Post-translational Regulation of Perilipin Expression

STABILIZATION BY STORED INTRACELLULAR NEUTRAL LIPIDS

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The perilipins are a family of polyphosphorylated proteins found exclusively surrounding neutral lipid storage droplets in adipocytes and steroidogenic cells. In steroidogenic cells, the cholesterol ester-rich lipid storage droplets are encoated with perilipins A and C. This study describes the dependence of perilipin levels on neutral lipid storage in cultured Y-1 adrenal cortical cells. The addition of fatty acids and cholesterol to the culture medium of Y-1 adrenal cortical cells greatly increased the storage of cholesterol esters and triacylglycerols concomitant with the formation of many new lipid storage droplets. The addition of fatty acids to the culture medium also produced a transient 6-fold increase in levels of perilipin A, but not C, mRNA, while much larger and stable increases in both perilipin A and C proteins were observed. The increases in perilipin protein levels were dependent upon the metabolism of fatty acids to triacylglycerol or cholesterol esters, since the incubation of cells with bromopalmate, a poorly metabolized fatty acid, failed to yield large increases in lipid content or perilipin levels. Constitutive expression of epitope-tagged perilipins in transfected Y-1 adrenal cortical cells was regulated by lipid similarly to expression of the endogenous perilipins despite an absence of untranslated perilipin mRNA sequences in the expression constructs. Epitope-tagged perilipin A mRNAs were efficiently loaded with polyribosomes whether or not fatty acids were added to the culture medium; therefore, the increase in perilipin levels in the presence of fatty acids is likely due to factors other than increased translational efficiency. We suggest that the large increase in cellular perilipin levels upon lipid loading of cells is the result of post-translational stabilization of newly synthesized perilipins by stored neutral lipids.

Many cells of the body, including steroidogenic cells, store excess cholesterol as cholesterol esters in lipid storage droplets. Steroidogenic cells use this stored cholesterol as a substrate for steroid hormone synthesis (1–3) or, as with other cells, for membrane synthesis (4). We have recently shown that the lipid storage droplets of steroidogenic cells are surrounded by perilipins (Ref. 5),1 a family of phosphorylated proteins encoded by a single gene and detected thus far only in adipocytes and steroidogenic cells. Steroidogenic cells such as Y-1 adrenal cortical cells have a different distribution of the perilipin isoforms from adipocytes; while adipocytes express predominantly perilipin A, with smaller amounts of perilipin B, Y-1 adrenal cortical cells express primarily perilipin A, with smaller amounts of a unique isoform, perilipin C, and trace quantities of perilipin B (5). Although the functions of the perilipins have yet to be determined, we propose a role in lipid metabolism based on the unique tissue distribution, subcellular localization, and metabolic properties. All cell types expressing the perilipins have a common mechanism of lipid hydrolysis; extracellular hormones stimulate production of cAMP, thus activating cAMP-dependent protein kinase. cAMP-dependent protein kinase catalyzes the phosphorylation of cholesterol ester hydrolase in steroidogenic cells or hormone-sensitive lipase in adipocytes; moreover, these hydrolytic enzymes are probably identical (6–8). Phosphorylation of the lipase facilitates its translocation to the surface of the lipid storage droplet (9),2 where hydrolysis of triacylglycerols and cholesterol esters occurs. cAMP-dependent protein kinase also mediates polyphosphorylation of the perilipins located in the limiting phospholipid monolayer surrounding the lipid storage droplet (10, 11), although the role of this event in lipid metabolism is unknown.

