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Post-translational Regulation of Adipose Differentiation-related Protein by the Ubiquitin/Proteasome Pathway

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Post-translational Regulation of Adipose Differentiation-related Protein by the Ubiquitin/Proteasome Pathway*

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Adipose differentiation-related protein (ADRP) is localized to lipid droplets in most mammalian cells. ADRP, proposed to regulate fatty acid mobilization and lipid droplet formation, is linked to lipid accumulation in foam cells of human atherosclerotic lesions. In this report, we show that ADRP protein accumulates in Chinese hamster ovary fibroblastic cells cultured in the presence of oleic acid but is destabilized when fatty acid sources are removed from culture serum. The latter effect was blocked by the proteasome inhibitor MG132, whereas inhibitors of other proteolytic processes were ineffective. Pulse-chase experiments confirmed that ADRP degradation is inhibited by MG132. Conditions that stimulate ADRP degradation also promoted the covalent modification of ADRP by ubiquitin, whereas the addition of oleic acid to culture media, which promotes triacylglycerol deposition, blunted the appearance of ubiquitinated-ADRP. Treatment with MG132 increased the levels of ADRP associated with lipid droplets, as well as throughout the cytosol. Finally, we demonstrate that the disappearance of ADRP protein after the onset of perilipin expression during adipocyte differentiation is due to degradation by proteasomes Thus, proteolytic degradation of ADRP mediated through the ubiquitin/proteasome pathway appears to be a major mode for the post-translational regulation of ADRP.

Intracellular neutral lipid storage droplets in eukaryotes are ubiquitous cellular organelles required for membrane biosynthesis, cholesterol metabolism, and energy balance (1, 2). Lipid droplets contain a triacylglycerol (TAG)[6] and cholesterol ester core surrounded by a phospholipid monolayer (3). Several related proteins localize to the surface of lipid droplets (4), including perilipin (5–8), adipose differentiation-related protein (ADRP) (9), TIP47 (10, 11), a putative cargo protein for the mannose 6-phosphate receptors (12), and S3–12 (13). These proteins share sequence homology within their PAT (perilipin/ADRP/TIP47) domains (4, 11, 14) and are derived from a common ancestral gene (15).

The localization to lipid droplets suggests that PAT proteins play a fundamental role in lipid metabolism (4, 16). To date, perilipin is the only family member for which a function in lipid metabolism has been firmly established (17), for review see Tansey *et al.* (18). Lipolysis in adipocytes is governed primarily by a protein kinase A-mediated reaction. Non-protein kinase A-phosphorylated perilipin protects TAG from hydrolysis and thus promotes TAG storage (19, 20). Indeed *perilipin*-null mice have reduced adipose TAG storage (21, 22). However phosphorylation of perilipin is required for the translocation of hormone-sensitive lipase from the cytosol to the lipid droplet surface (17). Because migration of hormone-sensitive lipase largely determines lipolytic activation in adipocytes, stimulation of lipolysis is strongly blunted in isolated primary adipocytes that lack perilipin or in whole *perilipin*-null animals (21).

ADRP, also termed adipophilin, is a 47-kDa protein that shares sequence similarity with perilipin (4). Unlike perilipin which is specific for adipocytes and steroidogenic cells (5–7), ADRP is expressed in most tissues (9, 23, 24). ADRP is suggested to selectively increase the uptake of long chain fatty acids and has a possible role in fatty acid transport (25–27). Cells cultured with fatty acids may have elevated *ADRP* mRNA and protein expression (26, 27). In lung tissue, the developmental expression pattern of *ADRP* mRNA and protein parallel TAG accumulation (28). ADRP not only serves as a sensitive marker of lipid accumulation (23, 29) but also has been reported to stimulate lipid accumulation and lipid droplet formation in murine fibroblasts (30). A recent study has revealed that *ADRP* mRNA is induced in human monocytes or macrophages by either oxidized (31) or enzymatically modified (29) low density lipoprotein and is highly expressed in a subset of lipid-rich foam cells of human atherosclerotic lesions (31). By contrast, *perilipin* mRNA is induced in foam cells of ruptured, but not of stable, human atherosclerotic plaques (32). Thus, the lipid droplet-associated proteins such as ADRP and perilipin are likely potential regulators for lipid accumulation of foam cells and destabilization of atherosclerotic plaque.

Although the PAT family proteins appear to play important roles in lipid metabolism, there are few studies on the regulation of these proteins; most available evidence points toward a post-translational regulation of ADRP. The ubiquitin/proteasome pathway is a potential mechanism for such regulation. Ubiquitin is an 8-kDa peptide of 76 amino acids that is ubiquitous in eukaryotes. Typically, ubiquitin or a polyubiquitin chain is covalently conjugated with the targeted protein to form polyubiquitinated protein complexes, which are delivered to and degraded by the 26 S proteasome, a 2000-kDa ATP-dependent proteolytic particle (33–35). If proteins fold improperly or form incorrect oli-

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[6] The abbreviations used are: TAG, triacylglycerol; ADRP, adipose differentiation-related protein; ALLN, *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; OA, oleic acid; Ad, adenovirus.

gomeric structures, they may become conjugated to ubiquitin and proteasomally degraded (33, 35). In the cellular response to environmental changes, the ubiquitin/proteasome system has been shown to regulate degradation of transcription factors, cell cycle proteins, tumor suppressors, and cell surface receptors, as well as proteins that are damaged or improperly localized (34). In this study, we report that a major mode for the post-translational regulation of ADRP is degradation via the ubiquitin/proteasome pathway.

