Live Cell Analysis and Targeting of the Lipid Droplet-binding Adipocyte Differentiation-related Protein\textsuperscript{*}\textsuperscript{,}\textsuperscript{**}

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Neutral lipid is stored in spherical organelles called lipid droplets that are bounded by a coat of proteins. The protein that is most frequently found at the surface of lipid droplets is adipocyte differentiation-related protein (ADRP). In this study, we demonstrate that fusion of either the human or mouse ADRP coding sequences to green fluorescent protein (GFP) does not disrupt the ability of the protein to associate with lipid droplets. Using this system to identify targeting elements, discontinuous segments within the coding region were required for directing ADRP to lipid droplets. GFP-tagged protein was employed also to examine the behavior of lipid droplets in live cells. Time lapse microscopy demonstrated that in HuH-7 cells, which are derived from a human hepatoma, a small number of lipid droplets could move rapidly, indicating transient association with intracellular transport pathways. Most lipid droplets did not show such movement but oscillated within a confined area; these droplets were in close association with the endoplasmic reticulum membrane and moved in concert with the endoplasmic reticulum. Fluorescence recovery analysis of GFP-tagged ADRP in live cells revealed that surface proteins do not rapidly diffuse between lipid droplets, even in conditions where they are closely packed. This system provides new insights into the properties of lipid droplets and their interaction with cellular processes.

In mammalian cells, lipid droplets serve as storage organelles, consisting primarily of cholesterol ester and triacylglycerols (reviewed in Ref. 1). Although long considered to be inert structures, there is now increasing evidence that they play active and diverse roles in the life cycle of cells. Recently, they have been implicated in maintenance of intracellular cholesterol balance and transport of lipids through association with caveolin proteins (2). Moreover, the lipid stored in droplets (3); mammary epithelial cells release milk fat globules from their apical surface that are directly derived from aggregated lipid droplets (4); lipid droplets appear to be the primary source of fatty acids that are converted into triacylglycerols and incorporated into very low density lipoprotein in hepatocytes (5). There is also a correlation between particular human diseases and accumulation of lipid droplets that suggests they are markers of pathological changes. Such diseases include atheroma, steatosis, obesity, and some cancers (6, 7). More recently, it has been suggested that aberrant targeting of the Nir2 protein to lipid droplets may induce changes to lipid transport that could correlate with retinal degeneration seen in Drosophila mutants (8). Indeed, lipid droplets may have important functions in viral and parasitic infections (9–13).

The surface of lipid droplets has a proteinaceous layer that is thought to prevent fusion with any adjacent lipophilic surface. However, analysis of the properties of surface proteins has received only limited attention. The most widely characterized lipid droplet-associated proteins are the perilipins, a family of polypeptides generated by alternative splicing from a single copy gene (14), and adipocyte differentiation-related protein (ADRP,\textsuperscript{1} also termed adipophilin) (6, 15–17). Expression of the perilipins appears restricted to adipocytes and steroidogenic cells (Refs. 18 and 19, reviewed in Ref. 20) whereas ADRP is detected in a broad range of different tissues (15). Examination of a variety of tissue culture cells also has revealed ADRP as a ubiquitous component of lipid droplets (6, 15). Moreover, enhanced expression of ADRP is a useful marker for pathologies that are characterized by increased accumulations of lipid droplets (6, 21–24). Hence, analysis of ADRP would provide valuable insight into the nature of proteins that associate with lipid droplets. In addition, ADRP offers potential for describing the characteristics of lipid droplets.

In this study, we have fused green and yellow fluorescent proteins (EGFP and EYFP) to the human and mouse ADRP coding sequences and examined the ability of the fusion proteins to associate with lipid droplets in tissue culture cells. This system was employed to identify sequences in ADRP that are required for localization to lipid droplets. ADRP tagged with fluorescent proteins also was used to analyze the behavior of lipid droplets in live cells and their interaction with other intracellular compartments.

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\textsuperscript{\textcircled{\textcircled{\textdagger}}} The abbreviations used are: ADRP, adipocyte differentiation-related protein; hADRP, human ADRP; mADRP, mouse ADRP; EGFP, enhanced green fluorescent protein; EYFP, enhanced yellow fluorescent protein; ORP, open reading frame; PBS, phosphate-buffered saline; RT, reverse transcriptase; HCV, hepatitis C virus; FRAP, fluorescence recovery after photobleaching.

\textsuperscript{1} The on-line version of this article (available at http://www.jbc.org) contains Videos 1–3.

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ADRP and Lipid Droplets

MATERIALS AND METHODS

Generation of Human and Mouse ADRP cDNA—ADRP gene-specific primers (primers 1 and 2, Table I) were designed from the human ADRP (hADRP) mRNA sequence (GenBank™ accession number BC005127). In conjunction with the Access RT-PCR System (Promega, United Kingdom), these primers generated hADRP cDNA from total RNA prepared from the human hepatoma cell line, HuH-7. Total RNA was made using Trizol reagent (Sigma) according to the manufacturer’s instructions. The hADRP cDNA was ligated to pGEM-T Easy (Promega) and, from the resultant progeny after transformation, plasmids were screened for the presence of hADRP sequences by restriction endonuclease digestion. Plasmid DNA from one positive bacterial clone, termed pLA1, was isolated and the nucleotide sequence of the hADRP cDNA was determined. For the mouse ADRP (mADRP) cDNA, an amplified product was generated using mRNA isolated from mouse L cells and primers 3 and 4 (Table I) that were derived from published murine sequences (GenBank™ accession number NM007408; Ref. 12). The PCR product was inserted into pCR1-TOPO (Invitrogen). A clone containing a mADRP cDNA insert, pCR1/mADRP, was selected by restriction enzyme analysis and the nucleotide sequence of the inserted fragment was determined.