The expression of the perilipins is closely linked to the storage of neutral lipids in adipocytes and steroidogenic cells. The perilipins are found surrounding the earliest detectable deposits of triacylglycerols in differentiating 3T3-L1 adipocytes.3 Inhibition of triacylglycerol deposition by biotin depletion of culture media concomitantly inhibits perilipin accumulation in differentiating 3T3-L1 adipocytes; fatty acid supplementation of these biotin-depleted culture media restores triacylglycerol synthesis and perilipin accumulation.3 The current study addresses the relationship between perilipin expression and neutral lipid storage in cultured Y-1 adrenal cortical cells. Y-1 adrenal cortical cells are a particularly attractive model for studying lipid regulation of perilipin expression, since they are maintained in culture as fully differentiated cells expressing lipid storage droplet proteins. The present study demonstrates that stored intracellular neutral lipids regulate levels of perilipins A and C in Y-1 adrenal cortical cells primarily by a post-translational mechanism.

EXPERIMENTAL PROCEDURES

Materials

Horse serum, fetal bovine serum, and fatty acid-free bovine serum albumin were purchased from Intergen. TRIZol and actinomycin D were purchased from Life Technologies, Inc. Cholesterol and oleic acid were purchased from Calbiochem; bromopalmate, cycloheximide, heparin,


and dithiothreitol were purchased from Sigma. PRIME RNase inhibitor was purchased from 5 Prime → 3 Prime (Boulder, CO). Ammonium sulfate-impregnated silica gel H thin layer chromatography plates were purchased from Analtech. The 12CA5 monoclonal antibody raised against an epitope from the hemagglutinin protein of influenza virus was purchased from BAbCo (Richmond, CA). The cDNA probe for c-myc (probe S107) was kindly provided by Dr. Beverly Mock; the cDNA probe for histone H4 was kindly provided by Dr. Hitoshi Kurumizaka.

Methods

Cell Culture and Fractionation—Y-1 adrenal cortical cells (12) were cultured in 100-mm dishes, as described previously (5). Fatty acids were coupled to tryptophan-free bovine serum albumin at a molar ratio of 6:1:1 molar oleic acid to albumin; the final fatty acid concentration was 400 μM in culture media. Cholesterol was added to culture media as an ethanolic solution to a final concentration of 130 μM cholesterol in 0.5% ethanol. Cells were collected and fractionated for immunoblot analysis essentially as described previously (10) but using a Beckman tube slicer (catalog number 303811) to facilitate removal of the floating cholesterol ester droplet fractions with as little contaminating supernatant as possible. Lipid storage droplets were further purified by a second centrifugation step of 27,000 × g for 30 min. Proteins in the floating lipid droplet fractions were precipitated in cold acetone and solubilized in Laemmli sample buffer (13). Samples were resolved on SDS-polyacrylamide gels (10% acrylamide and 0.2% N,N'-methylene bisacrylamide) and transferred to nitrocellulose for immunoblotting. Sample loads were equalized by loading lipid storage droplet fractions from equivalent cell numbers determined by DNA quantitation (14).

Immunoblotting—Polyclonal antibodies with reactivity against full-length perilipin A were affinity-purified from rabbit antisera raised against perilipin A purified from fat cakes of primary rat adipocytes (10). These antibodies also recognize perilipin C. Immunoblots were developed using enhanced chemiluminescence procedures with reagents from Amersham Corp. or Pierce.

Lipid Analysis—Cellular lipid content was determined by extracting cells with 2:1 chloroform:methanol (15) and spotting lipid extracts onto ammonium sulfate-impregnated silica gel H thin layer chromatography plates. Plates were developed in 90:10:1 hexane:diethyl ether:formic acid and charred at 160 °C for 1–2 h. Spots corresponding to the various lipid classes were quantitated by densitometry using a Molecular Dynamics computing densitometer; relative spot densities were calculated using ImageQuant software (Molecular Dynamics) and compared with lipid standards resolved on the same plates.

Northern Blot Analysis—RNA was extracted from cultured Y-1 adrenal cortical cells with TRIzol according to the protocol of the manufacturer. Total RNA was electrophoresed on 1% agarose gels containing formaldehyde, and the RNA was transferred to nitrocellulose or Hybond N+ (Amersham Corp.). High stringency Northern blot analysis was performed as described previously (16) using a 32P-labeled cDNA probe corresponding to the full-length coding region of perilipin A; this probe recognizes perilipin A, B, C, and D, the four murine perilipin mRNAs (5). Other cDNA probes included probes for c-myc and histone H4 where noted.