EXPERIMENTAL PROCEDURES

Reagents—Unless otherwise noted, all chemical reagents were obtained from Sigma. Proteasome or protease inhibitors MG132, lactacystin, ALLN, calpeptin, and leupeptin were purchased from Calbiochem. Geneticin (G418) and DNA transfection reagents came from Invitrogen. [^{35}S]Methionine and an enhanced chemiluminescence (ECL) detection kit were products of Amersham Biosciences. Methionine-free DMEM is a product of BioFluids (Rockville, MD). Rabbit and goat anti-mouse ADRP polyclonal sera were raised against a peptide composed of the N-terminal 26 amino acid residues of murine ADRP. Rabbit anti-ubiquitin antibody, agarose-conjugated protein A/G plus mixture, and horseradish peroxidase-conjugated IgG were obtained from Santa Cruz Biotechnology. Fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG were purchased from Jackson Immunoresearch.

Cells Culture and Expression of ADRP in CHO Cells—Chinese hamster ovary fibroblastic cells (CHO-K1, ATTC) were maintained in F12 medium (Invitrogen) containing 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 units/ml streptomycin at 37 °C in an atmosphere of 5% CO_2. Full-length mouse *ADRP* cDNA was subcloned in the pcDNA3.1 vector and transfected into CHO cells using the Lipofectamine Plus reagent (Invitrogen). After selection in 1500 μg/ml G418 (Invitrogen) for four generations, cells that stably expressed ADRP (CHO-ADRP) were maintained in 10% FCS-F12 medium in the presence of 300 μg/ml G418. For transient ADRP expression, CHO cells were infected with a recombinant adenovirus-expressing murine ADRP (Ad-ADRP). This vector was constructed according to a previously described method (17). For lipid loading, oleic acid was added to the culture medium at 400 μmol as a 5:1 molar complex with bovine serum albumin. For delipidation experiments, cells were cultured in medium containing 10% charcoal-delipidated FCS (Sigma).

Immunoblot Analysis—Cells were washed twice, lysed in sample buffer, diluted 1:1 with 2× SDS loading buffer, and heated to ~95 °C for 5 min. Equivalent amounts of protein were loaded, separated by 10% SDS-PAGE, and transferred to nitrocellulose membranes. The membrane was blocked using 5% nonfat dry milk in TBS buffer containing 150 mM NaCl, 0.05% Tween 20, and 20 mM Tris-HCl, pH 7.2, and incubated for 1 h with primary antibodies against ADRP followed by incubation for 1 h with specific secondary antibodies conjugated to horseradish peroxidase. Blots were developed using an enhanced chemiluminescence (ECL) detection kit.

Northern Blot Analysis—CHO cells were grown to 80% confluence, and total cellular RNA was extracted using TRIzol reagent (Invitrogen). Thirty μg of total RNA was loaded in each lane, separated in 1% formaldehyde denaturing agarose gel, and transferred to nylon membranes. ^{32}P-Labeled *ADRP* cDNA probe was prepared with the Rediprime DNA random labeling system (Amersham Biosciences), and unincorporated nucleotides were removed through a G-50 desalting spin column. The membrane was baked for 2 h at 80 °C prior to prehybridization. The hybridization procedures were performed in the presence of ^{32}P-labeled *ADRP* cDNA probe in Ultrahyb solution (Ambion) at 42 °C. *ADRP* mRNA bands were detected by autoradiography.

Immunoprecipitation—Cells grown in 60-mm diameter dishes were washed twice with PBS buffer and lysed in 1 ml of cold RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 2 mM EDTA) with proteases inhibitors (2 mM phenylmethylsulfonyl fluoride, 2 μg/ml ALLN, 2 μg/ml leupeptin, and 2 μg/ml aprotinin). After addition of the lysis buffer, the dishes were incubated on ice for 20 min. The cell lysates were centrifuged at 13,000 rpm at 4 °C for 20 min to pellet cell debris. The supernatant was transferred to a new tube and precleared with 30 μl of agarose-protein A/G plus mixture for 1 h at 4 °C. The immunoprecipitation reaction was performed at 4 °C for 2 h on a rotator in 1 ml of the precleared supernatant upon addition of rabbit anti-ADRP antibody at 1:500 or rabbit anti-ubiquitin at 1:200 and 30 μl of agarose-protein A/G plus mixture. Rabbit polyclonal serum against hormone-sensitive lipase or rabbit preimmune serum was used as the negative control. The immunoprecipitated complex was pelleted by centrifugation at 5000 rpm at 4 °C for 5 min, washed four times with cold RIPA buffer, and finally mixed with sample buffer containing 4% SDS and 10 mM dithiothreitol. After boiling at 100 °C for 5 min, proteins in the samples were loaded and separated by SDS-PAGE. For pulse-chase experiments, [^{35}S]methionine-labeled ADRP in immunoprecipitates was detected directly by autoradiography after separation by SDS-PAGE and exposure of the dried gel to X-film for 24 h. For subsequent immunoblot analysis, the immunoprecipitated proteins were separated on SDS-PAGE and transferred to nitrocellulose membrane followed by immunoblotting with the antibodies against ADRP or ubiquitin, respectively.

