Insulin Controls Subcellular Localization and Multisite Phosphorylation of the Phosphatidic Acid Phosphatase, Lipin 1*

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Brain, liver, kidney, heart, and skeletal muscle from fatty liver dystrophy (fld/fld) mice, which do not express lipin 1 (lipin), contained much less Mg^{2+}-dependent phosphatidic acid phosphatase (PAP) activity than tissues from wild type mice. Lipin harboring the fld^{Gly84→Arg} (Gly^{84} → Arg) mutation exhibited relatively little PAP activity. These results indicate that lipin is a major PAP in vivo and that the loss of PAP activity contributes to the fld phenotype. PAP activity was readily detected in immune complexes of lipin from 3T3-L1 adipocytes, where the protein was found both as a microsomal form and a soluble, more highly phosphorylated, form. Fifteen phosphorylation sites were identified by mass spectrometric analyses. Insulin increased the phosphorylation of multiple sites and promoted a gel shift that was due in part to phosphorylation of Ser^{106}. In contrast, epi- nephrine and oleic acid promoted dephosphorylation of lipin. The PAP-specific activity of lipin was not affected by the hormones or by dephosphorylation of lipin with protein phosphatase 1. However, the ratio of soluble to microsomal lipin was markedly increased in response to insulin and decreased in response to epinephrine and oleic acid. The results suggest that insulin and epinephrine control lipin primarily by changing localization rather than intrinsic PAP activity.

Lipin is a M_{r} ~ 100,000 Mg^{2+}-dependent phosphatidic acid phosphatase (PAP)^2 (1). This enzyme generates pools of diacylglycerol utilized in the synthesis both of TAG and of the phospholipids, phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine (2, 3). Lipin is encoded by the Lpin1 gene,3 which is mutated in mice with fatty liver dystrophy (fld) (4). Consequently, lipin was known to have an important role in lipid metabolism before the PAP activity of the protein was discovered.

The fld allele arose spontaneously as a result of a complex genetic rearrangement of the Lpin1 gene (4). Thus, fld/fld mice express neither lipin 1a nor lipin 1b, a slightly larger protein, which arises from alternative mRNA splicing (4, 5). Within a few days of birth fld/fld mice develop hypertriglyceridemia and hepatic steatosis (6). These abnormalities resolve after a few weeks, but several long lasting derangements develop, including glucose intolerance, insulin resistance (7), and defective peripheral myelination (8). A striking abnormality of the fld/fld mice is the absence of normal adipose tissue (7). Another mutant Lpin1 allele, designated fld^{Gly}, encodes a protein identical to wild type lipin, except that Arg replaces Gly at position 84 (4). The phenotype of fld^{Gly} mice is indistinguishable from that of fld/fld mice. The effect of the Gly^{84} → Arg mutation on the enzymatic activity of lipin has not been described.

The proteins encoded by Lpin1 are expressed in a wide variety of tissues, with the highest levels found in adipose tissue (4, 5). Lipin 1b increases markedly as fat cells differentiate, and it is the prevalent isoform in mature adipocytes (9). Two other isoforms, lipin 2 and lipin 3, are encoded by different genes (4). Conservation of sequence among the lipin isoforms is highest in domains, referred to as NLIP and CLIP, which are located in the NH_{2}- and COOH-terminal regions, respectively, of the proteins (4). These domains are found in lipin proteins from other species, including the Saccharomyces cerevisiae lipin 1 (1, 10), Pah1p, and the Schizosaccharomyces pombe protein, Ned-1 (11). Imbedded in the CLIP domain is a signature sequence of the HAD superfamily of hydrolases (12). In HAD family phosphatases, the phosphate group from the substrate is transferred to the NH_{2}- and COOH-terminal regions, respectively, of the proteins (4). These domains are found in lipin proteins from other species, including the Saccharomyces cerevisiae lipin 1 (1, 10), Pah1p, and the Schizosaccharomyces pombe protein, Ned-1 (11). Imbedded in the CLIP domain is a signature sequence of the HAD superfamily of hydrolases (12). In HAD family phosphatases, the phosphate group from the substrate is transferred to the NH_{2}- and COOH-terminal regions, respectively, of the proteins (4). These domains are found in lipin proteins from other species, including the Saccharomyces cerevisiae lipin 1 (1, 10), Pah1p, and the Schizosaccharomyces pombe protein, Ned-1 (11).

Activity measurements in hepatocytes and adipocytes indicate that the subcellular distribution of the Mg^{2+}-dependent PAP is controlled by hormones and fatty acids (14, 15). Incubating adipocytes with insulin increased the proportion of soluble phosphatase activity, whereas incubating the fat cells with norepinephrine or fatty acids, such as palmitic or oleic acid, increased the proportion of activity that fractionated with intracellular membranes upon centrifugation (15). The membrane-associated phosphatase has been proposed to be the form that is active in TAG synthesis (16); however, there is not...
Activity and Phosphorylation of Lipin

A strict relationship between rates of TAG synthesis and membrane association of the phosphatase in adipocytes (15). Fatty acids directly inhibit PAP activity in vitro (17, 18). It would be premature to conclude that the activities that have been measured in different subcellular fractions are due to lipin, since other PAP enzymes exist. Overexpressed green fluorescent protein-tagged lipin has been detected in both the nucleus and cytoplasm of HEK 293 cells (4); however, information on the subcellular localization of the lipin protein is limited.