Expression of Epitope-tagged Perilipin A in Y-1 Adrenal Cells—The polylinker sequence of pCMV (Invitrogen) was removed between the HindIII and ApoI restriction sites. Cassettes containing the nucleotide sequence for the 9 amino acids of the influenza virus hemagglutinin protein epitope recognized by the 12CA5 monoclonal antibody (17) 5' or 3' to a new polylinker containing HpaI, SacI, BanEl, NolI, and XhoI restriction sites were inserted into the cut vector using 5' HindIII and 3' ApoI restriction sites. The coding nucleotide sequence of perilipin A was amplified by the polymerase chain reaction using oligonucleotide primers containing HpaI (for 5' "sense" primer) or XhoI (for 3' "anti-sense" primer) restriction site sequences. The amplified product was cleaved with HpaI and XhoI and ligated into the appropriately cleaved vectors to create expression vectors encoding perilipin A with epitope tag sequences on either the amino or the carboxyl terminus. The fidelity of amplification of perilipin A cDNA by Vent DNA polymerase (New England BioLabs) was confirmed by dyeoxy sequencing with Sequenase (U.S. Biochemical Corp.).

DNA Transfections of Cells—Plasmids containing the epitope-tagged perilipin A sequences were transfected into Y-1 adrenal cells by electroporation. Stable transfecnts were selected with 0.3 or 0.6 mg/ml of active genetin (Life Technologies, Inc.), and cultures were maintained in 0.3 mg/ml active genetin. Polysonome Profiles—Polysonome profiles (18, 19) were obtained from 10–50% sucrose gradients of postmitochondrial supernatants of control and lipid-loaded Y-1 adrenal cells expressing perilipin A epitope-tagged at the carboxyl terminus. RNA extracts from equal volumes of fractions from the sucrose gradients were subjected to high stringency Northern blot analysis.

Immunofluorescence Microscopy—Cells were prepared for immunofluorescence microscopy (20) and stained with affinity-purified polyclonal antibodies against perilipin A and/or the 12CA5 monoclonal antibody against the epitope tag. Rhodamine-conjugated second antibodies (Jackson ImmunoResearch Laboratories, Inc.) were used to visualize perilipin staining, and fluorescein-conjugated second antibodies were used to visualize the epitope tag sequence.

Neutral lipids were visualized by staining paraformaldehyde-fixed cells for 10 min with 0.01% Nile Red (Molecular Probes, Inc., Eugene, OR) in phosphate-buffered saline. Cells were viewed with a Nikon Optiphot microscope equipped with a Bio-Rad MRC-1024 confocal imaging system (20).

RESULTS

Incubation of Y-1 Adrenal Cortical Cells with Free Fatty Acids and Cholesterol Dramatically Increases Intracellular Lipid Storage—Densely subconfluent Y-1 adrenal cortical cells were incubated in culture medium containing 200 μM cholesterol ester contributed by the serum component or in medium supplemented with 130 μM cholesterol and 400 μM oleic acid coupled to fatty acid-free bovine serum albumin. By 48 h, the cellular content of neutral lipids, triacylglycerol, and cholesterol esters, increased by 3.5-fold when compared with cells incubated for the same period of time in unsupplemented culture medium (Table I). Although cholesterol esters increased modestly, the most dramatic change was the selective increase in triacylglycerol content; cells grown in medium supplemented with fatty acids and cholesterol showed a 12-fold increase in triacylglycerol content when compared with cells grown in unsupplemented medium. The selective increase in triacylglycerol content may have been due in part to more efficient uptake of fatty acids than of cholesterol by the cells. We did not attempt to increase the efficiency of cholesterol uptake using cholesterol-rich lipoproteins. Cells grown in medium supplemented with cholesterol alone showed increased cholesterol ester storage (data not shown), while cells grown in medium supplemented with fatty acids alone showed increased triacylglycerol storage (see Table II). Nile red staining of cells grown in medium supplemented with oleic acid and cholesterol showed many very small brightly stained lipid storage droplets compared with few stained lipid droplets in cells grown in culture medium without additions (Fig. 1). In contrast to adipocytes, which accumulate neutral lipid in large storage droplets during differentiation, coalescence of lipid droplets into successively

