKA Cα and AKAP121/149 Are Involved in LPL Translation Inhibition—To determine whether AKAP is functionally involved with the inhibition of LPL translation, we used HT31 to inhibit cellular AKAP-PKA binding. HT31 is derived from the consensus peptide motif on AKAPs that bind to the R subunit of PKA (25). LPL was immunoprecipitated from cells treated with myristylated HT31 with and without the presence of epinephrine, and labeled with [^{35}S]methionine. As shown in Fig. 5, epinephrine inhibited LPL synthesis, and this inhibition was disrupted by HT31. Indeed, the translation of LPL was up-regulated in both control and epinephrine-treated cells, suggesting that AKAPs are involved in a constitutive inhibition of LPL even in control cells. Hence, this experiment demonstrated

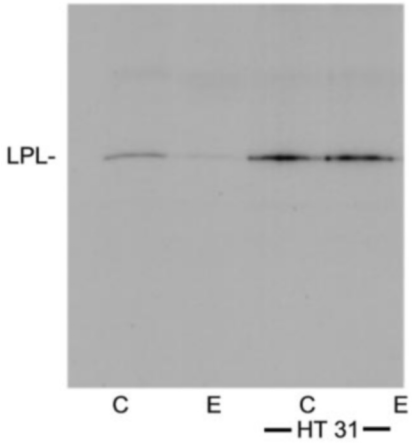

FIG. 5. **Effect of inhibition of AKAP-PKA binding on epinephrine-mediated inhibition of LPL translation.** As described under "Materials and Methods," both control and epinephrine-treated 3T3-F442A adipocytes were treated with HT 31 prior to pulse labeling with [^{35}S]methionine. LPL was immunoprecipitated from the cells with specific antibody. *C*, control cells. *E*, epinephrine-treated cells. This figure is representative of two experiments.

a functional role of the AKAPs in LPL translation inhibition by epinephrine.

To determine whether PKA Cα and AKAP149/121 were associated with each other as a complex, co-precipitation experiments were performed. Cell lysates from epinephrine-treated adipocytes were immunoprecipitated with either anti-Cα antibodies or anti-AKAP149/121 antibodies, followed by either Western blotting with the other antibody or ligand blotting with ^{32}P-regulatory subunit. No association between AKAP121/149 and C subunit was detected using this method (data not shown). Thus, both PKA Cα and AKAP149/121 appeared to be binding to the LPL 3′-UTR, but were not associated with each other.

The kinase activity of the PKA C subunit may be important in mediating LPL mRNA binding. To determine whether PKA Cα kinase activity was important, we treated cells with H89 (10 μM), which is a specific inhibitor of the Cα ATP binding site (15). This treatment would permit cAMP mediated release of the Cα subunit, but the subunit would be catalytically inactive. As shown in Fig. 6, a gel-shifted band was present in epinephrine-treated cells (a weaker gel-shifted band was present in control cells, which likely represents a small amount of baseline PKA activation). The addition of H89 to the cells 15 min prior to epinephrine treatment resulted in a reduced ability to form an RNA binding complex, as illustrated by the diminished gel shift associated with the epinephrine-treated cell extract (Fig. 6). Thus, PKA Cα kinase activity was necessary for the formation of the gel-shift RNA binding complex.

To further examine the effects of PKA kinase inhibition, *in vitro* translation experiments were performed with the cell extracts from control, epinephrine-treated, and H89-epinephrine-treated cells. The *in vitro* translation reactions were allowed to proceed for increasing time periods from 10 to 35 min. As shown in Fig. 7, all the cell extracts yielded some inhibition of translation when compared with the addition of no extract. This is consistent with the constitutive presence of the RNA binding complex. The epinephrine-treated cell extract yielded the most inhibition of LPL translation, and extract from cells treated with both epinephrine and H89 demonstrated *in vitro* translation that was similar to that of control cells. Thus, these data suggest that PKA kinase activity is important to both RNA binding, and translation.

