Isolation of cDNAs for perilipins A and B: Sequence and expression of lipid droplet-associated proteins of adipocytes

(cDNA cloning/gene expression/adipocyte differentiation/lipolysis)

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ABSTRACT The major cAMP-dependent protein kinase (A-kinase) substrate in adipocytes is perilipin, a protein found exclusively at the surface of the lipid storage droplets. Using anti-perilipin serum, we have isolated two related classes of full-length coding cDNAs, designated perilipin A and B, from a rat adipocyte cDNA expression library. The two cDNAs derive from two mRNA species that arise by differential splicing. The mRNAs are predicted to encode perilipins A and B, proteins of 517 aa (56,870 Da) and 422 aa (46,420 Da), respectively, which share a common 406-aa N-terminal sequence. The predicted perilipin A contains peptides present in proteolytic digests of the purified 62-kDa form of perilipin from rat adipocytes, as well as the requisite consensus A-kinase phosphorylation sites. Like perilipin A, the B form is expressed in adipocytes and is associated with lipid storage droplets. Modeling of predicted secondary structures fails to reveal an underlying basis for the tenacious association of perilipins with lipid droplets. These proteins exhibit a significant sequence relationship (~65% similarity through 105 aa) with only one other known protein, the adipocyte differentiation-related protein (ADRP). Like the perilipins, ADRP appears to be adipocyte-specific, which suggests that they interact in a related intracellular pathway. The molecular probes for perilipins A and B described here will permit detailed analyses of their functional role(s) in lipid metabolism.

The major reservoir of stored energy in animals is the triacylglycerol pool in lipid storage droplets of adipose cells. As reviewed briefly (1), little is known about the biochemical composition of the droplet surface. Recently, we described an adipocyte phosphoprotein, perilipin, that fractionates exclusively with the fat cake of rat adipocyte homogenates (1). Immunofluorescence studies reveal that perilipin is located at the periphery of lipid storage droplets in differentiated 3T3-L1 murine adipocytes (1). In lipolytically quiescent cells, perilipin is constitutively phosphorylated and migrates during SDS/PAGE with an apparent molecular mass of 62 kDa (1, 2). In response to lipolytic stimuli, the intracellular cAMP concentration is elevated, cAMP-dependent protein kinase (A-kinase) is activated, and up to six additional phosphates are added to perilipin, which then migrates as a 65/67-kDa doublet (1, 2). Given its adipocyte pattern of expression, subcellular location, and acute phosphorylation in response to hormonal stimulation, we have speculated that perilipin has a role in the packaging of lipid or in the trafficking of lipid metabolites across the lipid droplet/cytosol interface.

To provide tools to study the function of this interesting lipid droplet-associated protein, we have isolated cDNAs for perilipin and have identified two mRNAs for rat perilipin that are predicted to encode distinct protein variants. Both forms have been identified in adipocytes and their expression appears to be associated with adipocyte differentiation.

MATERIALS AND METHODS

Cells and Animals. Male Sprague–Dawley rats (~200 g; Taconic Farms) were used for all animal studies. Mouse 3T3-L1 preadipocytes were grown to confluence in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. Adipocyte differentiation was initiated with 0.5 mM 3-isobutyl-1-methylxanthine and 0.1 μM dexamethasone for 2 days. Then the cells were incubated with 1 μM insulin for an additional 2 days and thereafter maintained without hormones. The medium was changed every other day. More than 95% of the cells acquired numerous, small lipid droplets within 5 days after confluence and prominent droplets by 10 days.

Perilipin Peptide Sequences. Rat epididymal adipocytes were isolated, incubated with [32P]orthophosphate, and homogenized to prepare fat cakes (1–4). Proteins were extracted from the fat cake with SDS and separated by SDS/PAGE; the 62-kDa phosphovariant of perilipin was located by wet-gel autoradiography and excised (1). The gel chip was minced and protein was eluted by two sequential incubations (4 hr each) at 37°C in 20 mM Tris-HCl, pH 7.8/0.1 mM benzamidine/1 mM EDTA with leupeptin at 5 μg/ml. The eluates were concentrated with a Centricon-10 cartridge (Amicon) and the SDS concentration was reduced by repeated dilution and concentration using Centricon-10; SDS was measured colorimetrically with the Bio-Rad protein assay kit. Perilipin recovery was quantitative and the SDS was reduced to <0.02%. N-terminal sequencing of perilipin was attempted as described (5).