### Table I

<table>
<thead>
<tr>
<th>Lipid content of Y-1 adrenal cortical cells grown in culture medium without additions or with 400 μM oleic acid and 130 μM cholesterol for 48 h</th>
<th>Lipid content of Y-1 adrenal cortical cells grown in culture medium without additions, with 400 μM bromopalmitate, or with 400 μM oleate for 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth conditions</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>No added lipids</td>
<td>0.035 ± 0.004</td>
</tr>
<tr>
<td>With added lipids</td>
<td>0.411 ± 0.096</td>
</tr>
<tr>
<td>Oleate</td>
<td>0.771 ± 0.080</td>
</tr>
</tbody>
</table>

Lipid content data are presented as means ± S.D. in μg of lipid/μg of DNA (n = 4).
The addition of oleic acid and cholesterol to the culture medium of Y-1 adrenal cortical cells increases the number of minute lipid storage droplets. Y-1 adrenal cortical cells were incubated in culture medium without additions in A or in the same medium supplemented with 400 μM oleate and 130 μM cholesterol for 48 h in B. The cells were fixed with 3% paraformaldehyde and then stained with 0.01% Nile Red. A and B are confocal images. A, few fluorescent Nile Red-positive lipid droplets are present in cells cultured under normal conditions. B, fluorescent Nile Red-positive lipid droplets appear as clusters of minute spherical droplets in cells cultured with oleic acid and cholesterol. Scale bar, 10 μm.

The Expression of Perilipin Increases Concomitantly with Increased Storage of Neutral Lipids—Incubation of Y-1 adrenal cortical cells in medium supplemented with fatty acids and cholesterol led to very large increases in perilipins A and C (Fig. 2). While the total neutral lipid content increased by approximately 3.5-fold (triacylglycerol by 12-fold), large increases in perilipin mass were observed; densitometric scanning of immunoblots showed increases of at least 30-fold for perilipin C and more than 140-fold for perilipin A. The increased perilipin in the lipid-loaded cells was found almost exclusively in the floating fat cake fractions of homogenized cells. We have previously reported that centrifugation of Y-1 adrenal cortical cell homogenates fractionates the vast majority of perilipin in a floating fat cake with a minor amount of perilipin in the supernatant and membrane pellet.

The large increases in cellular perilipin levels with lipid loading and the localization of perilipin to lipid droplet surfaces were confirmed using immunofluorescence microscopy (Fig. 3). Cells grown in unsupplemented medium and stained with antibodies raised against perilipin showed few brightly stained lipid droplets arrayed singly or in small clusters of 2–6 droplets, while cells grown in medium supplemented with fatty acids and cholesterol showed large clusters of dozens of very small brightly stained lipid storage droplets. Staining of the perilipins appeared as very small rings around the perimeters of phase dense lipid storage droplets and nowhere else in the cells.