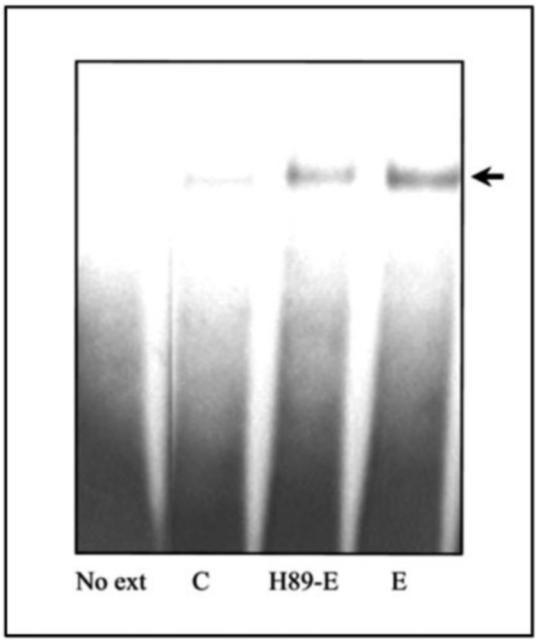

FIG. 6. **Effect of H89, an inhibitor of Cα kinase activity, on RNA binding complex formation.** ^{32}P-RNA corresponding to the 3′-UTR of LPL was incubated with control (*C*) and epinephrine (*E*)-treated cell extract from cells that had been treated with H89 (H89-E, 10 mM), followed by a gel-shift experiment as described under "Materials and Methods." *Arrow* indicates the shifted band from the RNA binding. These data are representative of three similar experiments.

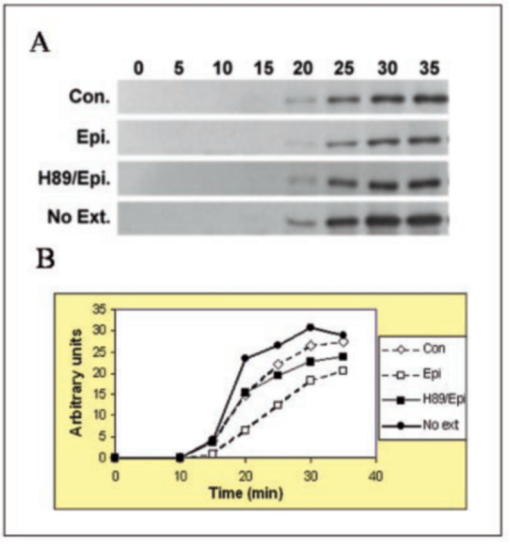

FIG. 7. **LPL *in vitro* translation in the presence of H89.** *In vitro* translation reactions using LPL mRNA were performed in the absence and presence of cell extracts from control (*Con*), epinephrine (*Epi*), and epinephrine-H89 treated cells, as described under "Materials and Methods." *A*, *in vitro* translation reactions were allowed to proceed for the indicated times. *B*, densitometric analysis of the image in *A*. These data are representative of two experiments.

DISCUSSION

LPL is an important enzyme in adipocyte biology and is highly regulated in response to numerous physiologic conditions and hormones (7). The mechanism of LPL regulation is complex, and may occur at the level of transcription, post-translational processing, or translation (23, 29–31). Changes in LPL translation have been demonstrated in response to the addition of epinephrine (10), glucose (32), and thyroid hormone (10). In addition, adipocyte LPL is translationally repressed in both humans and rats with diabetes (33–35). In previous stud-

ies, we examined the inhibition of LPL translation by epinephrine, and found that the important region of the LPL mRNA was in the first 24 nucleotides of the 3′-UTR (11). The identification of this region of the 3′-UTR was accomplished by *in vitro* translation with different RNA constructs, along with RNase protection assays, and transient transfection experiments (12). Subsequent studies of UV cross-linking the LPL mRNA binding region to an epinephrine-treated cell extract identified a 30-kDa protein as a likely candidate for the regulatory RNA-binding protein (12). The studies described in this paper were performed to better characterize the protein(s) involved in binding to the LPL 3′-UTR.

The initial identification of the RNA-binding protein utilized an RNA affinity column containing 123 nucleotides of the LPL RNA that is involved in translational regulation. The predominant protein that eluted from this column was at 30 kDa, and sequencing determined that this protein was the Cα subunit of PKA. The presence of PKA Cα subunit in the column eluate does not by itself demonstrate a role in LPL translational regulation. However, further experiments demonstrated that removal of the Cα subunit prevented translation inhibition *in vitro*, and prevented the gel shift caused by the epinephrine-treated cell extract. Thus, the presence of the Cα subunit of PKA in the cell extract was not coincidental, but was important to translational regulation. Although removal of Cα subunit from the extract removed the translation inhibition, and diminished the gel shift, the addition of the Cα subunit to purified LPL mRNA did not cause a gel shift. Thus, the Cα subunit by itself was not sufficient to cause translation inhibition. This information, along with the presence of other proteins in the 3′-UTR column elution, suggests the presence of an RNA binding complex.