Pulse-Chase Experiments—Cells were grown to 90% confluence in 60-mm diameter dishes and preincubated for 30 min in methionine-free 10% FCS-DMEM to deplete cold methionine from the cells. The cells were washed once with PBS and then pulse-labeled for 5 h with 0.1 mCi/ml [^{35}S]methionine in warmed methionine-free DMEM-10% FCS. Pulse-labeling reaction was terminated by the addition of a 1,000-fold excess of cold methionine. After one wash with PBS, cells were subsequently chased for 0 or 5 h in serum-free F12 culture medium in the presence or absence of 400 μM oleic acid or 10 μM MG132 as indicated. The [^{35}S]methionine-labeled ADRP reached its maximum level at the end point of labeling and then declined continually during chase. At the end point of the chase, the chase medium was removed, and the cells were washed once with PBS and lysed in 1 ml of cold RIPA buffer. [^{35}S]Methionine-labeled ADRP was immunoprecipitated with rabbit anti-ADRP antibodies and then separated by SDS-PAGE and detected by autoradiography. The density of bands was quantified by densitometry and analyzed using the NIH Image program.

Immunofluorescence and Confocal Imaging—Cells were treated in glass slide chambers and fixed for 15 min with 3% paraformaldehyde and 0.1% Triton X-100 in PBS buffer at room temperature followed by three washes for 10 min each with PBS. Nonspecific binding sites in the cells were blocked with 2% donkey serum for 30–60 min and washed for three times for 5 min each. The cells were incubated with rabbit polyclonal serum against ADRP at 1:500 overnight at 4 °C and subsequently with fluorescein isothiocyanate-conjugated donkey anti rabbit IgG at 1:100 for 1 h in the dark at room temperature (17). Intracellular lipid storage droplets were easily visible under Nomarski optics/differential interference contrast, and the immunofluorescent signal of ADRP was observed using a Zeiss SCM510 confocal microscope.

Quantification of Total Triacyglycerols—Cells grown in 6-well plates were treated with or without 2.5 μM MG132 for 24 h, rinsed with PBS buffer, and scraped into extraction buffer containing 20 mM Tris-HCl, pH 7.4, and 1 mM EDTA. The cells were lysed with three freeze-thaw cycles, and total lipids were extracted with chloroform and methanol
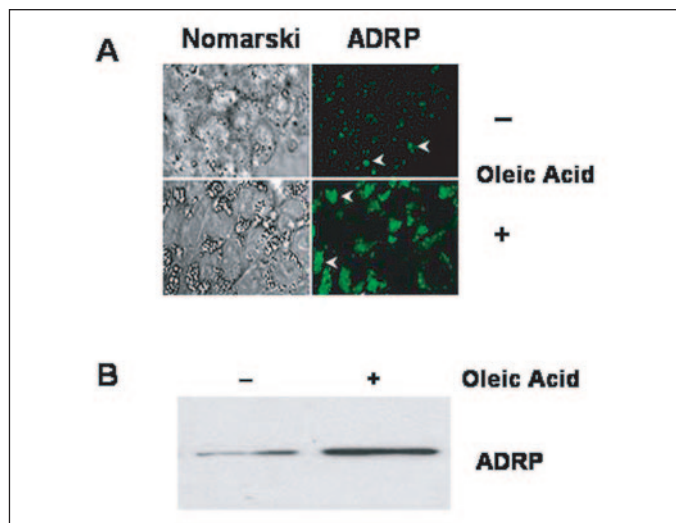
FIGURE 1. **Oleic acid increases intracellular lipid storage droplets and elevates ADRP protein levels in CHO cells.** *A*, CHO cells were grown for 48 h in the absence or presence of 400 μM oleic acid. The cells were immunostained with rabbit α-ADRP antibody and fluorescein isothiocyanate-conjugated second antibody and imaged by fluorescent confocal microscopy. Intracellular lipid storage droplets were visible under Nomarski interference optics, and ADRP (*arrowheads*) appeared in association with the lipid droplets. Note that incubation with oleic acid elevates intracellular lipid droplets and fluorescence of ADRP. *Bars* represent 10 μm. *B*, immunoblotting with α-ADRP antibodies was performed after CHO cells were incubated for 48 h with or without 400 μM oleic acid. *A* and *B* are representative of at least five separate experiments.

FIGURE 2. **Delipidated culture serum leads to a decrease in ADRP protein by a process prevented by the proteasome inhibitor MG132.** *A*, CHO cells transfected with Ad-ADRP were incubated for 24 h in normal or delipidated medium (*Delipid.*) with or without 400 μM oleic acid or with 5 μM MG132. MG132 was administrated at 6 h after transfer to delipidated medium and maintained for 18 h. Immunoblotting was performed using α-ADRP antibody. *B*, CHO cells transfected with Ad-ADRP were cultured in F12 medium supplemented with 10% normal calf serum and treated for 18 h with varying concentrations of MG132 as indicated. The blots shown in *A* and *B* are representative of three separate experiments.
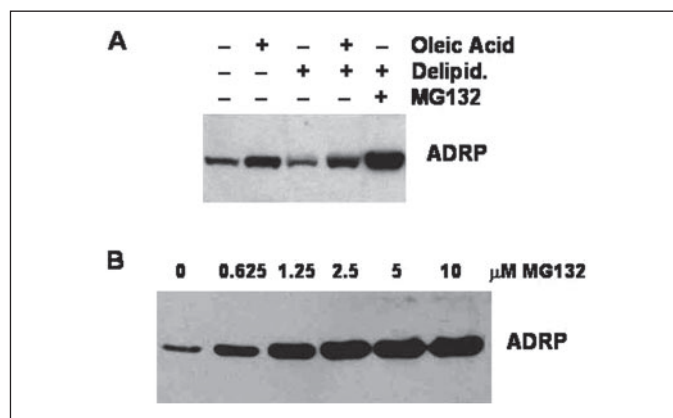
(2:1) as described previously (36). The mass of total triacylglycerols from triplicate samples was measured by OD at 340 nm using a commercial kit (Sigma) and normalized to total cellular protein content measured by the bicinchoninic acid method (Pierce).