Synthesis of Triacylglycerols or Cholesterol Esters Is Required for the Large Increases in Cellular Perilipin Levels—To test whether fatty acids or their metabolic products are required for the increased expression of perilipins, α-bromopalmitate, a poorly metabolized fatty acid (21), was added to cultures of Y-1 adrenal cortical cells. Bromopalmitate has previously been shown to mimic fatty acid effects in regulating gene expression (21, 22) but provides a poor substrate for fatty acyl-CoA synthase; hence, it is minimally incorporated into triacylglycerols or cholesterol esters. In this study, bromopalmitate was less efficiently metabolized to triacylglycerols than oleate in cultured Y-1 adrenal cortical cells (Table II). Supplementation of culture medium with 400 μM bromopalmitate increased triacylglycerol storage in Y-1 adrenal cortical cells by less than 4-fold when compared with normal culture conditions, while 400 μM oleate increased triacylglycerol levels by greater than 10-fold over unsupplemented culture medium. Correspondingly, bromopalmitate supplementation of culture medium increased perilipin A protein levels slightly while failing to increase perilipin C levels; oleate addition to culture medium increased levels of both perilipins A and C dramatically (Fig. 4). The increases in perilipin levels with bromo-
FIG. 3. Increases in neutral lipid storage in Y-1 adrenal cortical cells are accompanied by increased staining for perilipins surrounding lipid storage droplets. Y-1 adrenal cortical cells depicted in A, B, and C were grown in culture medium without additions, while cells depicted in D, E, and F were grown in medium supplemented with 400 μM oleic acid and 130 μM cholesterol for 48 h. Cells were fixed in 3% paraformaldehyde and then stained with affinity-purified polyclonal antibodies raised against perilipin A. A, C, D, and F are fluorescent confocal images obtained with a rhodamine filter. B and E are phase contrast images of the cells depicted in A and D, respectively. A and D show perilipin immunostaining. In C and F, perilipin immunostaining can be resolved as rings surrounding minute lipid storage droplets arrayed singly and in small (C) or large (F) clusters. The scale bar in E applies to A, B, D, and E (10 μm). The scale bar in F applies to C and F (5 μm).
Stabilization of Perilipins by Stored Lipids

The metabolism of fatty acids is necessary for the large increases in levels of perilipins A and C that occur during lipid loading. The figure shows an immunoblot of lipid storage droplet proteins from Y-1 adrenal cortical cells grown for 48 h in culture medium without additions (left lane), with 400 μM bromopalmitate (center lane), or with 400 μM oleate (right lane). The immunoblot was probed with affinity-purified antibody raised against perilipin A. The strong doublet at approximately 60 kDa represents phosphorylation variants of perilipin A, while the band at approximately 42 kDa is perilipin C. The band induced by loading cells with oleate at 52 kDa is probably perilipin B, while the band in all lanes at 56 kDa is a nonspecific band. Proteins loaded in each lane are from lipid storage droplets from cells containing 500 μg of DNA.

Bromopalmitate - + -
Oleate - + +

FIG. 4. The metabolism of fatty acids is necessary for the large increases in levels of perilipins A and C that occur during lipid loading. The figure shows an immunoblot of lipid storage droplet proteins from Y-1 adrenal cortical cells grown for 48 h in culture medium without additions (left lane), with 400 μM bromopalmitate (center lane), or with 400 μM oleate (right lane). The immunoblot was probed with affinity-purified antibody raised against perilipin A. The strong doublet at approximately 60 kDa represents phosphorylation variants of perilipin A, while the band at approximately 42 kDa is perilipin C. The band induced by loading cells with oleate at 52 kDa is probably perilipin B, while the band in all lanes at 56 kDa is a nonspecific band. Proteins loaded in each lane are from lipid storage droplets from cells containing 500 μg of DNA.

Lipid loading of Y-1 adrenal cortical cells increases the levels of perilipins A and C. The figure depicts densitometric scanning data from the Northern blots in A. Sample load variation was normalized using the density of signals for glyceraldehyde-3-phosphate dehydrogenase. Open and closed circles are data for the perilipin A signal for cells incubated with oleic acid and cholesterol, respectively. Open and closed triangles are data for the perilipin C signal for cells incubated with oleic acid and cholesterol, respectively. Each data point is from a single sample out of duplicates; this experiment was repeated several times.