Because of this evidence for an RNA binding complex, we examined the epinephrine-treated cell extract for other PKA-associated proteins. Using both a ligand blot and specific antibodies, we identified AKAP121/149 as another component of the RNA binding complex from epinephrine-treated cells. Treatment of cells with Ht31 eliminated epinephrine-mediated inhibition of LPL translation, indicating that the linkage of PKA to AKAPs was critical to LPL physiologic regulation.

AKAPs include several families of PKA anchoring proteins, which function to immobilize PKA at specific intracellular locations (13, 36). All AKAPs contain a PKA R subunit binding site, along with a targeting domain that determines the subcellular location. AKAP149 and AKAP121 are part of a family of AKAPs that are expressed in germ cells, thyroid, heart, and skeletal muscles (26, 27, 37). The AKAPs 149 and 121 are highly homologous and both contain a consensus KH domain, giving these proteins potential RNA binding properties. However, no previous study has clearly linked AKAPs 149/121 to RNA binding, and no previous study has described AKAP149/121 expression in adipose tissue. AKAPs 149 and 121 are designated by their apparent molecular weight on protein gels, even though their predicted molecular weight is lower (13). Previous studies have suggested that AKAP149 is the human homologue of AKAP121 (19). The experiments described in this report involved 3T3-F442A adipocytes, which is a mouse cell line. Hence, one would expect to find AKAP121 in these cells, rather than AKAP149. Although we cannot be certain which member of the AKAP family has been identified, the AKAP species migrates at ~121 kDa and interacts with R subunit in the ligand blot and antibodies to AKAP121/149, and we have referred to this protein as AKAP149/121.

Because the inactivation of Cα kinase activity with H89 prevented the gel shift with the LPL mRNA, Cα subunit kinase activity was important, perhaps through phosphorylation of AKAP121/149, which was also phosphorylated. These data are most consistent with a complex role for PKA Cα subunit in the regulation of LPL translation, where the protein is part of a multimeric RNA binding complex involving AKAP121/149, and perhaps other proteins which have not yet been characterized, and whose RNA binding activity is dependent on PKA kinase activity. As shown in Fig. 8, the PKAs consist of a heterotetrameric holoenzyme containing two C subunits bound to a regulatory R subunit dimer (13, 38, 39). In the absence of cAMP, the heterodimer is inactive, and upon binding of two molecules of cAMP to each R subunit, the two C subunits are released to phosphorylate serine and threonine residues on many different protein substrates. There are numerous isozymes of PKA because of different R and C subunits, and adipose tissue expresses mainly the RIIβ, Cα, and Cβ 1 subunits (40). AKAPs are important in directing PKA activity to specific cellular sites, and likely play additional roles in coordinating PKA tissue-specific functions. Our data suggest that AKAP149/121 and PKA Cα are both involved in the LPL RNA binding complex. However, co-precipitation experiments indicated that the C subunit was not directly associated with AKAP. It is possible that AKAP149/121 and the C subunit bind to different regions of the LPL RNA. Alternatively, another protein may be involved in the RNA binding complex. In addition, the relatively large amount Cα that was detected on the colloidal blue-stained gel (Fig. 1) may suggest that multiple copies of C subunit are involved in the complex. Finally, the PKA regulatory subunit was not eluted from the LPL 3′-UTR column, suggesting that the AKAP became separated from the PKA complex, or perhaps was lost in the purification scheme.

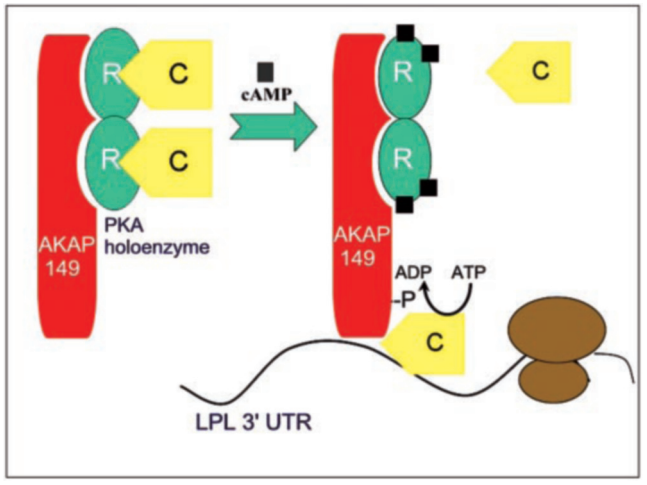

FIG. 8. **Conceptualization of C subunit and AKAP121/149 involvement with LPL translational regulation.**

Although no previous study has demonstrated PKA mediated RNA binding, there are numerous instances of PKA mediated stimulation of RNA-binding proteins. Previous studies have demonstrated cAMP-mediated activation of RNA-binding proteins that control mRNA stability through binding to the 3′-UTR of target RNAs, including Glut1, the Na^+/glucose cotransporter, lactate dehydrogenase, and phosphoenolpyruvate carboxykinase (41–45). In many instances, the cAMP-dependent RNA-binding protein binds to an AU-rich region on the target mRNA, although there are no clear homologies or consensus sequences among either the cis-acting RNA elements or the trans-acting binding proteins.