Differentiation of 3T3-L1 Adipocytes—3T3-L1 (ATCC) fibroblastic cells (preadipocytes) were grown to confluence in 12-well plate and maintained for another 24 h in DMEM containing 10% FCS. The confluent 3T3-L1 cells (day 0) were differentiated for 3 days in 10% FCS-DMEM containing 0.5 mM IBMX (3-isobutyl-1-methyl-xanthine), 10 μM dexamethasone, 1 μg/ml insulin, and 8 μg/ml biotin. The differentiation medium was changed daily, and replaced with regular medium at day 3 after initiating the differentiation program. The differentiated 3T3-L1 adipocytes were maintained in fresh 10% FCS-DMEM. At day 8 of differentiation, the 3T3-L1 adipocytes were treated for 18 h with 5 μM MG132.

Statistical Analysis—Data are expressed as the means ± S.E. A one-way analysis of variance Tukey's test was performed for statistical analysis using GraphPad Prism version 4.0, and *p* values < 0.05 were considered statistically significant.

RESULTS

Fatty Acid Loading Promotes Intracellular Lipid Droplet Formation and ADRP Accumulation—We showed previously that CHO cells cultured with the free fatty acid oleic acid (OA) increased TAG storage, with little deposition of OA into other lipid fractions (20). To determine whether these growth conditions additionally altered the expression of ADRP, we cultured CHO cells for 48 h in the absence or presence of 400 μM OA and assessed ADRP protein levels by immunofluorescent imaging and confocal microscopy as well as by immunoblotting. Relatively few and tiny lipid droplets (Fig. 1*A*) were present in CHO cells cultured in the absence of OA. After incubation for 48 h with 400 μM OA, both the number and size of the intracellular lipid storage droplets were significantly increased. We also observed enhanced ADRP immunofluorescence associated with the lipid droplets in cells cultured with OA

(Fig. 1*A*). Increased ADRP protein expression was confirmed by immunoblot analysis (Fig. 1*B*). Increases in intracellular lipid droplet storage and ADRP accumulation were observed by 12 h after the loading with OA (data not shown). Thus, increases in ADRP protein accumulation paralleled the increase in neutral lipid deposition.

Delipidation Induces Down-regulation of ADRP by a Process Blocked by Proteasomal Inhibitors—Delipidation of serum removes lipid precursors and deprives cells of fatty acid precursors that are required for lipid droplet accumulation (9). Results from Fig. 1 indicate that ADRP protein accumulation was promoted by the increase in intracellular lipid storage. We were interested to determine whether ADRP protein levels would be reduced upon the removal of lipid precursors from the culture media. To enhance ADRP detection, we utilized CHO cells that express ADRP from an adenovirus vector. The linkage of lipid deposition and ADRP expression is highlighted in Fig. 2*A*. Incubation of the cells for 24 h in culture medium containing 10% calf serum that had been delipidated with charcoal led to a substantial decrease of ADRP protein in comparison with nondelipidated control cells. By contrast, culturing cells in delipidated medium that was supplemented with 400 μM oleic acid prevented the decline in ADRP protein (Fig. 2*A*). Thus, ADRP appears to be degraded when TAG accumulation is inhibited. To determine whether proteasomal activity is involved in ADRP degradation, we treated the cells with MG132, a specific inhibitor of the 26 S proteasome. Clearly, MG132 promoted ADRP accumulation in cells deprived of lipid precursors (Fig. 2*A*). Moreover, under nondelipidation conditions, we observed that MG132 elevated ADRP protein in a concentration-dependent manner (Fig. 2*B*). The MG132-stabilizing effect was evident at 0.625 μM MG132 and maximal at ~2 μM (Fig. 2*B*). These results provide strong support that ADRP is degraded through a proteasomal pathway.

We investigated the effects of ADRP degradation using a series of protease inhibitors (37). It has been well established that MG132 specifically inhibits the 26 S proteasome and that lactacystin is specific for the 20 S proteasome (37, 38). ALLN is an inhibitor of both the 20 S and 26 S proteasomes and a partial inhibitor of the calpain protease. Calpeptin is specific for calpain, leupeptin inhibits trypsin-like and cysteine proteases, and chloroquine and NH_4Cl are lysosomal protease inhibitors. As shown in Fig. 3*A*, addition of the three proteasome-specific inhibitors, MG132, lactacystin, and ALLN, significantly increased