Construction of Plasmids Expressing DNase I, Human and Mouse ADRP Fused to Fluorescent Proteins—The hADRP ORF was excised from pLA6 and ligated to pEGFP-C1 (Clontech) to generate a plasmid termed pLA7 that encoded a fusion protein consisting of the hADRP ORF and the green fluorescent protein (GFP). For the mouse ADRP (mADRP) DNase I fusion protein, the same strategy was employed to fuse the hADRP ORF to mADRP (mDNase I). For expression of mADRP, a pGFP-mADRP—HuH7 and Vero cells were propagated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, non-essential amino acids, and 100 IU/ml penicillin/streptomycin. To generate cells constitutively expressing GFP-mADRP, a 10% EphGFP-mADRP was transfected with pGFP-mADRP. Cells expressing GFP-mADRP were selected first by pooling GFP fluorescent cell populations isolated by fluorescent-activated cell sorting followed by growth of individual colonies. Colonized cell lines were maintained in media containing 500 µg/ml G418.

Preparation of Cell Extracts, Polyacrylamide Gel Electrophoresis, and Western Blot Analysis—To prepare extracts, transfected HuH-7 cells were harvested by removing growth medium and washing the cell monolayers with PBS. Cells were solubilized in sample buffer, consisting of 160 mM Tris-HCl, pH 6.7, 2% SDS, 700 mM β-mercaptoethanol, 10% glycerol, and 0.004% bromphenol blue, at a concentration of ~2 x 10^6 cells/ml sample buffer. The samples were heated at 100 °C for 5 min to denature proteins and nucleic acids prior to electrophoresis through a 10% polyacrylamide gel prepared using 0.1% acrylamide, 0.1% bisacrylamide (37.5:1) stock solution (Bio-Rad). For Western blot analysis, proteins, separated on polyacrylamide gels, were transferred to nitrocellulose membrane. After blocking with PBS containing 5% milk powder (Marvel) and 0.05% Tween 20, membranes were incubated with the Living Colors™ full-length A. v. polyclonal antibody (Clontech), diluted 1:2000 in PBSA containing 5% milk powder (Marvel) and 0.05% Tween 20. After washing, bound antibody was detected using protein A-peroxidase (Sigma) followed by enhanced chemiluminescence.

Indirect Immunofluorescence, Staining of Lipid Droplets, and GFP Fluorescence—Cells on 13-mm cover slips were fixed with 3% paraformaldehyde in PBS for 20 min at room temperature. Following washing with PBSA/C (PBS containing 1% newborn calf serum), cells were incubated with primary antibody (diluted in PBSA/C at 1:200 and 1:500 for Living Colors™ and ADRP antiserum, respectively) for 2 h at room temperature. Cells were washed extensively with PBSA/C and then incubated with conjugated secondary antibody (either anti-rabbit or anti-sheep IgG) for 2 h at room temperature. After washing with PBSA/C and PBS, cells were rinsed finally with H2O before mounting on slides using Citifluor (Citifluor Ltd.). Samples were analyzed using a Zeiss LSM confocal microscope. ADRP antiserum was raised in sheep against a synthetic peptide comprised of amino acid residues 5-27 of hADRP conjugated to keyhole limpet hemocyanin.

In vitro syntheses of lipid droplets were stained with oil red O (Sigma) in paraformaldehyde-fixed cells as described previously (11, 12). For staining of lipid droplets with Bodipy 558/568 C18 (Molecular Probes Europe BV, The Netherlands), cells were incubated with the dye at a final concentration of 20 µg/ml for 45 min at 37 °C in PBS, rinsed to remove excess stain followed by a further incubation of 60 min at 37 °C in cell culture media. Cells were either viewed live or fixed and mounted onto glass coverslips with the Zeiss LSM510 confocal microscope to provide standard viewing conditions. The intracellular distribution of GFAP was determined for 100 cells expressing each of the GFP-hADRP variants.

Acquisition of Live Cell Data—To obtain still images of live cells and
for FRAP analysis, coverslips were placed inverted on a microscope slide and analyzed using a Zeiss LSM510 confocal microscope. For photobleaching studies, selected regions of cells were bleached at 100% laser power (488 nm laser line). Before and after photobleaching, images were taken at 7-s intervals using 2% laser power. Time-lapse imaging was performed with an Ultraview Realtime Confocal imaging system (PerkinElmer Life Sciences) in which the microscope was contained within a temperature-controlled chamber. Imaging was performed at 37 °C in pre-warmed minimal essential medium lacking phenol red supplemented with 30 mM HEPES.