The mechanisms involved in the control of lipin are still poorly defined. In previous 32P-labeling experiments, we discovered that lipin was phosphorylated in a rapamycin-sensitive manner in response to insulin in rat adipocytes (5), indicating that lipin was a target of the mTOR signaling pathway. The present study was conducted to investigate control of the activity and phosphorylation of lipin.

**EXPERIMENTAL PROCEDURES**

**Animals**—Mice homozygous for the fld allele were generated by mating heterozygous BALB/cBy-fl/fld+ mice (Jackson Laboratories). Five-month-old wild type and fld/fld progeny from this cross were used in experiments. Where indicated, some of the mice were fasted overnight. Otherwise, the mice were allowed free access to food and water.

**Cell Culture**—3T3-L1 fibroblasts in 10-cm diameter plates were differentiated into adipocytes (19), which were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Adipocytes were used 7–14 days after adding the differentiation medium. For experiments, the culture medium was replaced with 145 mM NaCl, 5.4 mM KCl, 1.4 mM CaCl2, 1.4 mM MgSO4, 25 mM NaHCO3, 5 mM glucose, 5 mg/ml bovine serum albumin, 0.2 mM sodium phosphate, and 10 mM HEPES, pH 7.4. Adipocytes were then incubated at 37 °C with insulin or other additions as indicated. 293T cells were cultured and assayed under the conditions of the assays, the amount of [32P]phosphate in 0.5 ml of the aqueous phase was determined by scintillation counting.

**Extract Preparation**—Incubations were terminated by rinsing with chilled phosphate-buffered saline (145 mM NaCl, 5.4 mM KCl, and 10 mM sodium phosphate, pH 7.4) and then immediately homogenizing the cells (1 ml of buffer per 10-cm diameter dish) in a glass tissue grinder with a Teflon pestle driven at 1,000 rpm. Homogenization buffer was composed of Buffer A (50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 10 mM sodium phosphate, and 50 mM β-mercaptoethanol, 10 μM pepstatin, 0.5 μM microcystin, and, where indicated, 1% Triton X-100 or Nonidet P-40. Unless otherwise stated, homogenates were centrifuged at 10,000 × g for 10 min, and the supernatants were retained for analyses.

**Subcellular Fractionations**—3T3-L1 adipocytes were fractionated by slight modification of the method described by Piper et al. (21). For each treatment, a 10-cm-diameter plate of cells was rinsed in PBS before the cells were homogenized as described above except in 1 ml of Buffer B (0.25 M sucrose, 50 mM NaF, 1 mM EDTA, and 50 mM Tris-HCl, pH 7.4). The homogenates were centrifuged at 16,000 × g for 20 min, and the pellets containing plasma membranes, mitochondria, and nuclei were retained. The supernatants were centrifuged at 175,000 × g for 1 h. The high speed supernatants containing soluble proteins were collected, and the pellets containing microsomes were suspended in 1 ml of Buffer B.

**Measurements of PAP Activities**—[32P]Phosphatidic acid was purified by thin layer chromatography after using γ-[32P]ATP and Escherichia coli diacylglycerol kinase to phosphorylate 1,2-dioleoyl-sn-glycerol (22). PAP activity was determined by using a modification of the method of Carman and Lin (22) to measure [32P]phosphate release from [32P]phosphatidic acid. Briefly, samples of cell or tissue extracts (5 μl), subcellular fractions (5 μl), or immune complexes (10 μl) were added to reaction mixtures (100 μl) containing 1 mM Triton X-100, 10 mM β-mercaptoethanol, 0.1 mM [32P]phosphatidic acid (10,000 cpm/nmol), 50 mM Tris-maleate (pH 7.0), and either no added Mg2+ or 2 mM MgCl2 as described below. After 20 min at 30 °C, the reactions were terminated by adding 0.5 ml of 0.1 N HCl in methanol. [32P]Phosphate was extracted by vigorous mixing after adding 1 ml of chloroform and 1 ml of 1 M MgCl2. The [32P]phosphate in 0.5 ml of the aqueous phase was determined by scintillation counting.

With tissue extract samples (see Fig. 1) or subcellular fractions of cells (see Fig. 7), Mg2+-dependent PAP activity was determined by subtracting activity measured in the absence of MgCl2 from activity measured in the presence of 2 mM MgCl2. To determine PAP activities attributable to overexpressed lipin proteins in extract samples (see Fig. 2), reaction mixtures were supplemented with 2 mM MgCl2, and the activities from cells transfected with a control plasmid were subtracted from the activities from cells overexpressing lipin. For lipin immune complexes (see Fig. 6), PAP activities were routinely measured only in the presence of 2 mM MgCl2. Results of control experiments revealed that PAP activity in these complexes was negligible without MgCl2.

Results of time course and dilution experiments verified that, under the conditions of the assays, the amount of [32P]phosphate released from [32P]phosphatidic acid was linearly related to the time of incubation and the amount of extract or immune complexes. In some experiments, corrections were made for the amount of lipin protein in immune complex assays. This was accomplished by dividing PAP activity by the relative amount of lipin estimated from lipin immunoblots.