ADRP levels after treatment for 18 h. By contrast, the other protease inhibitors were ineffective in increasing ADRP protein levels (Fig. 3*A*). These data indicate that ADRP can be degraded through the proteasome pathway.
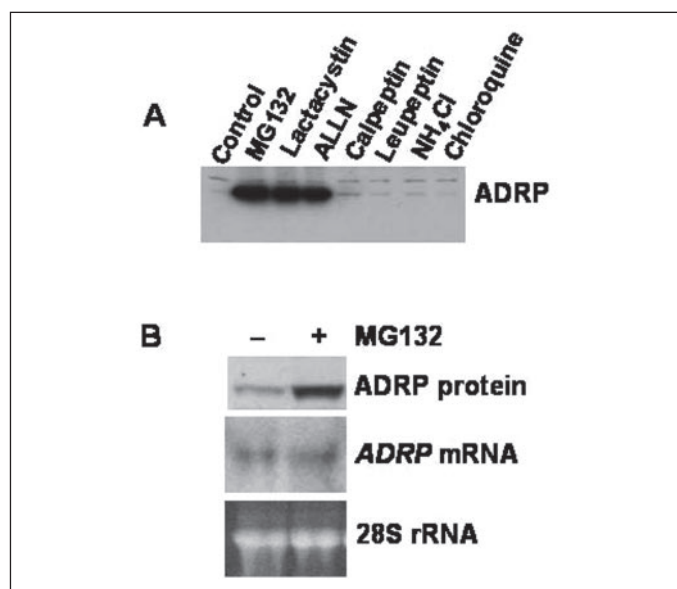
We also examined whether proteasomal inhibitors increase ADRP protein indirectly by elevating *ADRP* mRNA levels. Although addition of 5 μM MG132 for 18 h significantly increased ADRP protein levels, addition of this agent did not alter *ADRP* mRNA levels under the same conditions (Fig. 3*B*). Thus, the proteasome inhibitors elevated ADRP protein by a post-transcriptional mechanism.

Proteasomal Degradation of ADRP—To examine ADRP turnover more directly, we performed pulse-chase experiments. Cells were labeled for 5 h with [^{35}S]methionine followed by a 5-h chase in the presence of unlabeled methionine with or without 10 μM MG132 or 400

FIGURE 3. **Proteasome inhibitors specifically increase ADRP protein accumulation without altering *ADRP* mRNA transcript levels.** *A*, CHO cells were treated for 18 h with different protease inhibitors. After lysis, 50 μg of whole cell protein was separated by SDS-PAGE and immunoblotted using α-ADRP antibody and horseradish peroxidase-conjugated second antibody. The reagents used were: 10 μM MG132, 5 μM lactacystin, 50 μM ALLN, 25 μM calpeptin, 25 μM leupeptin, 2.5 mM NH$_4$Cl, and 100 μM chloroquine. *B*, CHO cells were treated with or without 5 μM MG132 for 18 h and harvested for immunoblotting and northern blotting. *Top panel*, immunoblot of ADRP protein; *middle panel*, Northern blot of ADRP RNA; *bottom panel*, 28 S rRNA control. Blots shown are representative of three separate experiments.

μM oleic acid. ^{35}S-Labeled ADRP was immunoprecipitated and detected by autoradiography. More than 50% of the labeled ADRP was lost during the 5-h control chase (Fig. 4, *A* and *B*). The specific proteasome inhibitors MG132 (Fig. 4*A*,B), lactacystin, and ALLN (data not shown), strongly (~85%) preserved the labeled ADRP during the chase period (Fig. 4, *A* and *B*), whereas calpeptin, leupeptin, chloroquine, and NH$_4$Cl had no effect on ADRP degradation (data not shown). These results confirmed that degradation of ADRP was specifically mediated by the proteasomal pathway. The addition of oleic acid only modestly blunted the loss of the ^{35}S-labeled ADRP (Fig. 4, *A* and *B*).

We also examined the effects of MG132 and oleic acid treatment on the stabilization of total cellular (*i.e.* lipid-droplet associated) ADRP following the removal of TAG precursors from the cell media (see Fig. 2). In contrast to the differing effects on newly synthesized ADRP, both MG132 and exogenous oleic acid fatty acid efficiently "preserved" total levels of ADRP protein during a similar time course as compared with cells cultured in the absence of lipid precursors (Fig. 4*C*). These results suggest that ADRP may be stabilized by a co-translational association with lipid droplets, whereas unassociated ADRP is preferentially subjected to proteasomal degradation.

Growth of Cells in the Presence of Fatty Acids Attenuates ADRP Polyubiquitination—Proteins that are targeted for proteasomal degradation are generally conjugated with polyubiquitin chains, forming the high molecular weight complexes that are delivered to proteasomes for further processing (38). Proteasome inhibitors do not prevent ubiquitination but block the ubiquitinated proteins from attaching to proteasome particles. We treated cells for 24 h with or without 10 μM MG132 or 10 μM lactacystin and performed immunoprecipitation using antibody against ubiquitin followed by immunoblotting with antibody against ADRP (Fig. 5*A*). In addition, ADRP was immunoprecipitated with α-ADRP and subsequently immunoblotted with α-ubiquitin (Fig. 5*B*). In both cases, polyubiquitin-conjugated ADRP complexes were detected as typically broad patterns from cells treated with MG132 or lactacystin (Fig. 5, *A* and *B*). Such immunoprecipitates were not recognized with rabbit preimmune serum or with rabbit polyclonal antibody against hormone-sensitive lipase, both of which served as negative controls (data not shown). Clearly, these results demonstrated that ADRP was conjugated with polyubiquitin prior to degradation by the proteasomes. Direct immunoblotting with α-ADRP (Fig. 5*C*) showed the appearance of several extra bands upon incubation with 10 μM MG132 for 24 h, which likely reflects polyubiquitinated ADRP. Such polyubiquitination was dramatically attenuated in the presence of oleic acid (Fig.