In our experiments, some gel-shift product was also present in control (no epinephrine treatment) cells. This suggests a constitutive expression of PKA Cα in the cells, and is consistent with previous studies by us and others. In a previous study, we

found that adipocytes from hypothyroid rats demonstrated increased LPL translation because of the absence of a constitutive LPL translation inhibitor (46). The hypothyroid state is known to decrease catecholamine sensitivity (47), which would be predicted to decrease cAMP stimulation of PKA. Thus, the LPL RNA inhibitor in these previous studies was likely a low level of constitutive PKA Cα, which was then decreased in the hypothyroid adipocytes.

In adipose tissue, hormones that cause elevated cAMP (β-adrenergic agonists, ACTH, and glucagon) result in the activation of PKA, which then reversibly phosphorylates HSL (2, 3), resulting in adipocyte lipolysis and the release of nonesterified fatty acids from adipocytes. Lipolysis is the physiologic reverse reaction to LPL-mediated triglyceride accumulation, and therefore PKA C is an appropriate candidate for an LPL-inhibitory signal. These data would suggest that cAMP stimulation results in a coordinated response to rapidly promote release of adipocyte lipid and inhibition of lipogenesis through post-transcriptional mechanisms.

Acknowledgments—We are pleased to acknowledge Annadell Fowler, Linda Bates, and Rami Kaakaji for technical assistance and Sarah Dunn for secretarial assistance.