**Generation of cDNA Constructs Encoding Lipin Proteins**—cDNA encoding lipin 1b (accession number AF412811) flanked by EcoRI sites was generated by PCR using pBluescript to generate pBluescriptlipin 1b. Next a DNA fragment encoding a triple HA epitope tag was added to the EcoRI site of pBluescriptlipin 1b. The resulting plasmid was then cut with NotI and KpnI, and the fragment containing HA-lipin 1b was ligated into the EcoRI site of pcDNA3 to generate pcDNA3HA-lipin 1b. The resulting plasmid was then cut with NotI and KpnI, and the fragment containing HA-lipin was introduced between the NotI and KpnI sites of pCDNA3 to generate pcDNA3HA-lipin 1b. cDNAs encoding lipin proteins having point mutations were generated by oligonucleoti-

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4 T. E. Harris and J. C. Lawrence, Jr., unpublished observations.
de-directed mutagenesis of pcDNA3\textsuperscript{HAb} by using a QuikChange II kit (Stratagene). Deletion constructs were generated by using PCR and appropriate primers. All constructs were cassette-subcloned, and the mutated fragments were sequenced and found to be free of errors.

**Electrophoretic Separation of 3T3-L1 Adipocytes and Transfection of 293T Cells**—Adipocytes (4–6 days postdifferentiation) were removed from the culture dishes (150-mm diameter) by incubating with trypsin (0.05%) for 5 min. The cells were collected by centrifugation (1000 \( \times \) g for 5 min), rinsed once with Buffer C, and suspended in Buffer C (350 \( \mu \)l/dish), which contained 120 mM KCl, 0.15 mM CaCl\(_2\), 2 mM EGTA, 5 mM MgCl\(_2\), 2 mM ATP, 5 mM glutathione, 10 mM potassium phosphate, and 25 mM HEPES, pH 7.6. Cells (350 \( \mu \)l) were added to cuvettes (4 mm) containing 50 \( \mu \)g of the appropriate pcDNA3 plasmid and then electrophorated with a single pulse from a Gene Pulser (Bio-Rad) set at 280 V and 960 microfarads. Transfection efficiency judged by electrophorating cells with a vector encoding green fluorescent protein was 40–50%. 293T cells (10-cm plate for each vector) were transfected with pcDNA3 vectors as described previously (19).

**Expression of HA-lipin in 3T3-L1 Adipocytes by Adenoviral-mediated Gene Transfer**—Viruses for expressing HA-lipin and A106 HA-lipin were prepared using procedures described by He et al. (24). Briefly, cDNAs encoding the HA-tagged lipin 1b proteins were excised with KpnI and NotI from the pBluescript constructs and inserted between the KpnI and NotI sites in the shuttle vector, pAdTrackCMV. The resulting plasmids were cotransformed with the adenoviral backbone plasmid, pAdEasy-1, into BJ5183 bacteria. Recombinant plasmids were selected and transfected into HEK 293 cells to generate virus, which was amplified and then purified by CsCl gradient centrifugation to create a high titer viral stock. 3T3-L1 adipocytes were infected essentially as described by Kotani et al. (25). The efficiency of infection judged by expression of green fluorescent protein, which is also encoded by the HA-lipin virus, was ~50%. The adipocytes were used 40–48 h after infection. Virus encoding \( \beta \)-galactosidase was used as a control.

**Antibodies**—The two lipin antibodies, LAb-1 and LAb-2, prepared by immunizing rabbits with peptide conjugates have been described previously (5). The peptide for LAb-1 corresponded to the sequence between Ser\textsuperscript{406} and Gly\textsuperscript{430} in mouse lipin 1b, and that for LAb-2 corresponded to the last 11 amino acids in the protein. Monoclonal antibodies to the HA epitope were purified from culture medium from 12CA5 hybridoma cells.

To generate antibodies that specifically recognize lipin phosphorylated in Ser\textsuperscript{106}, rabbits were immunized with the following phosphopeptide coupled to keyhole limpet hemocyanin: Cys-Tyr-Leu-Ala-Thr-Ser(P)-Pro-Ile-Leu-Ser-Glu (where Ser(P) represents phosphorylated Ser). To select the phosphospecific antibodies, the serum was first incubated with the nonphosphorylated peptide that had been coupled to SulfoLink resin (Pierce). Next, the fraction that did not bind to the nonphosphorylated peptide resin was incubated with the phosphopeptide coupled to SulfoLink. After washing the column, the phosphospecific antibodies were eluted at pH 2.7, immediately neutralized, and affinity-purified using protein A-agarose.

**Immunoprecipitations**—Adipocyte extract samples (800 \( \mu \)l) were incubated with lipin antibodies (2 \( \mu \)g) bound to protein A-agarose beads (15 \( \mu \)l) or with the anti-HA antibody, 12CA5 (2 \( \mu \)g), bound to protein G-agarose beads (15 \( \mu \)l), at 4°C for 12 h with constant mixing. As a control for specificity, rabbit or mouse nonimmune IgG was substituted for the lipin antibodies or 12CA5, respectively. The beads were then washed four times with Buffer A supplemented with 1% Triton X-100.