FIG. 5. Treatment of Y-1 adrenal cortical cells with oleic acid induces a transient increase in the levels of perilipin A mRNA. Y-1 adrenal cortical cells were grown in culture medium supplemented with 400 μM oleic acid (left side of A) or 130 μM cholesterol (right side of A). RNA was extracted at the times indicated. Northern blots of total RNA (15 μg/lane) were probed with a perilipin A cDNA probe (top portion of A) or a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (lower portion of A). Perilipins B and D are minor mRNAs in steroidogenic cells (5).

Lipid Loading of Y-1 Adrenal Cortical Cells Increases the Levels of Perilipins A and C

To determine whether the effect of fatty acids on perilipin A mRNA levels was due to changes in mRNA stability, we examined the decay of perilipin mRNA levels in the absence of continued RNA synthesis. Northern blot analysis of RNA samples from actinomycin D-treated cells revealed little decay of perilipin mRNAs from cells incubated in the presence or absence of oleic acid for 10 h (Fig. 6). By contrast, the degradation of a control (c-myc) mRNA showed a short half-life of less than 1 h (29). The perilipin A mRNA degradation rates for both lipid loaded and unsupplemented conditions were too low to account for the observed transient increase in perilipin A mRNA levels over 12 h. These data imply that fatty acid-induced increases in perilipin A mRNA levels are likely due to transcriptional control. However, consistent with the exceedingly low levels of perilipin mRNAs in steroidogenic cells (less than 0.01% of total mRNA; see Ref. 5), we were unable to reliably detect perilipin-specific hybridization to radiolabeled RNA synthesized in nuclei isolated from Y-1 adrenal cortical cells incubated with or without fatty acids.
At the beginning of the experiment, culture medium containing 10 μg/ml actinomycin D with no further additions (top portion of A) or with 400 μM oleic acid (middle portion of A) was added. RNA was extracted at the times indicated. Northern blots of total RNA (20 μg/lane) were probed with a perilipin A cDNA probe (A, top and middle portions). The lower portion of A shows the blot from the top portion of A (no supplementation) reprobed with a c-myc probe (probe S107) as a control for an mRNA with a short half-life. B depicts densitometric scanning data from the Northern blots in A. No corrections for sample load variability were made. Open circles are densitometry data for the perilipin signal for cells incubated without added lipids. Closed circles are densitometry data for the perilipin A signal for cells incubated with added oleic acid. Open squares are densitometry data for the c-myc signal for cells incubated without added lipids.

**Content of Epitope-tagged Perilipin A**—The above data indicated that fatty acids contribute to a post-transcriptional control mechanism. To address whether this additional mechanism was due to control of translation or a post-translational mechanism, we studied the effects of fatty acids on the expression of epitope-tagged perilipin A driven by a constitutive cytomegalovirus promoter. Perilipin A was expressed as a fusion protein with an epitope tag from the hemagglutinin protein of influenza virus added to the carboxyl terminus. These constructs were constitutively and stably expressed in Y-1 adrenal cortical cells using a vector containing the cytomegalovirus promoter and a selectable marker for neomycin resistance. Only the coding sequence of the perilipin A cDNA was used in preparing these constructs; hence, potential 5′ and 3′ regulatory elements were eliminated. The mRNA encoding the fusion protein was easily detected in stably transfected cells and was approximately 100-fold stronger than that of endogenous perilipin A mRNA in cells incubated in the absence of supplemental lipids. B shows an immunoblot of triplicate samples of lipid storage droplet proteins from Y-1 adrenal cortical cells expressing the perilipin fusion protein grown for 48 h in culture medium without additions (three left lanes) or supplemented with 400 μM oleic acid and 130 μM cholesterol (three right lanes). Immunoblots were probed with affinity-purified polyclonal antibodies raised against perilipin A. The triplet of bands at approximately 60 kDa represents overlapping doublets of phosphorylation variants of endogenous perilipin A (lower and middle band; indicated by closed triangles) and epitope-tagged perilipin (middle and upper band; indicated by open triangles; identified by reprobing the blot with the 12CA5 monoclonal antibody against the epitope tag). Proteins loaded on each lane are from lipid storage droplets from cells containing 500 μg of DNA. Additional exposure of this blot revealed faint perilipin signals in the lanes of proteins from cells grown in culture medium without added lipids (left three lanes).
Stabilization of Perilipins by Stored Lipids