For peptide analysis, 5 μg of perilipin was digested to completion with 0.2 μg of trypsin for 2 hr at 37°C in 20 mM Tris-HCl, pH 7.8/0.5 mM CaCl2. After centrifugal ultrafiltration of the digest in a Centricon-10 cartridge, the filtrate was treated sequentially with KCl and guanidine (6) to remove residual traces of SDS. The filtrate was applied to a Vydac 214TP column (2.1 × 250 mm) equilibrated with 0.1% trifluoroacetic acid (TFA) and a Hewlett-Packard Model 1090 M HPLC instrument with a column oven (60°C) was used for peptide separations. The column was washed with 0.1% TFA for 10 min at 250 μl/min. The flow rate was

Abbreviation: RT–PCR, reverse transcription–coupled PCR.

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reduced to 150 µl/min and peptides were eluted with 0.085% TFA in acetonitrile (7). The column effluent was monitored at 215 nm and fractions were collected manually, immediately placed on dry ice, and stored at −80°C. Several of the peak fractions were subjected to another separation on an Applied Biosystems Aquapore RP-300 column (250 x 1 mm); the flow rate was 100 µl/min with a 0–100% gradient of acetonitrile over 30 min. The homogeneous peptides thus obtained were analyzed with an Applied Biosystems model 477A sequencer equipped with a model 120A phenylthiohydantoin analyzer. Initially, the Normal-1 cycles were used, but the modifications recommended by Tempst and Riviere (8) and Speicher (9) were incorporated for the majority of the studies. Data analyses were aided by the Applied Biosystems model 610A software.

Phospho Amino Acid Analysis. 32P-labeled 62-kDa and 65/67-kDa forms of perilipin were purified from quiescent and stimulated cells, respectively, as described above. Phospho amino acids were determined according to Cooper et al. (10).

RNA Isolation and Gel Analysis. RNA was isolated from rat and human primary adipocytes and from 3T3-L1 preadipocytes and adipocytes by 1% SDS/phenol extraction (11). To ensure complete solubilization of lipid during the extraction procedure, aqueous SDS/cell pellet volume ratios were >3:1. RNA was separated by denaturing gel electrophoresis, blotted onto filters, and hybridized with cDNA probes in 50% formamide/0.8 M Na+ at 37°C (12).

Screening of the cDNA Library. Poly(A)+ RNA was prepared from rat adipocytes and used to construct a λgt11 cDNA library (13). The library was screened initially with total and affinity-purified polyclonal antisemir against 62-kDa perilipin (14). Three overlapping clones were selected from an initial screen of 100,000 recombinants. Inserts were subcloned into plasmids and sequenced by the dideoxynucleotide method as applied to double-stranded DNA (15). Labelled inserts from these clones were used to rescreen the cDNA library.

Polymerase Chain Reaction (PCR). The Geneamp RNA PCR kit (Perkin–Elmer/Cetus) was used for reverse transcription-coupled PCR (RT–PCR) amplification of RNA sequences. Oligonucleotides were prepared with an Applied Biosystems DNA synthesizer.

Databank Search. The nucleic acid and derived amino acid sequences of the perilipin cDNAs were compared with known sequences using searches by BLAST (16) and FASTA (17) algorithms provided by the National Center for Biotechnology Information (Bethesda, MD); PROSITE (18) was used for relevant structure and domain analyses based on primary amino acid sequences.

RESULTS

Isolation of Two Perilipin cDNA Classes. A λgt11 rat adipocyte cDNA expression library was constructed and probed with total and affinity-purified polyclonal antisera to perilipin. The affinity-purified antisemir reacts specifically with perilipin on protein blots (1). Several cDNAs that encode epitopes which crossreacted with both antisera. cDNA inserts were subcloned into plasmids and used as probes to rescreen the library to obtain full-length protein-coding recombinants. Two classes of cDNAs were identified that are predicted to encode distinct, but related, proteins (Fig. 1). The two cDNAs are identical both in their 5′ untranslated regions and in the first 1218 nt (406 aa) of their coding regions; beyond, they diverge. One, perilipin A, is predicted to encode a protein of 517 aa, and the other, perilipin B, a protein of 422 aa.