**Electrophoretic Analyses**—Protein samples were subjected to electrophoresis in 8.75% polyacrylamide gels in the presence of SDS (30). Relative amounts of \( {\text{32P}} \) were determined using a PhosphoImager (Amersham Biosciences). Immunoblots were prepared as described previously (5), except with different antibodies, and signals were detected by using either film or a CCD camera system (Fujifilm LAS3000). Band intensities were determined by using the volume integration function of Image-Quant (Amersham Biosciences).

**Mass Spectrometry**—After SDS-PAGE and staining with Coomassie Blue, the band containing lipin was sliced from the gel. In-gel digestion of the protein with trypsin was performed as described previously (26). An aliquot of the resulting peptides was analyzed by nanoflow HPLC coupled to microelectrospray ionization on an LCQ Deca ion trap mass spectrometer (Thermo Electron Corp., San Jose, CA) (27). MS/MS spectra acquired were searched against a human-rat-mouse data base (National Center for Biotechnology Information, available on the World Wide Web at www.ncbi.nlm.nih.gov/) using SEQUEST (28). Peptide sequence and phosphorylation site assignments and were validated by manual interpretation of the MS/MS spectra.

**Other Materials**—Insulin and rapamycin were obtained from Calbiochem NovoBiochem. 1,2-Dioleoyl-sn-glycerol and 1,2-dioleoyl-sn-glycerol-3-phosphate were from Avanti Polar Lipids. Diacylglycerol kinase, epinephrine, and most commonly used chemicals were from Sigma. Recombinant PPl\(_C\) was generously provided by Anna DePaoli-Roach (Indiana University).

**RESULTS**

**Fatty Liver Dystrophy Mutations**—Mg\(^{2+}\)-dependent PAP activities were measured in extracts of brain, heart, kidney, lung, skeletal muscle, and liver from wild type and \( \text{fld}/\text{fld} \) mice that had been fed \text{ad libitum} (Fig. 1). Most tissues from \( \text{fld}/\text{fld} \) mice contained dramatically lower levels of Mg\(^{2+}\)-dependent PAP activity than the corresponding tissues from wild type mice (Fig. 1A). An exception was the liver, where the activity from \( \text{fld}/\text{fld} \) mice was approximately half of that measured in wild type liver. Thus, enzymes other than lipin account for much of the hepatic Mg\(^{2+}\)-dependent PAP in fed mice. Although differing amounts of Mg\(^{2+}\)-independent activity were observed in different tissues (Fig. 1B), the Mg\(^{2+}\)-independent activities from the \( \text{fld}/\text{fld} \) tissues were not significantly different from the wild type activities.

Consistent with the previously observed effect on increasing lipin protein (29), fasting increased Mg\(^{2+}\)-dependent PAP activity in livers from wild type animals by ~2-fold (Fig. 1A). In contrast, fasting did not change Mg\(^{2+}\)-dependent PAP activity in livers of \( \text{fld}/\text{fld} \) mice. Thus, lipin appears to account for most,
Activity and Phosphorylation of Lipin

FIGURE 1. Influence of the fld mutation on PAP activity. A, samples of brain (B), heart (H), kidney (K), lung (Lu), skeletal muscle (M), and liver (L) from wild type (WT) or fld/fld mice that had been fed ad libitum and liver samples from mice that had been fasted overnight (Fast) were homogenized in Buffer A plus 1.0% Triton X-100. Mg$^{2+}$-dependent (A) and Mg$^{2+}$-independent (B) PAP activities were measured in extracts and expressed relative to extract protein. Except for the liver activities, which are means ± S.E. from three mice, the results are means ± S.E. from five animals.

if not all, of the Mg$^{2+}$-dependent PAP activity induced in the liver by fasting.

To investigate the effect of the Gly$^{84}$ → Arg mutation found in fld$^{2j}$ mice, HA-tagged forms of wild type and mutant lipin proteins were overexpressed in HEK 293 cells, and Mg$^{2+}$-dependent PAP activities were measured in cell extracts. Approximately equal amounts of lipin 1b and lipin 1a were expressed in the cells, and the PAP activities of these proteins were very similar (Fig. 2A). Less of the R84 lipin accumulated compared with the wild type lipin 1b, but even after correcting for the expression levels, the PAP activity of the mutant lipin was only ~20% that of the wild type enzyme (Fig. 2A). By comparison, the activity of the lipin protein in which the catalytic Asp in the HAD domain was mutated to Glu was negligible.

Removing the COOH-terminal 15 amino acids from lipin did not significantly affect the PAP activity (Fig. 2B, 1–909). However, removing an additional 37 amino acids (Fig. 2, 1–872) resulted in a striking reduction in activity. Deleting the entire CLIP domain by COOH-terminal truncation abolished PAP activity (Fig. 2, 1–641), which was expected, since this truncated protein lacks the HAD motif that is involved in catalysis. Interestingly, deleting the NLIP domain (Fig. 2, Δ106) also dramatically decreased PAP activity. Thus, both NH$_2$- and COOH-terminal regions of lipin are required for full PAP activity.

Phosphorylation of certain sites in lipin decreases the mobility of the protein in SDS-PAGE, explaining, at least in part, the multiple electrophoretic forms of the proteins that were detected (Fig. 2, A and B). R84 exhibited less electrophoretic heterogeneity than the other lipin proteins, and the average mobility of the mutant protein was higher (Fig. 2A), suggesting that it was not as highly phosphorylated as the wild type lipin. Lipin proteins with COOH-terminal truncations of up to 283 amino acids still exhibited electrophoretic heterogeneity indicative of multisite phosphorylation (Fig. 2B). Consistent with their smaller size, the average mobilities of the truncated proteins were higher than wild type lipin.