Data Analysis—Quantitative analysis of photobleaching experiments was performed using Zeiss LSM510 software. Images captured using the Ultraview system were converted to 8-bit TIFF files using a routine written in Interactive Data Language (created by T. Zimmerman, EMBL) and thereafter analyzed with ImageJ software (National Institutes of Health, Bethesda, MD). Quicktime videos were assembled using ImageJ and still images compiled using Adobe Photoshop version 6 and Adobe Illustrator version 10.

On-line Supplemental Material—Videos 1 and 2 were assembled from images of HuH-7 cells expressing GFP-mADRP taken at a frame rate of 1 per s and an exposure time of 250 ms. Video 3 was assembled from images of Vero cells that had been microinjected with plasmids expressing GFP-hADRP (pLA4) and GFP-DNase X. 8 h after injection, images were taken at 6-s intervals with an exposure time of 600 ms. Cells were maintained in a humidified atmosphere at 37 °C during the imaging process. Imaging was performed using an Ultraview Realtime Confocal imaging system (PerkinElmer Life Sciences).

RESULTS

ADRP Fused to EGFP Associates with Lipid Droplets—To clone both the human and mouse cDNA sequences for ADRP, primers were derived from the nucleotide sequences flanking the coding region for human and mouse ADRP (Table I). Template mRNA for reverse transcription was prepared from human hepatoma HuH-7 and mouse L cells. Following reverse transcription, amplification by PCR and cloning, full-length copies of the coding region for both human and mouse ADRP were obtained. Sequence analysis of the entire coding region for one clone of both human and mouse ADRP revealed only a single nucleotide change in each that was different from sequences deposited in the GenBank data base but these did not alter the predicted amino acid sequences. For localization studies, DNA fragments encoding human and mouse ADRP were ligated in-frame to the 3′ end of EGFP, thereby generating GFP-hADRP (expressed by plasmid pLA4) and GFP-mADRP fusion products. Previous studies have shown that ADRP is located at the surface of cytoplasmic lipid droplets (6, 15). To examine whether the GFP-ADRP products for both species retained the capacity to associate with these structures, constructs expressing the fusion proteins were introduced into HuH-7 cells by transfection. In the case of GFP-mADRP, transfected cells were selected by G418 selection to isolate clonal cell lines that constitutively expressed the fusion protein. HuH-7 cells contain numerous lipid droplets that can be identified by staining with lipophilic dyes and detection by antisera that is specific for ADRP at the surface of the structures (Fig. 1A, panels i and v). Compared with EGFP, which shows staining throughout HuH-7 cells (Fig. 1A, panels ii, iii, and iv), GFP-mADRP was located at the surface of spherical intracellular structures in live cells (Fig. 1A, panel iii). To demonstrate that these structures represented lipid droplets, fixed cells were probed with Living Colors antiserum and an antiserum that could recognize the endogenous human form of ADRP but not the corresponding mouse protein that was a component of the GFP fusion product (Fig. 1A, panels ii–vi, and data not shown). The localization for endogenous hADRP and the GFP-mADRP products coincided (Fig. 1A, panel vi), indicating that GFP-mADRP was located at the surface of lipid droplets. This result also demonstrated that it was possible to detect tagged and untagged ADRP on the same lipid droplet. Therefore, sufficient attachment sites are present on lipid droplets to accommodate both forms of ADRP. To verify the localization for GFP-mADRP, cells expressing the fusion protein were incubated with Bodipy 558/568 dodecanoic acid, a fluorescent fatty acid analogue that is sequestered in lipid droplets (26, 27). This dye was selected because its spectral properties allow discrimination from EGFP by confocal microscopy and staining of droplets does not require treatment of cells with alcohols, which is necessary for oil red O staining and induces fusion of lipid droplets. Results in both live cells (data not shown) and cells fixed with paraformaldehyde showed specific localization of GFP-mADRP at the surface of lipid droplets stained with the Bodipy dye (Fig. 1A, panels vii–ix). The apparent molecular weight for GFP-mADRP was estimated at about 80,000, which approximates to the predicted size for the fusion protein (Fig. 1B, lane 1). GFP-hADRP, produced by plasmid pLA4, also was detected at the surface of lipid droplets and had an estimated size that agreed with its predicted molecular weight (Figs. 2A, panel i, and 3, lane 2). Localization of human and mouse ADRP fusion proteins was neither influenced by the methods used to introduce plasmids into cells (e.g. microinjection and electroporation; data not shown) nor by cell type (Fig. 5). Therefore, linking EGFP to the N terminus of ADRP does not affect its intracellular localization. Attempts to fuse EGFP to the C terminus of ADRP gave few cells that produced fluorescence of the chimeric protein, and the fluorescent protein that was expressed was not present at the surface of lipid droplets (data not shown). Consequently, ADRP with EGFP fused to the N terminus was used in further studies of the human and mouse forms of the protein.