The predicted protein sequence of perilipin A (Fig. 2A) includes seven peptides present in purified rat 62-kDa perilipin; the predicted molecular mass, 56,870 Da, is similar to the ~60-kDa size of dephosphorylated perilipin (1). Perilipin A possesses six consensus A-kinase sites, consistent with the number of phosphates added to perilipin by A-kinase during lipolytic activation of adipocytes (1, 2). Each of the putative A-kinase sites includes serine as the phosphate acceptor, and in further experiments (data not shown) we found that only serine residues of the 65/67-kDa perilipin were phosphorylated following the elevation of A-kinase activity in rat adipocytes. By contrast, in unstimulated cells, most 32P of the 62-kDa form of perilipin was on threonine. No phosphotyrosine was detected.

The site of divergence between the perilipin B and perilipin A cDNAs incorporates a consensus donor RNA splice site, GTNNGT (19), suggesting that alternative RNA splicing gives rise to the two variants (Fig. 2B, see below). Perilipin B translation terminates after aa 422 and is predicted to yield a protein of 46,420 Da, ~10.5 kDa smaller than perilipin A. Perilipin B contains five of the seven peptide sequences identified in perilipin A and three of the six consensus A-kinase sites.

Perilipin Sequence Analyses. The perilipin nucleic acid and predicted amino acid sequences were analyzed by BLAST and FASTA searches of the GenBank, EMBL, and Swiss-Prot databases (search date, April 1993) (16, 17). Only one listing, the mouse adipocyte differentiation-related protein, ADPR, exhibited a significant (P < 0.0003) sequence relationship with perilipin (20). Amino acids 17–121 of perilipin A and B are ~40% identical (~65% similar) in sequence to aa 9–113 of ADPR (Fig. 2C). Combined Garnier–Osguthorpe–Robsons (21) analyses of perilipin amino acid sequences indicated three regions (aa 142–157, 247–269, and 348–368) of perilipins A and B with moderately positive hydrophobic indices, although none of these is predicted to form membrane-spanning domains (22).

Attempts to perform N-terminal sequence analysis of purified perilipin have been unsuccessful, suggesting that the N-terminal amino acid is modified. We have no biochemical data regarding the presumed blocking group. There are several potential N-myristoylation sites located within the interior of perilipin. However, since the size of perilipin is similar to that predicted from cDNA sequencing, the protein may not be processed in a manner that places any of the putative myristoylation sites at the N terminus, as is required for the acyl modification (23). The amino acid sequence of perilipin also includes a potential N-glycosylation site (24) at aa 42, but apart from potential phosphorylation sites, no additional modifications are predicted and no homologies to...
**A**

**Fig. 2.** Sequence analyses of perilipin A and perilipin B. (A) Protein-coding sequences and untranslated regions of perilipin A cDNA. Numbers at left and right indicate amino acids and nucleotides, respectively. The six consensus A-kinase sites are underlined twice. The seven peptide sequences presented in bold type with a single underline correspond to peptides found in the proteolytic digest of purified 62-kDa perilipin. Three additional peptides did not match perilipin A; these may derive from another ~60-kDa adipocyte protein that, as with perilipin, cofractionates with the fat cake. (B) Region of divergence between the perilipin A and B cDNA sequences. Amino acid positions are noted by numbers. Translation of perilipin B terminates after aa 422. For perilipin A, translation continues through aa 517. The amino acids that are identical in perilipin A (Peri A) and B (Peri B) are in bold type and indicated by broken, vertical lines. A consensus donor splice junction in perilipin B cDNA is in bold type and underlined. The 3′ untranslated sequence and orientation of oligonucleotide 1 from perilipin A and oligonucleotide 2 from perilipin B that were used for RT-PCR (see Fig. 4) are indicated. (C) Sequence comparison between N-terminal regions of perilipin and ADRP. Amino acids 17–121 of perilipin and 9–113 of ADRP are aligned. Amino acid identities are indicated by double dots, and similarities by single dots.