Sites in Both the NH$_2$- and COOH-Terminal Regions of Lipin Are Phosphorylated—Endogenous lipin from 3T3-L1 adipocytes also exhibited electrophoretic heterogeneity, indicative of multisite phosphorylation (Fig. 3A). Incubating $^{32}$P-labeled adipocytes with insulin increased the phosphorylation of lipin, as evidenced both by a gel shift and by an increase in $^{32}$P-labeled lipin (Fig. 3A). The effects of insulin were attenuated by rapamycin, implicating mTOR in the hormonal response. Inhibiting PI 3 kinase, which signals upstream of mTOR (30), with wortmannin also blocked the effects of insulin. These results indicate that lipin phosphorylation is controlled by insulin in a similar manner in 3T3-L1 adipocytes and in primary rat adipocytes, where the hormone was previously shown to increase lipin phosphorylation (5).

To facilitate the analyses of lipin phosphorylation, adenoviral vectors were developed to allow overexpression of epitope-tagged forms of lipin in adipocytes. Insulin stimulated the phosphorylation of overexpressed HA-lipin (see Fig. 5B). Indeed, when adjusted for the amount of lipin protein estimated by immunoblotting, the amounts of $^{32}$P incorporated into the endogenous and overexpressed lipin were very similar (4).

As a first step in identifying regions of lipin phosphorylated in response to insulin, $^{32}$P-labeled HA-lipin from adipocytes was incubated briefly with trypsin, and the resulting fragments were immunoprecipitated with HA antibodies or the lipin peptide antibodies, LAb-1 or LAb-2 (Fig. 3B). Several $^{32}$P-labeled peptides were immunoprecipitated by the three antibodies, but as would be expected from the different locations of the epitopes, the labeled peptides exhibited distinctly different mobilities when subjected to SDS-PAGE. A fragment of appa-
Activity and Phosphorylation of Lipin

Several phosphorylation sites were identified in the NH$_2$-terminal region of lipin. Mutational analyses of lipin were conducted to determine which of these NH$_2$-terminal sites represented the major site of insulin-stimulated phosphorylation found in the $M_r \sim 30,000$ peptide generated by limited trypsin proteolysis. Results with $\Delta 106$ lipin provided an important clue. Deleting the first 106 amino acids of lipin but also abolished the shift in electrophoretic mobility produced by insulin (Fig. 5A). This finding suggested that the site responsible for the gel shift resided in the first 106 amino acids of lipin. Ser$^{106}$ was the only site identified by mass spectrometry in this region, and introducing a Ser$^{106}$→Ala mutation clearly limited the gel shift produced by insulin (Fig. 5A), indicating that Ser$^{106}$ is phosphorylated in response to the hormone.

FIGURE 3. Peptide mapping by limited trypsin proteolysis indicates that the NH$_2$-terminal region of lipin is phosphorylated in response to insulin. A, 3T3-L1 adipocytes were incubated for a total of 135 min in medium supplemented with 0.2 mCi/ml [32P]phosphate. During this time, the cells were incubated as follows: no additions, 10 milliunits/ml insulin (INS) for the last 15 min, 20 nM rapamycin (RAP) for the last 75 min, rapamycin for 60 min followed by insulin plus rapamycin for the final 15 min, 100 nM wortmannin (Wm) for the last 75 min, and wortmannin for 60 min followed by wortmannin plus insulin for the last 15 min. $^{32}$P-Labeled lipin was immunoprecipitated and subjected to SDS-PAGE. A phosphor image and a lipin immunoblot are shown. B, HA-lipin, 3T3-L1 adipocytes were incubated for a total of 2 h in medium supplemented with 0.2 mCi/ml [32P]phosphate. During this time, the cells were incubated as follows: no additions (CON), 10 milliunits/ml insulin (INS) for the last 15 min, 20 nM rapamycin (RAP) for the last 1 h, or rapamycin for 30 min followed by insulin plus rapamycin for the final 30 min (RAP + INS). After preparing extracts, HA-lipin was immunoprecipitated by using HA antibodies. The immune complexes were washed twice (1 ml/wash) with a solution containing 1 mM dithiothreitol and 10 mM sodium phosphate, pH 7.4, and suspended in 25 μl of the same solution. After adding trypsin (100 ng), the samples were incubated at 37°C for 4 min. The digests were terminated by incubating the samples at 95°C for 5 min after adding 50 μl of a stop solution containing 2% SDS, 1 mM dithiothreitol, 10 μg/ml soybean trypsin inhibitor, and 10 mM sodium phosphate, pH 7.4. The volume of the entire sample was adjusted to 1 ml by adding stop solution (minus SDS) that had been supplemented with 1.2% Triton X-100, and fragments were immunoprecipitated with antibodies to HA, LAb-1 (1), or LAb-2 (2). Samples were subjected to SDS-PAGE, and an autoradiogram of the $^{32}$P-labeled fragments was prepared. Binding sites of HA, LAb-1, and LAb-2 in lipin are depicted in the model. C, the relative amounts of $^{32}$P recovered in the $M_r \sim 30,000$ NH$_2$-terminal fragment (HA-IP) and the $M_r \sim 45,000$ COOH-terminal fragment (LAB-2 IP) were determined by phosphorimaging. The results are expressed as percentages of the control values and are means ± S.E. from three experiments.