Efficient Localization to Lipid Droplets Requires Discontinuous Sequences within the ADRP Coding Region—In the absence of any previous analysis of the sequences required for lipid droplet association, we made a series of constructs that removed portions of the hADRP coding region and examined

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**Table I**

| Oligonucleotide | Sequence (5′→3′) | Position in ADRP nucleotide sequence | Application |
|-----------------|-----------------|------------------------------------|-------------|
| Primer 1        | GGGGCAGGTATTAAATGACTTATG | 1385–1408<sup>a</sup> | RT-PCR amplification of hADRP cDNA |
| Primer 2        | CCCAGGAAGAAAATGGCAGTCGTT | 74–94<sup>b</sup> | RT-PCR amplification of hADRP cDNA |
| Primer 3        | CTATGGCAGGACGAGCTGACGCCGTC | 79–102<sup>b</sup> | RT-PCR amplification of mADRP cDNA |
| Primer 4        | TCATCTGGCCACCAACATCTGCT | 1375–1398<sup>b</sup> | RT-PCR amplification of mADRP cDNA |
| mADRP1          | AGCTGGATCTCCAGTGCGACGATGTA | 79–96<sup>b</sup> | Restoration of 5′ coding sequences for mADRP |
| Mut1            | TCTCTAGCTCTCAATGGAGG | 662–682<sup>b</sup> | Generation of pLA10 |
| Mut2            | GTCTAGACCATCTACATCAG | 584–604<sup>b</sup> | Generation of pLA9 |
| Mut3            | CCCCCGTTTGCATGCTGCACTC | 494–514<sup>a</sup> | Generation of pLA13 |
| Mut4            | TGATGTGACGAGCAAGAAGGT | 224–244<sup>a</sup> | Generation of pLA11 |
| Mut5            | GACCTACGTTCTACAC | 164–184<sup>a</sup> | Generation of pLA12 and pLA17 |
| Mut6            | AGACAGAGGCGGCACTGCTAAAC | 737–757<sup>a</sup> | Generation of pLA11, pLA12, and pLA17 |

<sup>a</sup> Sequences were derived from GenBank™ accession number BC005127.<br>
<sup>b</sup> Sequences were derived from GenBank™ accession number NM007408.
the intracellular distribution of the resultant GFP-hADRP variants by fluorescence microscopy. From observations over a series of experiments, three distinct patterns of GFP-hADRP fluorescence were identified and these were used to categorize the behavior of the fusion proteins (Table II). Cells with Class A distribution represented association of GFP-hADRP with the surface of lipid droplets with little or no fluorescence elsewhere within the cell (Fig. 2A, panel i). For Class C distribution, fluorescence was observed throughout the cell with no specific staining around lipid droplets (Fig. 2A, panel iii). Class B distribution was a combination of fluorescence at the surface of lipid droplets and diffuse intracellular fluorescence (Fig. 2A, panel ii). It was assumed that this distribution was a partial phenotype and that targeting was less efficient but not abolished with such GFP-hADRP species. The three types of distribution were not time-dependent as identical patterns of fluorescence were observed over a series of experiments. Cells with Class A distribution represented association of GFP-hADRP with the surface of lipid droplets with little or no fluorescence elsewhere within the cell (Fig. 2A, panel i). For Class C distribution, fluorescence was observed throughout the cell with no specific staining around lipid droplets (Fig. 2A, panel iii). Class B distribution was a combination of fluorescence at the surface of lipid droplets and diffuse intracellular fluorescence (Fig. 2A, panel ii). It was assumed that this distribution was a partial phenotype and that targeting was less efficient but not abolished with such GFP-hADRP species. The three types of distribution were not time-dependent as identical patterns of

**Fig. 1. Localization and detection of GFP-mADRP in HuH-7 cells.** A, cells constitutively expressed EGFP (panel ii) and GFP-mADRP (all panels except for panel ii). Panels show cells viewed under the following conditions: panel i, cells were fixed with paraformaldehyde and stained with oil red O; panels ii and iii, cells were viewed live and protein was detected directly by GFP fluorescence; panels iv–vi, cells were fixed with methanol and probed with anti-Living Colors™ (panel iv) and anti-ADRP antisera (panel v); panels vii and viii, cells were incubated with Bodipy 558/568 C12 and then fixed with 4% paraformaldehyde prior to analysis of GFP (panel vii) and Bodipy fluorescence (panel viii). Panels vi and ix are merged images of panels iv and v and panels vii and viii, respectively. Cells in panels i–vi are at the same scale and the size bar in panel i represents 10 μm. The size bar in panel vii represents 5 μm. B, Western blot analysis of GFP-mADRP and EGFP expressed in HuH-7 cells. Extracts from cells constitutively producing GFP-mADRP (lane 1) and EGFP (lane 2) were run on a 12% polyacrylamide gel and the proteins were transferred to nitrocellulose membrane following electrophoresis. The membrane was probed with anti-Living Colors™ antiserum. Protein size markers are indicated to the left of the blot and bands corresponding to GFP-mADRP and EGFP are indicated by filled boxes.
target to lipid droplets with roughly equal proportions of cells showing either Class A or Class B distribution and only 8% of cells with a Class C distribution (Table II). By contrast, pLA14 produced a protein that had diffuse fluorescence alone in 94% of cells examined, and only a small proportion of cells with some targeting to lipid droplets. Hence, the N-terminal 222 residues of hADRP are sufficient for localization with lipid droplets with an efficiency that is only slightly lower than that for the full-length protein. To further define the regions of ADRP responsible for this distribution, three other C-terminal truncated GFP-hADRP constructs, pLA10, pLA9, and pLA13, were generated that had stop codons inserted following nucleotides encoding amino acid residues 195, 169, and 139, respectively (Fig. 2B). The fusion protein made by pLA10 behaved identically to that made by pLA5 (Table II). However, there was a change in the overall distribution with the protein produced by pLA9 such that 57% of cells displayed only diffuse fluorescence (Table II). Targeting to lipid droplets along with diffuse fluorescence was found in 43% of cells but no cells displayed exclusive localization of GFP-tagged protein on lipid droplets. Further truncation of the coding region to amino acid residues 139 (pLA13) effectively abolished targeting to lipid droplets (Fig. 2B, Table II). We conclude from this data that sequences between amino acids 139 and 195 have a role in the association of ADRP with lipid droplets.