**Multiple Perilipin mRNAs in Rat Adipocytes.** Rat adipocyte RNA blots were hybridized with probes from the region common to perilipins A and B and from regions unique to the A or B cDNA (Fig. 1). The common, C probe recognized two mRNAs of ~3.9 and ~3.0 kb. Whereas only the larger, less abundant mRNA was recognized by the B probe, both mRNAs hybridized to the A probe (Fig. 3). Since the sequence data suggested that perilipin A and B mRNAs arise by differential splicing of a common precursor (see Fig. 2B), it seemed possible that the 3.9-kb mRNA contained the unique regions from both A and B cDNAs and was an effective precursor to the 3.0-kb mRNA. A predicted structure for the 3.9-kb mRNA is depicted in Fig. 4A. None of the perilipin B clones contained complete 3′ sequences. However, we confirmed the organization of the 3.9-kb RNA by RT-PCR analyses of rat adipocyte RNA.

RNA from rat primary adipocytes was reverse-transcribed using an oligonucleotide (oligo 1) located within the perilipin
A cDNA and 3' to the presumptive acceptor splice junction (see Figs. 2B and 4A). The resulting cDNA product was subjected to PCR using an additional oligonucleotide (oligo 2) located 3' to the donor splice junction but within perilipin B only (see Figs. 2B and 4A). Under these conditions, only the 3.9-kb mRNA is predicted to yield an amplified product. No PCR fragment will be produced from the 3.0-kb mRNA. The reaction generated a single DNA fragment of ≈0.8 kb that, as expected, hybridized with B sequences but not with sequences common to both mRNAs or with A sequences (Fig. 4B). The size of the fragment approximates the length difference between the two perilipin mRNAs. The 3.9-kb mRNA is predicted to encode perilipin B, whereas the 3.0-kb mRNA encodes perilipin A.

**Tissue and Developmental Specificity of Perilipin mRNA Expression.** We previously detected perilipin only in adipocytes by immunoblot analysis of proteins isolated from a variety of rat tissues (1). The complex pattern of perilipin mRNA organization prompted a reexamination of tissue specificity by RNA blot hybridization (Fig. 5). The autoradiogram of the RNA blot was deliberately overexposed in order to detect potential faint hybridization of perilipin probes to RNA isolated from other rat tissues. The 3.9- and 3.0-kb perilipin mRNAs were not detected in any of the non-adipose tissues. Further, we did not observe novel cross-hybridizing species that might result from alternative RNA processing and encode additional variants. If perilipin mRNA is present in non-adipose tissue it must be ≤0.002% of total mRNA. On the other hand, we have detected perilipin RNA in mouse brown fat and in mouse and human white adipocytes.

Since perilipin expression appeared to be confined to adipocytes, we examined expression of perilipin during adipocyte differentiation in a tissue culture system. RNA from 3T3-L1 murine preadipocytes and differentiated adipocytes was hybridized on blots with a probe containing sequences common to rat perilipin A and B. Perilipin mRNA was detected only after adipocyte differentiation was initiated and, as in rat adipocytes, the differentiated 3T3-L1 cells expressed multiple mRNAs (Fig. 6).

**DISCUSSION**

On the basis of their expression of epitopes that react with affinity-purified antibodies, two related classes of cDNAs have been isolated from an Agt11 rat adipocyte cDNA expression library. The predicted 56,870-Da product of perilipin A cDNA includes seven peptide sequences obtained from proteolytic digestion of purified 62-kDa perilipin and six consensus A-kinase phosphorylation sites, consistent with the number of phosphates added to perilipin upon activation of A-kinase in the cell. These data indicate that perilipin A corresponds to the previously identified 62-kDa phosphoprotein of adipocytes (1).