ent $M_r \sim 30,000$ was the predominant $^{32}$P-labeled species in HA immunoprecipitates. Considering the size of the fragment and the location of the HA tag at the NH$_2$ terminus of lipin, the $M_r$ ~30,000 fragment contains the NH$_2$-terminal region of lipin, including the conserved NLIP domain. A fragment of apparent $M_r \sim 45,000$ was the major $^{32}$P-labeled species recovered with LAb-2. Since LAb-2 binds the COOH-terminal 11 amino acids of lipin, this $M_r \sim 45,000$ fragment includes the conserved CLIP domain.

Based on $^{32}$P labeling of the fragments recovered with the three antibodies, the effect of insulin on increasing phosphorylation was most pronounced in the NH$_2$-terminal region of lipin. When corrected for the amounts of peptide present, the results indicate that the phosphorylation of the NH$_2$-terminal sites were increased by ~2-fold in response to the hormone, whereas the COOH-terminal sites were modestly affected (Fig. 3C). Likewise, the effects of rapamycin on decreasing phosphorylation were much more pronounced on the NH$_2$-terminal region of lipin than on sites in the COOH-terminal region of the protein.

Mass Spectrometric Analysis of Phosphorylation Sites—To identify phosphorylation sites, HA-lipin peptides generated by exhaustive proteolysis with trypsin were resolved by reverse phase HPLC and analyzed by mass spectrometry. Phosphorylated peptides were identified from the mass contributed by phosphate (80 atomic mass units). The MS/MS spectrum derived from a representative phosphopeptide is shown in Fig. 4A. In this case, fragments of the peptide that contained Ser(P)$^{106}$ (pS in sequence YLATpSPILSEGAAR) had the mass predicted from the amino acid composition plus 80 atomic mass units. Fragments differing by 98 atomic mass units result from the loss of phosphoric acid during fragmentation. Thus, Ser$^{106}$ is a site of phosphorylation in lipin.

Including Ser$^{106}$, 15 phosphorylation sites were unambiguously assigned based on the MS/MS spectra of the phosphopeptides (Fig. 4B). The location of the sites with respect to other features in lipin 1b is shown in Fig. 4C. Four phosphopeptides were detected in which the position of the phosphorylated residue could not be assigned. In these peptides, two potential sites were closely spaced (Ser$^{281}$ and Thr$^{282}$, Ser$^{353}$ and Ser$^{356}$, Ser$^{647}$ and Ser$^{648}$, Ser$^{720}$ and Thr$^{722}$). Although the MS/MS spectra definitively placed phosphate in the pair, we were unable to determine which residue in the pair was phosphorylated. Thus, results of the mass spectrometric analyses of lipin indicated that there were at least 19 phosphorylation sites.

Insulin-stimulated Phosphorylation of Ser$^{106}$—Several phosphorylation sites were identified in the NH$_2$-terminal region of lipin. Mutational analyses of lipin were conducted to determine which of these NH$_2$-terminal sites represented the major site of insulin-stimulated phosphorylation found in the $M_r \sim 30,000$ peptide generated by limited trypsin proteolysis. Results with $\Delta 106$ lipin provided an important clue. Deleting the first 106 amino acids of lipin but also abolished the shift in electrophoretic mobility produced by insulin (Fig. 5A). This finding suggested that the site responsible for the gel shift resided in the first 106 amino acids of lipin. Ser$^{106}$ was the only site identified by mass spectrometry in this region, and introducing a Ser$^{106}$→Ala mutation clearly limited the gel shift produced by insulin (Fig. 5A), indicating that Ser$^{106}$ is phosphorylated in response to the hormone.
Activity and Phosphorylation of Lipin

The phosphorylation of Ser\textsuperscript{106} was confirmed by immunoblotting lipin with a phosphospecific antibody directed against the site. Insulin caused a severalfold increase in reactivity of this antibody with lipin (Fig. 5B). The antibody failed to react with A106 lipin or with lipin that had been dephosphorylated with PP1\textsubscript{C} (Fig. 6A), providing additional support for the specificity of the antibody for Ser(P)\textsuperscript{106}. Incubating cells with rapamycin abolished the reactivity of lipin with the phosphospecific antibody (Figs. 5B and 6A), indicating that phosphorylation of Ser\textsuperscript{106} is dependent on the mTOR signaling pathway.

The finding that insulin decreases the electrophoretic mobility of most of the lipin protein (e.g. see Fig. 5A) indicates that Ser\textsuperscript{106} is highly phosphorylated. However, this finding does not mean that other sites are not phosphorylated in response to insulin. To estimate the contribution of Ser\textsuperscript{106} phosphorylation to the overall effect of insulin, \textsuperscript{32}P-labeling experiments were conducted after overexpressing HA-tagged forms of wild type and A106 lipins. Insulin increased the \textsuperscript{32}P labeling of both lipin proteins (Fig. 5B). After correcting for the amount of lipin protein recovered, \textsuperscript{32}P incorporation into wild type lipin was found to be increased \textasciitilde{2}-fold by insulin (Fig. 5C). The \textasciitilde{2}-fold increase in A106 phosphorylation was only slightly lower, indicating that sites other than Ser\textsuperscript{106} are phosphorylated in response to insulin.