The above results identified the C-terminal boundary for sequences that are sufficient to direct ADRP to lipid droplets. To examine whether the N-terminal region of ADRP had a role in targeting, two constructs, pLA11 and pLA12, which lacked sequences for amino acids 1–28, respectively, were

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**Fig. 3.** Apparent molecular weight and relative abundance of wild-type and variant forms of GFP-hADRP. HuH-7 cells were transfected with plasmid DNA for 18 h, stained with Bodipy 558/568 C12, and then fixed with 4% paraformaldehyde. Plasmids used for transfection were: panels i and iv, pLA4; panels ii and v, pLA17; panels iii and vi, pLA12. Panels i–iii show GFP fluorescence and Bodipy staining is illustrated in panels iv–vi. Arrows indicate corresponding regions in each cell with lipid droplets. All images are at the same scale and the size bar in panel i represents 10 μm. B, schematic diagram of the regions of hADRP expressed in each of the plasmids. The segments that correspond to PAT-1 (dark boxed region), PAT-2 (light boxed region), and that have sequence identity with tandem repeats in the S3–12 membrane protein are shown. Numbers indicate amino acid residues in the hADRP coding region.

| Construct   | Class A (%) | Class B (%) | Class C (%) |
|-------------|-------------|-------------|-------------|
| pEGFP-C1    | 0           | 0           | 100         |
| pLA4        | 70          | 26          | 4           |
| pLA5        | 47          | 45          | 8           |
| pLA14       | 0           | 6           | 94          |
| pLA10       | 49          | 46          | 5           |
| pLA9        | 0           | 43          | 57          |
| pLA13       | 0           | 3           | 97          |
| pLA11       | 0           | 0           | 100         |
| pLA12       | 0           | 0           | 100         |
| pLA17       | 0           | 59          | 41          |
| pLA22       | 66          | 31          | 3           |
| pLA29       | 16          | 64          | 20          |

* Percentage of cells with exclusive association with lipid droplets.  
* Percentage of cells with a mixture of association with lipid droplets and diffuse fluorescence.  
* Percentage of cells with only diffuse fluorescence.
derived from plasmid pLA5 (Fig. 2B). Proteins made by these constructs failed to show any association with lipid droplets and were distributed throughout the cell (Table II). The requirement for N-terminal sequences in targeting was further tested by restoring the C-terminal coding region to plasmid pLA12, generating pLA17 (Fig. 2B). The resultant protein was found at the surface of lipid droplets in 59% of cells examined but diffuse fluorescence was also present in these cells (Table II). The remaining 41% of cells showed no targeting to lipid droplets. We conclude that sequences within the N-terminal 28 amino acids have a role in efficient association of ADRP with lipid droplets. However, the data also suggest that there is redundancy in the sequences that are necessary for lipid droplet association. In particular, it would appear that there are targeting elements in the C-terminal domain but these sequences alone are insufficient to direct ADRP to lipid droplets.

Our results above showed that the signals required for efficient association of ADRP with lipid droplets were distributed along the protein and it was possible that these were organized as a contiguous targeting element. Therefore, pLA22 was constructed that lacked amino acids 61–130 (Fig. 2B). The protein made by pLA22 behaved almost identically to pLA4, which expressed full-length hADRP. 66% of cells had GFP-hADRP made by pLA22 exclusively on lipid droplets and a lower proportion (31%) in which the protein was both present on lipid droplets and diffuse within the cell (Table II). This showed that the sequences for targeting to lipid droplets are not continuous within ADRP. As a further test of a role for amino acids between residues 131 and 220 in directing efficient association with lipid droplets, we made plasmid pLA29, which lacked amino acids 61–220 (Fig. 2B). The resultant protein was found with lower abundance at the surface of lipid droplets but targeting was not abolished (Table II). This highlights again the redundancy in the sequences within ADRP that are needed for efficient association with lipid droplets because the results with pLA13 would predict that removal of a region between amino acids 139 and 220 should abrogate targeting.

To verify that any reduced association of the variant forms of GFP-hADRP with lipid droplets was not a consequence of proteolysis, the fusion proteins produced by each of the constructs were examined by Western blot analysis using Living Colors™ antiserum to estimate their apparent molecular weights (Fig. 3). Our analysis showed that the apparent molecular weights of the proteins correlated with their predicted molecular weights. We conclude that any disruption to the ability of GFP-hADRP mutants to associate with lipid droplets is not a consequence of cleavage within the fusion proteins that may remove targeting sequences. The differences in abundance of GFP-hADRP between individual mutants was because of either variable transfection efficiency or different levels of transcription for individual plasmids because the amount of RNA detected in transfected cells correlated with the relative abundance of the proteins seen in Fig. 3 (data not shown).