FIGURE 4. **Effects of MG132 and oleic acid on ADRP protein stability.** *A*, CHO cells transfected with Ad-ADRP were pulse-labeled for 5 h with [^{35}S]methionine and chased for 5 h in serum-free medium in the absence or presence of 10 μM MG132 or 400 μM oleic acid. ^{35}S-labeled ADRP was immunoprecipitated (*IP*) using α-ADRP antibodies and detected by autoradiography. The image shown is representative of four separate experiments. *B*, densitometry analysis of *A*. The values are presented as percentage of ^{35}S-labeled ADRP prior to chase (means $\pm$ S.E.) from four separate experiments. *C*, CHO cells transfected with Ad-ADRP were grown for 5 h and transferred to delipidated medium in the absence or presence of 10 μM MG132 or 400 μM oleic acid. Total ADRP protein was examined by immunoblotting (*IB*) with α-ADRP antibodies. The blot shown is three separate experiments.
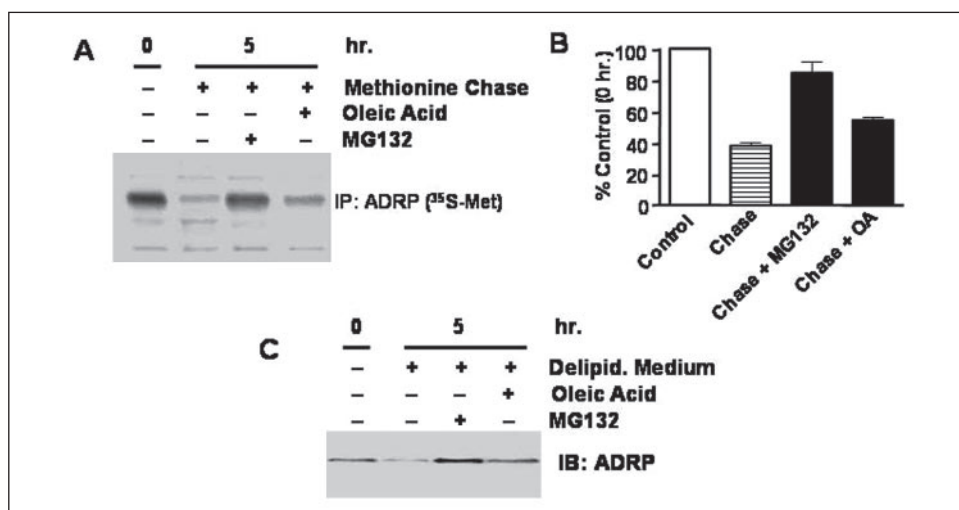
FIGURE 5. **ADRP conjugation to ubiquitin is attenuated by oleic acid.** *A* and *B,* CHO cells transfected with Ad-ADRP were incubated for 24 h with or without 10 μM MG132 or 10 μM lactacystin (*Lact*). Cellular lysates were prepared and immunoprecipitated (*IP*) using α-ubiquitin (α-*Ub*) antibodies followed by immunoblotting (*IB*) with α-ADRP antibodies (*A*) or immunoprecipitated using α-ADRP antibodies followed by immunoblotting with α-ubiquitin antibodies (*B*). *C,* Ad-ADRP infected CHO cells were treated for 12 h with or without MG132 at the indicated concentrations in the absence or presence of 400 μM oleic acid. ADRP protein in the cell lysates was probed with the antibodies against ADRP. The *arrows* indicate presumptive polyubiquitinated ADRP; their appearance was reduced by the addition of oleic acid. All of the blots shown represent at least three individual experiments.
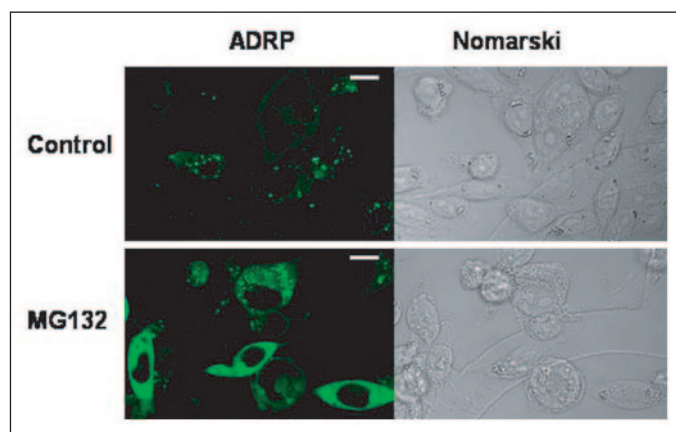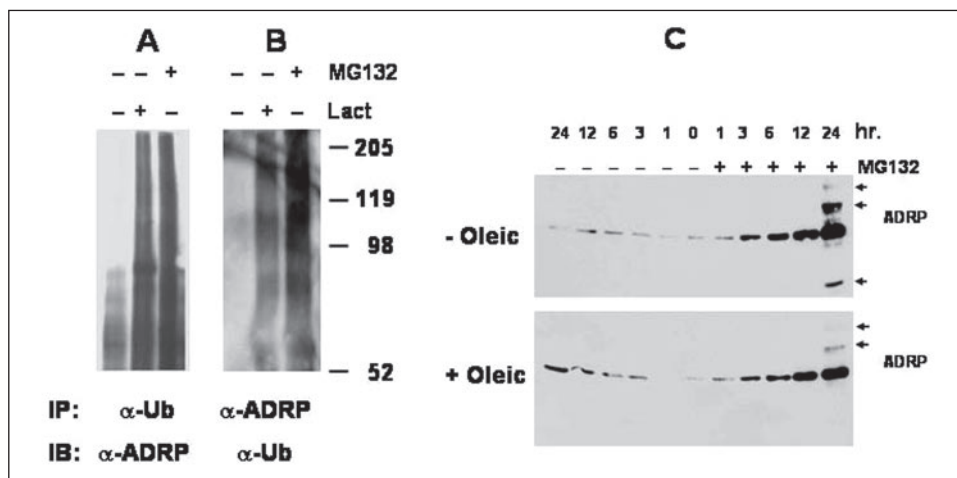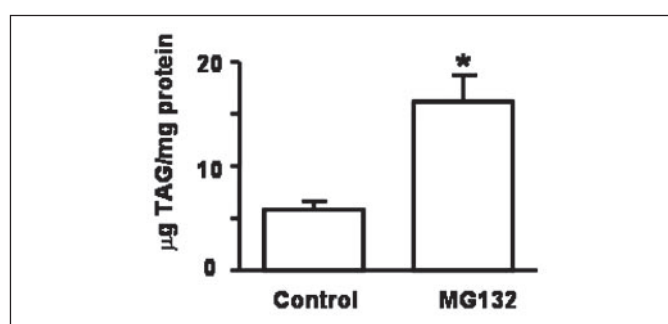