Reciprocal Changes in Lipin Phosphorylation Produced by Insulin and Epinephrine Are without Effect on PAP Activity—In contrast to insulin, both epinephrine and oleic acid increased the electrophoretic mobility of lipin (Fig. 6A), indicative of dephosphorylation. Incubating cells with insulin, rapamycin, epinephrine, or oleic acid was without effect on PAP activity measured in immune complexes isolated using lipin antibodies (Fig. 6B). An implication is that changes in the phosphorylation of lipin were insufficient to change PAP activity, at least
under the conditions of these assays. To investigate further the possible effect of lipin phosphorylation, immune complexes were incubated with recombinant PP1C, the protein phosphatase 1 catalytic subunit, before measuring PAP activities. After incubating with the phosphatase, lipin collapsed into a single band of relatively high mobility in SDS-PAGE (Fig. 6A), confirming that the electrophoretic heterogeneity of lipin was due to differences in phosphorylation. PP1C also abolished reactivity with the phosphospecific antibody (Fig. 6A), providing additional evidence of the efficiency of dephosphorylation. However, PP1C had no effect on PAP activity, supporting the conclusion that the activity of lipin was not affected by changes in its phosphorylation state.

Insulin and Epinephrine Promote Reciprocal Changes in Lipin Localization Correlated with Changes in Phosphorylation—Several agents have been shown to affect the subcellular distribution of Mg$^{2+}$-dependent PAP. To investigate the effects of insulin on the subcellular distribution of lipin, cells were homogenized in a detergent-free solution containing 0.25 M sucrose. The homogenates were fractionated by differential centrifugation to yield fractions containing the following: plasma membrane/mitochondria/nuclei, microsomal membranes, and soluble proteins. Relatively little lipin or Mg$^{2+}$-dependent PAP activity was detected in the plasma membrane/mitochondria/nuclear fraction. Insulin markedly increased the amounts of lipin (Fig. 7A and C) and PAP activity (Fig. 7D) in the soluble fraction. Since the effect of insulin appeared to be too rapid to be explained by synthesis of new lipin protein, it seemed more likely that insulin was promoting a change in the subcellular distribution of lipin. As expected, insulin decreased both lipin protein (Fig. 7A and C) and PAP activity (Fig. 7D) in the microsomal fraction.

The relative amount of soluble Mg$^{2+}$-dependent PAP in control 3T3-L1 adipocytes was lower than that observed in previous experiments in untreated rat adipocytes (~28% versus 56%) (15). Although it would not be unreasonable to expect differences between cultured 3T3-L1 adipocytes and primary fat cells, other factors might also have contributed to the different amounts of soluble activity measured. For example, our
Activity and Phosphorylation of Lipin

![Diagram](image)

FIGURE 7. Changes in lipin phosphorylation produced by insulin, epinephrine, and oleic acid are associated with redistribution of lipin between soluble and membrane-bound forms. A, 3T3-L1 adipocytes (10 cm dish/treatment) were incubated for 2 h, and the following: no additions (C), 10 milliliters/ml insulin for the last 60 min (I), 20 nM rapamycin for 120 min (R), and rapamycin for 60 min followed by insulin plus rapamycin for 60 min (RI). Cells were homogenized in 1 ml of Buffer B, and a pellet fraction containing nuclei, mitochondria, and plasma membranes was prepared by centrifuging the homogenates at 16,000 \( g \) for 20 min. The supernatant was centrifuged at 175,000 \( g \) for 1 h to obtain a supernatant fraction containing soluble proteins and a pellet fraction containing microsomal membranes, which were suspended in 1 ml of Buffer B. Samples (40 \( \mu l \)) of the fractions were subjected to SDS-PAGE, and lipin immunoblots were prepared. B, 3T3-L1 adipocytes were incubated without additions (C), with decreasing concentrations (1 mM, 330 \( \mu M \), and 100 \( \mu M \)) of oleic acid (O) or with 10 \( \mu M \) epinephrine (E). After 30 min, fractions containing soluble and microsomal proteins were isolated, and a lipin immunoblot was prepared. C and D, adipocytes were incubated without additions and with insulin and rapamycin as described in A. In addition, cells were incubated as follows: 330 \( \mu M \) oleic acid (O) for the last 30 min, insulin for 30 min followed by insulin plus oleic acid for the final 30 min (IO), 10 \( \mu M \) epinephrine (E) for the last 30 min, and insulin for 30 min followed by insulin plus epinephrine for the final 30 min (IE). C, the relative amounts of lipin in the soluble and microsomal fractions were estimated from immunoblots. Results are expressed as percentages of the total lipin from control cells. D, Mg\(^{2+}\)-dependent PAP activities in the soluble and microsomal fractions expressed as percentages of the total control activity, which averaged 15.9 ± 2.3 nmol/min/10-cm plate of cells. The results in C and D are mean values ± S.E. from three experiments.

homogenization buffer was supplemented with protease inhibitors and protein phosphatase inhibitors, including 50 mmol NaF, and it had a higher ionic strength than that used in the previous study (15). Based on earlier findings (31), the higher ionic strength would be expected to stabilize binding of Mg\(^{2+}\)-dependent PAP to membranes. Moreover, in contrast to the previous study (15), we did not routinely include albumin in the homogenization buffer. In control experiments, we found that adding 10 mg/ml fatty acid-free albumin to the buffer used to homogenize 3T3-L1 adipocytes increased the relative amount of soluble lipin by only 15 ± 4% (\( n = 3 \) experiments). Importantly, removing free fatty acids with albumin did not block the effects of insulin on the distribution of Mg\(^{2+}\)-dependent PAP between the soluble and membrane-bound forms.