Mobility of Lipid Droplets in Live Cells—Previous studies in live baby hamster kidney cells using an EGFP-tagged caveolin mutant indicated that lipid droplets are relatively immobile structures (2). Given that droplets can provide lipid precursors for specific purposes in certain cell types, including hepatocytes, their behavior may differ between cell types. Hence, we sought to re-examine their mobility using GFP-mADRP as a marker protein in stably transfected HuH-7 cells. The majority of lipid droplets, particularly those that were larger in size, tended to oscillate around a point of origin but did not migrate by any substantial distance (Fig. 4B, panels i–x; lipid droplets indicated by open arrows; Supplemental Materials, Videos 1 and 2). However, a small number of droplets did display rapid, unidirectional movement that was transient (Fig. 4, A and B, indicated by filled arrows; Supplemental Materials, Videos 1 and 2). For example, the lipid droplet indicated in Fig. 4B moves rapidly for a period of 5 s (panels ii–vi), remains relatively immobile for 2 s (panels vii–viii) and then moves again for a further 2 s (panels viii–x). We estimate that the speed of such droplets is in the range 2–2.5 μm/s and indicates motion along a cellular transport network. In cells treated with nocodazole, we failed to detect any similar rapid movements of lipid droplets (data not shown). Hence, we conclude that such movement is most likely mediated by interaction with microtubules.

Association of Lipid Droplets with the ER—It was considered likely that most lipid droplets, which moved only within a limited area, were attached to another subcellular network other than microtubules. Recent evidence has indicated association between lipid droplets and the ER (2). To examine this possibility directly under live cell conditions, HuH-7 cells were microinjected with plasmids expressing a commercially available YFP-tagged protein that targets the ER and YFP-hADRP. Constructs expressing YFP-tagged proteins were utilized because no plasmid expressing EGFP-ER marker protein was available. Given that the ER and lipid droplets have distinct morphologies, the targeting of the different YFP-tagged proteins was readily discriminated. Imaging in HuH-7 cells indicated that, whereas it was difficult to identify individual strands of the ER membrane in this cell type (Fig. 5, panel i), there was close association between ER and lipid droplets (Fig. 5, panels iii and iv). By comparison, strands of the ER membrane were more readily identified in Vero cells (Fig. 5, panel v) and the cells contained fewer lipid droplets (Fig. 5, compare panels ii and vi). Co-expression of YFP-hADRP and the YFP-ER targeting protein in Vero cells again revealed co-localization of lipid droplets with the ER (Fig. 5, panels vii and viii). This co-localization was particularly evident at peripheral regions of the cell where packing of the ER and lipid droplets was less dense (Fig. 5, panel viii). We further analyzed the extent of this association between lipid droplets and the ER by time-lapse microscopy. Bleaching of EYFP is more rapid than that for EGFP and therefore we used plasmids expressing GFP-hADRP (pLA4) and a GFP-DNase X fusion protein that is
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Fig. 5. Association of lipid droplets with the ER membrane. Live images were taken by confocal microscopy of HuH-7 (panels i–iv) and Vero cells (panels v–viii) microinjected with plasmids that expressed YFP fusion proteins. Cells were microinjected with the following constructs for 6 h before imaging: panels i and r, YFP-ER (Clontech); panels ii and vi, YFP-hADRP; panels iii and vii, YFP-ER and YFP-hADRP. Panels iv and viii show magnified images of the boxed regions in panels iii and vii, respectively. Images in panels i–iii and v–viii are at the same scale and the size bar in panel iv represents 10 μm. Images in panels iv and viii are at the same scale and the size bar in panel iv represents 5 μm.

directed to the ER. Analysis of individual images along with animations from Vero cells expressing both proteins indicated concurrent movement of both the ER and attached lipid droplets (Fig. 6, Supplemental Materials, Video 3; data not shown). This reveals that there is intimate association between the ER and lipid droplets in live cells.

ADRP Does Not Diffuse Rapidly between Adjacent Lipid Droplets—Organelles such as the ER, Golgi, and plasma membrane form continuous intracellular structures that permit rapid diffusion of proteins along membranes. Frequently, lipid droplets are found in tightly packed clusters (see Fig. 5, boxed regions 1 and 4, respectively). After photobleaching, there was little recovery of fluorescence (Fig. 7B); the small amount of recovered fluorescence was observed also in fixed cells and therefore represents refolding of GFP-mADRP (data not shown). This lack of fluorescence recovery was consistent in all other experiments, including conditions where fluorescence was measured up to 10 min after bleaching (data not shown). Hence, we conclude that ADRP does not readily diffuse between droplets.

DISCUSSION

ADRP is a component of lipid droplets found in a broad range of cultured cells and tissues. Accordingly, it is a useful model protein to examine the properties of proteinaceous components of lipid droplets and the innate behavior of these organelles. In our study, we have employed fusion of both human and mouse forms of ADRP with EGFP to study both of these aspects.