FIGURE 6. **Inhibition of proteasomal degradation with MG132 enhances the immunofluorescent signal of ADRP and increases intracellular lipid droplet formation.** CHO-ADRP cells were treated for 18 h with or without 5 μM MG132. Immunofluorescent staining was performed using antibody against ADRP. *Top*, control cells; *bottom*, 5 μM MG132. *Bars* represent 10 μm in *both panels*. The picture shown is representative of at least five separate experiments.

FIGURE 7. **Inhibition of proteasomal degradation of ADRP is accompanied with an increase in total cellular triacylglycerols.** CHO cells transfected with Ad-ADRP were grown in 6-well plates treated with or without 2.5 μM MG132 for 24 h. Total lipids were extracted from cells with chloroform and methanol and the mass of total triacylglycerols was measured in triplicate and normalized with total cellular protein content. Data are presented as the means $\pm$ S.E. of four separate experiments performed in triplicate. *, $p < 0.02$ compared with control.

5*C, bottom*) suggesting that lipid droplet stabilization of ADRP reduces ubiquitination.

Elevation of ADRP Accumulation by MG132 Is Associated with an Increase in Total Cellular Triacylglycerols—We have demonstrated that ADRP protein levels are regulated by the cellular capacity to store TAGs. We thus sought to determine whether, reciprocally, the up-regulation of ADRP promoted intracellular lipid storage. Treatment with MG132 greatly increased ADRP protein levels and the cellular immunofluorescence of ADRP (Fig. 6). We also noticed a change from the specific association of ADRP with intracellular lipid droplets to a wider distribution throughout the cytosol. This broad distribution may reflect the association of ADRP with very small lipid droplets, an increased association with an elevated pool of intracellular of fatty acids (25, 27), or the accumulation of unassociated, polyubiquitinated ADRP. However we observed that cells that were treated with 2.5 μM MG132 for 24 h (Fig. 7) and that possess elevated ADRP protein accumulated significantly higher levels of total TAG (~2.8-fold) as compared with untreated controls. Thus, increasing ADRP protein by inhibiting its degradation may promote intracellular TAG synthesis, data that are consistent with observations that link ADRP and lipid accumulation (30).

Disappearance of ADRP Protein during the Differentiation of 3T3-L1 Adipocytes Is Mediated by Proteasomal Degradation—*ADRP* mRNA expression is up-regulated early during differentiation of 3T3-L1

adipocytes (39). Shortly thereafter *perilipin* gene expression is induced, and the intracellular lipid droplets acquire a coating of perilipin. Although *ADRP* mRNA levels remain elevated, ADRP protein is no longer detected (9). We speculated that perilipin association with lipid droplets precludes the binding of ADRP, thus promoting its proteasomal degradation. To test this hypothesis, we added the specific proteasome inhibitor MG132 to differentiating 3T3-L1 adipocytes at day 8 of differentiation, a time when perilipin expression is elevated and ADRP protein has disappeared (Fig. 8). Clearly, the proteasome inhibitor promoted the accumulation of ADRP protein in the maturing 3T3-L1 adipocytes (Fig. 8), indicating that the disappearance of ADRP protein late during differentiation results from proteasome-mediated degradation.