REFERENCES

1. Goldberg, I. J. (1996) *J. Lipid Res.* **37,** 693–707
2. Stralfors, P., Bjorgell, P., and Belfrage, P. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 3317–3321
3. Holm, C., Langin, D., Manganiello, V., Belfrage, P., and Degerman, E. (1997) *Methods Enzymol.* **286,** 45–67
4. Osuga, J., Ishibashi, S., Oka, T., Yagyu, H., Tozawa, R., Fujimoto, A., Shionoiri, F., Yahagi, N., Kraemer, F. B., Tsutsumi, O., and Yamada, N. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 787–792
5. Kelley, D. E., and Simoneau, J. A. (1994) *J. Clin. Invest.* **94,** 2349–2356
6. Unger, R. H. (1995) *Diabetes* **44,** 863–870
7. Eckel, R. H. (1987) in *Lipoprotein Lipase* (Borensztajn, J., ed) pp. 79–132, Evener, Chicago
8. Londos, C., Brasaemle, D. L., Schultz, C. J., Adler-Wailes, D. C., Levin, D. M., Kimmel, A. R., and Rondinone, C. M. (1999) *Ann. N. Y. Acad. Sci.* **892,** 155–168
9. Ramsay, T. G. (1996) *Endocrinol. Metab. Clin. North Am.* **25,** 847–870
10. Ong, J. M., Saffari, B., Simsolo, R. B., and Kern, P. A. (1992) *Mol. Endocrinol.* **6,** 61–69
11. Yukht, A., Davis, R. C., Ong, J. M., Ranganathan, G., and Kern, P. A. (1995) *J. Clin. Invest.* **96,** 2438–2444
12. Ranganathan, G., Vu, D., and Kern, P. A. (1997) *J. Biol. Chem.* **272,** 2515–2519
13. Feliciello, A., Gottesman, M. E., and Avvedimento, E. V. (2001) *J. Mol. Biol.* **308,** 99–114
14. Wion, K. L., Kirchgessner, T. G., Lusis, A. J., Schotz, M. C., and Lawn, R. M. (1987) *Science* **235,** 1638–1641
15. Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Toshioka, T., and Hidaka, S. (1990) *J. Biol. Chem.* **265,** 5267–5272
16. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
17. Furusawa, M., Ohnishi, T., Taira, T., Iguchi-Ariga, S. M., and Ariga, H. (2001) *J. Biol. Chem.* **276,** 36647–36651
18. Tso, J. Y., Sun, X. H., Kao, T. H., Reece, K. S., and Wu, R. (1985) *Nucleic Acids Res.* **13,** 2485–2502
19. Chen, Q., Lin, R. Y., and Rubin, C. S. (1997) *J. Biol. Chem.* **272,** 15247–15257
20. Ranganathan, G., Kaakaji, R., and Kern, P. A. (1999) *J. Biol. Chem.* **274,** 9122–9127
21. Hausken, Z. E., Coghlan, V. M., and Scott, J. D. (1997) in *Protein Targeting Protocols* (Clegg, R. A., ed) pp. 47–64, Humana Press, Totowa, NJ
22. Xie, G., and Raufman, J. P. (2001) *Am. J. Physiol.* **281,** G1051–G1058
23. Ong, J. M., Kirchgessner, T. G., Schotz, M. C., and Kern, P. A. (1988) *J. Biol. Chem.* **263,** 12933–12938
24. Goers, J. F., Petersen, M. E., Kern, P. A., Ong, J., and Schotz, M. C. (1987) *Anal. Biochem.* **166,** 27–35
25. Carr, D. W., Hausken, Z. E., Fraser, I. D., Stofko-Hahn, R. E., and Scott, J. D. (1992) *J. Biol. Chem.* **267,** 13376–13382
26. Lin, R. Y., Moss, S. B., and Rubin, C. S. (1995) *J. Biol. Chem.* **270,** 27804–27811
27. Trendelenburg, G., Hummel, M., Riecken, E. O., and Hanski, C. (1996) *Biochem. Biophys. Res. Commun.* **225,** 313–319
28. Ashley, C. T. J., Wilkinson, K. D., Reines, D., and Warren, S. T. (1996) *Science* **262,** 563–566
29. Ong, J. M., Simsolo, R. B., Saffari, B., and Kern, P. A. (1992) *Endocrinology* **130,** 2310–2316
30. Ong, J. M., and Kern, P. A. (1989) *J. Clin. Invest.* **84,** 305–311
31. Doolittle, M. H., Ben-Zeev, O., Elovson, J., Martin, D., and Kirchgessner, T. G. (1990) *J. Biol. Chem.* **265,** 4570–4577
32. Ong, J. M., and Kern, P. A. (1989) *J. Biol. Chem.* **264,** 3177–3182
33. Simsolo, R. B., Ong, J. M., Saffari, B., and Kern, P. A. (1992) *J. Lipid Res.* **33,** 89–95
34. Tavangar, K., Murata, Y., Pedersen, M. E., Goers, J. F., Hoffman, A. R., and Kraemer, F. B. (1992) *J. Clin. Invest.* **90,** 1672–1678
35. Ranganathan, G., Li, C., and Kern, P. A. (2000) *J. Biol. Chem.* **275,** 40986–40991
36. Diviani, D., and Scott, J. D. (2001) *J. Cell Sci.* **114,** 1431–1437
37. Feliciello, A., Rubin, C. S., Avvedimento, E. V., and Gottesman, M. E. (1998) *J. Biol. Chem.* **273,** 23361–23366
38. Walsh, D. A., Perkins, J. P., and Krebs, E. G. (1968) *J. Biol. Chem.* **243,** 3763–3765
39. Francis, S. H., and Corbin, J. D. (1999) *Crit. Rev. Clin. Lab. Sci.* **36,** 275–328
40. McKnight, G. S., Cummings, D. E., Amieux, P. S., Sikorski, M. A., Brandon, E. P., Planas, J. V., Motamed, K., and Idzerda, R. L. (1998) *Recent Prog. Horm. Res.* **53,** 139–159
41. Tian, D., Huang, D., Brown, R. C., and Jungmann, R. A. (1998) *J. Biol. Chem.* **273,** 28454–28460
42. Stephens, J. M., Carter, B. Z., Pekala, P. H., and Malter, J. S. (1992) *J. Biol. Chem.* **267,** 8336–8341
43. Hod, Y., and Hanson, R. W. (1988) *J. Biol. Chem.* **263,** 7747–7752
44. Christ, B., Heise, T., and Jungermann, K. (1991) *Biochem. Biophys. Res. Commun.* **177,** 1273–1282
45. Peng, H., and Lever, J. E. (1995) *J. Biol. Chem.* **270,** 23996–24003
46. Kern, P. A., Ranganathan, G., Yukht, A., Ong, J. M., and Davis, R. (1996) *J. Lipid Res.* **37,** 2332–2340
47. Bilezikian, J. P., and Loeb, J. N. (1983) *Endocr. Rev.* **4,** 378–396