The current study demonstrates a close relationship between cellular levels of stored neutral lipid and perilipins. Levels of perilipins in Y-1 adrenal cortical cells in culture increase dramatically with the addition of fatty acids and cholesterol to the culture medium. Furthermore, metabolism of fatty acids to triacylglycerols or cholesterol esters is required for the observed increases in perilipin levels. Thus, the abundance of perilipins in cells reflects the availability of substrates for neutral lipid synthesis and, hence, the amount of deposited neutral lipid.

A number of observations in the present study provide evidence that neutral lipids regulate perilipin levels via a post-transcriptional mechanism. Lipid loading of Y-1 adrenal cortical cells produces large increases in cellular levels of perilipin A and C proteins, while comparatively small increases are observed in levels of perilipin A mRNA: perilipin C mRNA is unchanged. Additionally, lipid-induced increases in epitope-tagged perilipin can be observed when the fusion protein ex-
pression is driven by a constitutive viral promoter. The perilipin cDNA in these constructs lacks all potential 5' or 3' untranslated regulatory sequences, and while levels of the exogenous transcript are unaffected by fatty acids, the fusion protein levels are acutely regulated by the availability of fatty acids. We have demonstrated that the metabolism of fatty acids to triacylglycerols or cholesterol esters is essential for the observed increases in perilipin levels; bromopalmitate, a poorly metabolized fatty acid, fails to increase perilipin C protein levels while increasing perilipin A protein levels only slightly. Additional evidence points to a post-translational regulatory mechanism. The loading of polyribosomes onto perilipin A mRNA was comparable whether or not cells were incubated with supplemental fatty acids. Hence, neither the initiation nor the elongation of translation of perilipin A appears to be affected by lipids. The most likely mechanism for the post-transcriptional regulation of perilipins A and C by fatty acids is post-translational stabilization of newly synthesized perilipins by neutral lipids. We have never been able to detect perilipins unaccompanied by triacylglycerol and cholesterol esters from cell extracts of either steroidogenic cells or adipocytes; thus, a lipid-protein association probably occurs co-translationally or very soon after translation. This observation suggests that cells do not accumulate the perilipins in a non-lipid-associated pool that is recruited when the cells begin to accumulate lipid. Rather, we suggest that perilipin synthesized in excess of that needed to associate with the available stored neutral lipid pool is degraded. Further evidence from adipocyte models suggests that once the perilipins are associated with lipid, they are quite stable in the cells; the half-life for perilipin A associated with lipid storage droplets in 3T3-L1 adipocytes is approximately 40 h.\(^5\) Based on the accumulated evidence, we propose the following model. Perilipins may exist in two intracellular pools: 1) a

\(^5\) C. J. Schultz and C. Londos, unpublished data.
free or non-lipid-associated pool that has an extremely short half-life, and 2) a stable, lipid-associated pool that degrades very slowly. The addition of substrates for neutral lipid synthesis may increase the partitioning of newly synthesized perilipins into the stable lipid storage droplet-associated pool. We are currently attempting to develop methods to detect the putative transient pool of free perilin.

The mechanism for post-translational stabilization of the perilipins by stored neutral lipids may be analogous to that observed for apolipoprotein B (apoB) synthesized by liver cells. Intracellular and secreted levels of apoB are acutely regulated by the availability of fatty acids in the culture medium of hepatoma cells (Refs. 24–26, and as reviewed in Refs. 27 and 28). Furthermore, the structure of apoB-containing lipoproteins is very similar to that of intracellular lipid storage droplets; both consist of a core of triacylglycerol and cholesterol ester surrounded by a monolayer of phospholipid and cholesterol into which proteins are embedded. ApoB is co-translationally inserted into the endoplasmic reticulum, where lipids rapidly associate with the nascent peptide sequences, thus nucleating lipoprotein formation. Triacylglycerol synthesis is essential to stabilize apoB; in the absence of adequate substrate for triacylglycerol synthesis, the majority of newly synthesized apoB is degraded (24–28). Proposed mechanisms include neutral lipid-mediated shielding of vulnerable residues on the nascent protein from endogenous proteases and the facilitation of translocation of nascent apoB through the secretory pathway and away from a protease-containing compartment.