DISCUSSION

In the present study we show that exogenous oleic acid enhances the accumulation of ADRP protein in CHO cells. This also promotes the deposition of TAG in intracellular neutral lipid droplets, which provide stabilization sites for ADRP association. Conversely, deprivation of fatty acids from the culture medium leads to a loss of ADRP, a process antagonized by MG132, a specific proteasome inhibitor. MG132 completely blocked the degradation of endogenous ADRP without changing *ADRP* mRNA levels, suggesting a post-translational regulation of ADRP. Only proteasome-specific inhibitors prevented ADRP degradation in either ADRP-overexpressing or native
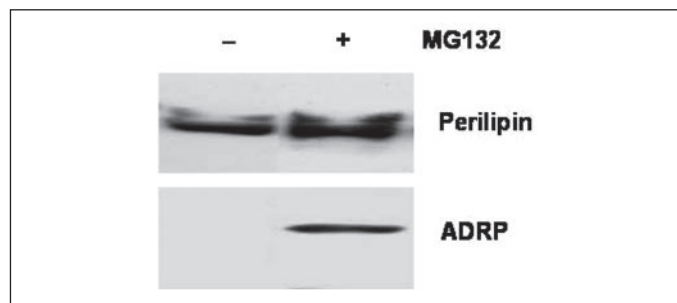
FIGURE 8. **Disappearance of ADRP in differentiated 3T3-L1 adipocytes is due to the proteasomal degradation of the protein.** 3T3-L1 fibroblast cells were grown to full confluence in 12-well plates and differentiated into adipocytes. At day 8 of differentiation, the cells were treated with or without 5 μM MG132 for 18 h. Adipocyte lysates were prepared, separated on SDS-PAGE, and subjected to immunoblotting using the primary antibodies against ADRP and perilipin. The blot shown represents three separate experiments.

CHO cells. Protease inhibitors directed at alternate degradative processes had no effect.[7]

In pulse-chase experiments, [^{35}S]methionine-labeled ADRP reached a maximum at the end of the pulse and then continually declined during the chase period. Under these conditions, newly synthesized ADRP has a half-life of ~2 h. This is in contrast to lipid droplet-associated perilipin, which has a half-life of nearly 12–24 h.[8] MG132 significantly blocked the loss of [^{35}S]methionine-labeled ADRP, confirming that ADRP is degraded through the proteasome pathway.

Polyubiquination is a step in targeting proteins for degradation by the 2000-kDa, ATP-dependent, 26 S proteasome complex. Ubiquitin is a small peptide that marks proteins through a covalent bond between glycine and lysine residues on the target protein. By regulating protein degradation through ubiquitination, cells can quickly eliminate the function of proteins in response to the changes in cellular environment (33–35). Proteasomal inhibitors do not prevent ubiquitination but block the binding of ubiquitinated proteins to proteasomes resulting in the accumulation of polyubiquitinated proteins. These usually exhibit a smear of high molecular mass by SDS-PAGE (37, 38). Deubiquitinating enzymes serve to rescue ubiquitinated proteins from degradation (35). As the amount of native ADRP is elevated upon addition of proteasome inhibitors, we detected the co-immunoprecipitation of ADRP and ubiquitin, confirming that ADRP is conjugated to polyubiquitin and targeted for degradation.

We showed previously that fatty acid loading of Y-1 adrenal cortical cells greatly increases perilipin protein without a corresponding increase in *perilipin* mRNA (8). We speculated that the increase in perilipin protein results from a post-translational stabilization of newly synthesized perilipin by association with the stored neutral lipids. Similarly, we reported that the addition of fatty acids to isolated rat lung lipofibroblasts leads to a large increase in ADRP protein without a change in *ADRP* mRNA (28), again suggesting post-translational regulation of ADRP protein. Fatty acids are precursors for the intracellular neutral lipid droplets that provide surface sites for ADRP binding (9). The present study shows that delipidation of the serum deprives the cells of fatty acid precursors. Under these conditions ADRP is degraded by proteasomes. The addition of oleic acid increases TAG accumulation and dramatically attenuates the appearance of polyubiquitinated ADRP.

In 3T3-L1 adipocytes, ADRP protein is present early during differentiation but disappears after the onset of perilipin expression, even in the face of elevated *ADRP* mRNA expression (9). Recent studies have revealed that ADRP and perilipin compete for space on the lipid droplet surface. We surmise that intracellular lipid droplets have limited surface space to bind either perilipin or ADRP. Perilipin attachment thus precludes the association of ADRP with the droplets (20, 40). Now we have demonstrated that as perilipin protein prevails in mature 3T3-L1 adipocytes, ADRP protein is degraded by proteasomes. ADRP stability can be preserved by proteasomal-specific inhibition. Thus, proteasomal degradation of ADRP under a variety of conditions in either fibroblasts or adipocytes is an essential mode of regulation. In additional experiments, we found that perilipin is also degraded by proteasomes, as evidenced by its ubiquitination and protection by proteasomal-specific inhibitors.[9] The regulation of lipid-associated proteins by the ubiquitin/proteasome pathway is not limited to the PAT family. Apolipoprotein B, which is co-translationally inserted into nascent lipoproteins, is also degraded by proteasomes in the absence of adequate lipid products (41–46). Similarly, lipoprotein A (47) and apolipoprotein E (48) are subject to proteasomal degradation. This regulatory pathway may be a common mode for modulation of lipid-associated proteins.

It has been reported that ADRP is able to bind stochiometrically to fatty acids and to transport long chain fatty acid into cells (25, 27). As a specific marker of lipid accumulation, ADRP is also proposed to play a role in the formation of intracellular lipid droplets (23, 29, 30). Inhibition of ADRP degradation with MG132 leads to enhanced ADRP association with lipid droplets and, suprisingly, increased ADRP in the cytosol. This increased cytosolic ADRP may represent an increase in lipid droplets (25, 27) or, alternatively, accumulation of polyubiquinated ADRP that does not associate with droplets. Currently there is no method of distinguishing between the intracellular localization of native ADRP and its ubiquitinated species, but we have demonstrated a 2.8-fold elevation of total TAG in the cells that possess increased ADRP upon the inhibition of proteasomal degradation. This suggests a role for ADRP to facilitate intracellular TAG synthesis and lipid accumulation.

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