Analysis of the sequences required to direct hADRP to lipid droplets revealed that targeting signals are distributed throughout the polypeptide coding region (Fig. 8). In the context of our study, we define targeting as the process through which ADRP is directed to lipid droplets and then forms a stable association with the organelles. Therefore, reduced targeting efficiency could result from the inability of the tagged proteins to attach stably to lipid droplets, possibly through competition with endogenous ADRP. We found that removal of the N-terminal 28 amino acids impaired lipid droplet localization and a second targeting element was located between residues 139 and 220. Sequences that can contribute to efficient association with lipid droplets also were present in the C-terminal region from amino acids 221 to 437 but this segment of the protein alone was not capable of directing the protein to droplets. These regions of the protein do not form a contiguous targeting element because sequences between amino acids 61 and 130 can be removed with no significant impact on the efficiency of lipid droplet association. Our results indicate that removing one of these targeting sequences can impair but not abolish lipid droplet localization, suggesting that there is redundancy in the sequence requirements for efficient association. Thus, identification of dispensable sequences has not been possible. A recent study also indicated redundancy within the sequences that were necessary for directing perilipin to lipid droplets (28). Moreover, simple targeting signals for peroxisomal membrane proteins have not been readily identified and it appears that more than one targeting element may reside within such proteins (29). Therefore, sequence redundancy may be a general feature for directing proteins to the surface of organelles such as peroxisomes and lipid droplets.

It has been proposed that ADRP can be separated into two domains, PAT-1 and PAT-2, based on sequence identity with other lipid droplet-associated proteins (14). PAT-1 is considered to consist of approximately the N-terminal 100 amino acids of the protein and the remaining C-terminal segment is PAT-2 (Fig. 8). Sequence identity between PAT-1 domains for lipid droplet proteins is greater than that for PAT-2. Indeed, using PAT-1 sequences, lipid droplet proteins have been identified putatively in Drosophila melanogaster and Bombyx mori (14). Our analysis shows that the N-terminal 28 amino acids of hADRP, which are necessary for efficient targeting, are contained within PAT-1. However, the other sequences that have been identified as important for efficient lipid droplet association are located in PAT-2. The region within perilipin that has been identified as important for localization to lipid droplets also lies in PAT-2 (28). This indicates that the higher sequence identity in PAT-1 between lipid droplet-associated proteins such as ADRP and perilipin does not necessarily represent conserved targeting sequences. Hence, the sequences that direct these proteins to lipid droplets may be distinct. It has also

3 J. F. Coy and A. Girod, unpublished results.
4 A. I. Taylor and J. McLauchlan, unpublished results.
been observed that a region in the PAT-1 domain of ADRP has sequence identity with a tandem repeat in the plasma membrane protein, S3–12 (20, 30). Our results show that removing this segment from ADRP has no effect on lipid droplet association (Fig. 8), leading us to conclude that any sequence identity with this tandem repeat is not related to targeting of ADRP.

The discontinuous nature of the targeting sequences in ADRP differs from the characteristics of other lipid droplet-associated proteins apart from perilipin. Association of HCV core protein with lipid droplets requires a domain of about 55 amino acids that is present also in a related virus, GB virus-B but not in pesti- and flaviviruses that share the same genomic organization as HCV (11, 12). Apart from a short stretch of amino acids, removal or substitution of amino acids along the length of the domain abolishes lipid droplet localization. In addition, plant oleosins have a central region of 85 amino acids that has all of the sequences required for efficient attachment in mammalian and plant cells; this central region is flanked by domains that appear to be dispensable for lipid droplet association (12, 31). Moreover, the targeting sequences in the HCV core and plant oleosin proteins are hydrophobic, whereas no corresponding segments with similar characteristics are found in either human or mouse ADRP. This suggests that processes, which are different from those for the viral and plant proteins, may guide localization of ADRP to lipid droplets.

In the case of HCV core, trafficking of the protein to lipid droplets involves initial targeting and cleavage of a precursor protein at the ER and core is found both at the ER membrane as well as at the surface of lipid droplets (32). It is likely that transfer of core between the two organelles is facilitated by the attachment of

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**Fig. 7.** FRAP analysis of GFP-mADRP attached to lipid droplets. A, an HuH-7 cell that constitutively produced GFP-mADRP was photobleached in boxed regions 1 and 4. Images were taken at the times indicated by confocal microscopy and bleaching was performed after the image was taken at 7 s. The scale bar in the first panel represents 5 μm. B, fluorescence intensities in photobleached and equivalent non-bleached areas (boxed regions 2 and 3) were measured and plotted against time. Intensity was quantified relative to the fluorescence measured at \( t = 0 \) s for each boxed region. Percentage fluorescence intensity was plotted for each region as follows: region 1, open circles; region 2, open boxes; region 3, closed boxes; region 4, closed circles.