Both similarities and some obvious differences exist between the pathways for the incorporation of apoB into developing lipoproteins and the association of the perilipins with nascent lipid storage droplets. Like apoB, the perilipins lack a recognizable signal sequence. Perilipin A that is epitope-tagged on the amino terminus targets to lipid storage droplets and remains intact, suggesting that proteolytic processing of a signal sequence on perilin is not required for its association with the lipid droplet. We do not yet know which organelle(s) is involved in assembly of lipid storage droplets, but these lipid droplets retain an intracellular localization and hence do not follow the same assembly pathway as secreted lipoproteins. Interestingly, the levels of endogenous and carboxyl terminally epitope-tagged perilin are similar in lipid-loaded cells despite higher levels of the epitope-tagged perilin mRNA relative to the endogenous perilin mRNA; this may suggest that perilipins modified at the carboxyl terminus are less efficiently targeted to or incorporated into lipid storage droplets.

The perilipins lack extensive sequences containing the motifs known to mediate lipid-protein associations (16); hence, the nature of the lipid-protein interactions required for lipid stabilization of newly synthesized perilin remains obscure. It is possible that the perilipins associate with a protein that embeds into the lipid storage droplet and that the observed lipid dependence of perilin expression is a function of the ability of lipids to stabilize this anchoring protein. If an anchor protein is required for perilin association, then the required protein-protein interactions must be quite strong, since isolating lipid storage droplets by centrifugation through 100 μL sodium carbonate, pH 11, fails to strip the perilipins from lipid droplets.6

It is probable that transcriptional control mechanisms account for a portion of the regulatory effects of fatty acids on perilin expression in steroidogenic cells. Levels of mRNA for perilipins A and C are regulated differently by fatty acids; while levels of perilipin A mRNA are increased approximately 6-fold by the addition of oleic acid to culture medium, perilipin C mRNA levels are unaffected by fatty acid. These increases in perilipin A mRNA in response to fatty acid are probably due to increased transcription rather than to lipid-mediated stabilization of the mRNA. Fatty acids are involved in the transcriptional regulation of a number of genes encoding proteins involved in lipid metabolism through activation of the transcription of members of the peroxisomal proliferator-activated receptor family of transcription factors (reviewed in Refs. 29 and 30). Further studies are needed to determine whether a member of the peroxisomal proliferator-activated receptor family is involved in the regulation of perilin expression in steroidogenic cells.

The addition of fatty acid and cholesterol to the culture medium of Y-1 adrenal cortical cells promotes the increased storage of neutral lipids leading to the formation of a multitude of minute lipid storage droplets encoated with perilin. This is in sharp contrast to the pattern of lipid storage droplet accumulation in differentiating adipocytes; as adipocytes store triacylglycerol during differentiation, small lipid droplets appear to coalesce into larger structures, eventually resulting in very large, often unilocular lipid storage droplets. The lipid droplets of cultured steroidogenic cells fail to coalesce into larger structures, even after extensive lipid loading over the course of a week.7 This morphological observation suggests that the lipid storage droplets of steroidogenic cells may lack a coalescence factor present in adipocytes or perhaps contain a factor that prevents coalescence of the droplets. Consequently to the increased surface area of the lipid storage droplets of steroidogenic cells may be the ability to respond very rapidly to trophic hormones; the extra surface area may facilitate the rapid hydrolysis of stored cholesterol esters to provide substrate for steroid hormone synthesis.

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