The second cDNA class is predicted to encode a 46,420-Da variant protein, perilipin B, which is identical to perilipin A in its N-terminal 406 aa. Perilipin B lacks three of the six consensus A-kinase sites found in the A form, and is likely to be the 50-kDa fat-associated A-kinase substrate (1). The affinity-purified anti-perilipin serum also recognized a 50-kDa fat-specific protein in rat (1) and, more recently, autoradiography and immunoblotting of proteins extracted from fat cakes indicated that the phosphoprotein and immunoreactive protein were identical (J. Theodorakis, personal communication). Perilipins A and B are phosphorylated in unstimulated cells and hyperphosphorylated upon elevation of A-kinase activity (1, 2). The hyperphosphorylation of peri-
lipin A, but not B, causes a marked alteration in migration during SDS/PAGE. It appears that the three additional A-kinase sites specific to the C-terminal region of perilipin A are responsible for this altered behavior during SDS/PAGE. Since immunoblot analysis of subcellular fractions indicate that both perilipin A and B fractionate exclusively with lipid, and since immunofluorescence studies detect reactive epitopes only in tight association with the lipid droplet, it appears that perilipin B, like perilipin A, is localized at the periphery of the droplet.

The perilipins exhibit sequence similarity with only a single additional protein (or gene) listed in the current databases (20). The highly significant, albeit limited, relationship (=65% similarity through 105 aa) between the perilipins and ADRP is intriguing, especially since they share a common tissue specificity. The perilipins associate exclusively with the lipid droplet surface, whereas ADRP is associated with a particulate fraction in or near the plasma membrane. Nevertheless, it is probable that the perilipins and ADRP are involved in related adipocyte processes. Perhaps the perilipin and ADRP interact with a common factor involved in lipid metabolism that substances to and from the lipid droplet.

Previously, we discussed (1) the difficulties encountered in separating perilipin from lipid and its insolubility in the absence of strong ionic detergents (e.g., SDS). Surprisingly, the primary sequences of the perilipins reveal no structural features that might underlie such behavior. Unlike other classes of lipid-associated proteins [e.g., the apolipoproteins (25) or plant oil-droplet proteins (26)], which possess extended hydrophobic regions and/or amphiphatic helices, similar structures are not predicted for the perilipins. Although the N terminus of perilipin appears to be modified, a typical N-terminal, glycol N-myristoylation site does not exist (23). There are several possible myristoylation sites at internal regions of the perilipins, but there is no evidence that cleavage of perilipin places any of these at the N terminus.

Molecular studies indicate that the 3.0- and 3.9-kB mRNAs, for perilipins A and B, respectively, derive from alternative splicing. Further, analyses of genomic sequences indicate that the perilipins are encoded by a single-copy gene (J. Gruia-Gray and A.R.K., unpublished work). Taken together, these data suggest that the differences in structure between the two mRNAs derive from an additional, internal sequence specific to the 3.9-kb form (see Fig. 4A). The two mRNAs may use identical transcription initiation and polyadenylation sites. The considerably greater abundance of perilipin A mRNA relative to that for perilipin B is consistent with the relative levels of A and B proteins as measured by immuno blotting and phosphate incorporation. RNA blot hybridization for various rat tissues reveals that the perilipins are expressed only in primary adipocytes. These data do not exclude low-level expression of perilipin in non-adipose cells which accumulate only a few, small lipid droplets.

The tissue specificity and developmental expression pattern of perilipin, its location at the surface of the lipid droplet, and its phosphorylation by A-kinase concomitant with activation of lipolytic activity suggest a role for this protein in lipid metabolism. Recently, we demonstrated that hormone-sensitive lipase, the rate-limiting enzyme of lipolysis, translocates to the lipid droplet surface upon lipolytic activation of adipocytes (27). One potential role for perilipin might be as a barrier to deny access of the lipase to the lipid of unstimulated cells, whereas, upon phosphorylation of perilipin by A-kinase, the lipid surface may become exposed. Alternatively, perilipin may serve as a “docking” protein for the lipase during stimulated conditions or may be required to establish or maintain the organization of the lipid droplet. Whether perilipin interacts with vimentin, an intermediate-filament protein that is reported to form cage-like structures surrounding newly forming lipid droplets (28), remains to be resolved. Also of interest is the question of possible distinct functions for perilipins A and B. This paper provides a basis for exploring the above questions and for determining the consequences of enhanced or blocked expression of perilipin in adipocyte and nonadipocyte systems.

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