**Fig. 8.** Schematic diagram showing regions in hADRP that contain targeting signals. The segments that correspond to PAT-1, PAT-2, and that have sequence identity with tandem repeats in the S3–12 membrane protein are shown. The hADRP coding region is represented by four domains (I-IV) and numbers indicate the amino acid residues that limit each domain. The contributions of combinations of domains I-IV to intracellular distributions that correspond to Class A (exclusive localization on lipid droplets), Class B (a combination of association with lipid droplets and diffuse localization throughout the cell), and Class C (no specific targeting to lipid droplets) are shown. Regions that were present (+) or absent (−) in the fusion proteins tested are indicated.
lipid droplets to the ER, which we describe from our live cell studies. However, for ADRP, no staining of the ER is found by either indirect immunofluorescence or live cell analysis. The mechanism that controls partitioning of ADRP is presumably a reflection of differences between either components or compositions of lipid droplets and the ER that favor interaction with lipid droplets. In addition, the literature indicates that ADRP is synthesized in a free and not ER-bound polysomes (20). A similar situation has been demonstrated for the perilipins (33). It may be that association of ADRP and perilipin with the ER is not favored to prevent fusion of lipid droplets with the ER after their formation.

Lipid droplets often occur as clusters in a number of cell types. This may indicate that they form a connected network in which transfer of macromolecules between droplets could occur. FRAP is gaining wider use to determine the mobility of GFP-tagged proteins in organelles with continuous surfaces such as the ER, Golgi, and plasma membranes (Refs. 34–36, reviewed in Ref. 37). More recently, FRAP has revealed that clusters of mitochondria are functionally distinct and unconnected (38). From our data, ADRP does not rapidly diffuse between lipid droplets that are apparently in contact with each other. This suggests that droplets exist as discrete entities and that there is little or no transfer of surface proteins between them. As a consequence, there may be little turnover of ADRP at the surface of lipid droplets after their formation.

Analysis of live cell offers advantages for determining the characteristics of lipid droplets. Lipid droplets are heterogeneous in size both between different cell types and even in individual cells. The factors that govern their size are not known but could be determined during their biogenesis. It is also possible that additional neutral lipid is added after formation, either through transfer from the ER or from other lipid droplets. In HuH-7 cells, lipid droplets were no greater than about 1 μm in diameter in live cells using GFP-ADRP as a marker. However, upon fixation and subsequent treatment with alcohol to stain lipid droplets with oil red O, their maximum size increased to 3 μm (data not shown). We propose that this discrepancy results from fusion of closely associated droplets and the size distribution in cells, which are stained using alcoholic solutions, is not authentic. Such an observation may have a bearing on the interpretation of lipid droplet size in cells that store large quantities of neutral lipid and in pathological conditions where lipid storage is assessed. For example, steatosis of the liver can be described as either micro- or macrovesicular depending on the diameter of lipid droplets (39). It is possible that the presence of lipid droplets with larger diameters do not reflect their size in vivo and is an artifact of the fixation process.

Formation of lipid droplets is considered to occur by a budding process at the ER (1, 40). From live cell studies, our results reveal that, following their biogenesis, droplets continue to attach to the ER membrane. As lipid droplets are a reservoir, their close association with the ER may be important for maintenance and expansion of the organelle during the cell cycle. Such an association also allows ready access to a storage reservoir under conditions where excess lipid is either synthesized or absorbed by cells. Thus, lipid droplets may be important to maintain lipid homeostasis and permit normal function of the ER in abnormal situations where lipid is either limiting or in excess. In HuH-7 cells, we observed that a proportion of lipid droplets had movements that were consistent with transient attachment to the microtubule network. Such trafficking was clearly distinct from the movement of droplets that were ER-associated. We presume that brief association of lipid droplets with microtubules indicates movement from one site on the ER to a second site. However, the factors that control lipid droplet association with microtubules are not known. We did not observe similar rapid movement in Vero cells. This cell type contains considerably fewer lipid droplets compared with HuH-7 cells and, because the proportion of lipid droplets that move rapidly in HuH-7 cells is low, it is possible that observations on longer time periods are necessary to identify microtubule-associated movement of lipid droplets in Vero cells. Alternatively, microtubule-related trafficking could be cell type-dependent. As well as acting as a source of lipid for membranes, lipid droplets have specialized functions in certain cell types, which may affect their interactions with cellular processes. For example, milk fat globules released by mammary epithelial cells are derived from lipid droplets secreted from the apical surface and it is proposed that movement of droplets to sites of secretion involve trafficking along microtubules (41). In addition, hepatocytes are a primary source for the production of very low density lipoprotein, and triglycerides stored in lipid droplets provide much of the lipid content in these particles (5). Mechanistically, this process is poorly understood but it may require mobilization of lipid droplets to regions of the ER where lipoprotein assembly occurs. Hepatocytes are the progenitor cells for the human hepatoma from which HuH-7 cells are derived and thus may maintain characteristics for the very low density lipoprotein assembly pathway that involve lipid droplets. Therefore, the association of lipid droplets with microtubules in HuH-7 cells may not be found more generally in other cell types.

In summary, we have identified sequences within ADRP that are important for association with lipid droplets and have demonstrated that the protein can be utilized to examine the properties of these organelles. This has allowed analysis of the interaction between lipid droplets and other cellular processes. From our work and other studies where association with mitochondria, intermediate filaments (6, 42), and peroxisomes (43) have been observed, it appears that lipid droplets are not inert sites of lipid storage but actively interconnect with other organelles. Further studies on the consequences of these interactions will help to elucidate the full extent of the contribution of lipid droplets to cellular metabolism.

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Live Cell Analysis and Targeting of the Lipid Droplet-binding Adipocyte Differentiation-related Protein
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