Rapamycin did not affect the subcellular distribution of lipin or PAP activity in either the absence or presence of insulin. In contrast to insulin, oleic acid and epinephrine decreased the amount of soluble lipin protein (Fig. 7, A and B) and PAP activity (Fig. 7D). Insulin blocked the responses to 330 \( \mu M \) oleic acid but was ineffective in counteracting the effects of epinephrine (Fig. 7C). None of the treatments significantly affected the total (microsomal plus soluble) PAP activity.

Interestingly, most of the membrane-associated lipin was found in a high mobility band that was essentially absent in the soluble fraction (Fig. 7, A and B). This band presumably represents a hypophosphorylated form of lipin, since its mobility was the same as that of lipin that had been dephosphorylated \textit{in vitro} (see Fig. 6A). Moreover, the increase in soluble lipin produced by insulin was associated with loss of the hypophosphorylated form (Fig. 7B), suggesting that phosphorylation of lipin releases the enzyme from intracellular membranes. Consistent with this hypothesis, oleic acid and epinephrine increased the electrophoretic mobility of lipin as well as the amount of microsomal lipin (Fig. 7B).

DISCUSSION

Mice homozygous for the \textit{fld} of the \textit{Lpin1} gene represent a useful model for evaluating the role of lipin \textit{in vivo} (4). The present finding that tissues from \textit{fld/fld} mice contained markedly less PAP activity than the corresponding wild type tissues indicates that lipin is a major Mg\(^{2+}\)-dependent PAP in mammalian cells (Fig. 1). The lack of PAP activity normally provided by lipin surely contributes to the failure of \textit{fld/fld} mice to accumulate TAG in fat cells, since PAP catalyzes the penultimate step in TAG synthesis (2). Loss of PAP activity may also partially explain the lipodystrophy of \textit{fld/fld} mice, since lipin protein harboring the \textit{fld}\(^{-}\) mutation (Gly\(^{84}\)→ Arg) exhibited only a fraction of the PAP activity of wild type lipin when overexpressed in cells (Fig. 2). Previous studies indicated that this mutant form of lipin was excluded from the nucleus (4). Recent evidence that lipin functions to regulate the activities of PGC-1\(\alpha\) and PPAR\(\alpha\) in hepatocytes (29) is consistent with the view that loss of nuclear functions of lipin also contributes to phenotypic changes in \textit{fld/fld} and \textit{fld}\(^{1}\)/\textit{fld}\(^{2}\) mice (4).

The phosphorylation of lipin was first observed in rat adipocytes, where it was shown to be increased in a rapamycin-sensitive manner in response to insulin (5). The present results demonstrate that insulin-stimulated phosphorylation of lipin occurs in 3T3-L1 adipocytes (Figs. 3, 5, 6, and 7). The results also show for the first time that the phosphorylation of lipin is decreased by epinephrine (Figs. 6 and 7). The reciprocal effects of insulin and epinephrine on lipin phosphorylation are consistent with the opposing actions of these hormones on TAG accumulation in adipocytes. However, changes in phosphorylation produced by the hormones did not significantly alter the intrinsic PAP activity of lipin (Figs. 6 and 7). Moreover, dephosphorylation of lipin \textit{in vitro} by using PPI\(_{C}\) did not change the PAP activity of the enzyme (Fig. 6), providing additional evidence that activity is not affected by the phosphorylation state of lipin.

Lipin was recovered primarily in the soluble and the microsomal fractions of 3T3-L1 adipocytes (Fig. 7). Insulin decreased the membrane-associated form, accounting for the increase in soluble lipin. Conversely, epinephrine decreased soluble lipin and increased the membrane-associated form. With both insulin and epinephrine, the changes in PAP activity in the two fractions exactly matched the changes in protein. These find-
ings indicate that changing subcellular localization is a major mechanism for controlling lipin function, supporting previous conclusions based on measurements of Mg$^{2+}$-dependent PAP activity that were made before the lipin protein was identified (14, 15).

Oleic acid also decreased soluble lipin and increased lipin in the microsomal fraction. Since epinephrine stimulates lipolysis in adipocytes, an increase in intracellular free fatty acids may be involved in the effects of the hormone on lipin. The interpretation that fatty acids increase the membrane localization of PAP has been called into question by the finding that adding oleic acid to a soluble, membrane-free, preparation of the PAP in vitro causes a portion of the enzyme to sediment upon centrifugation (32). Such an action cannot explain the present finding that the increase in microsomal lipin produced by oleic acid was associated with dephosphorylation of the protein (Fig. 7).

The soluble form of lipin was more highly phosphorylated than the membrane-bound form, as evidenced by its lower electrophoretic mobility in SDS-PAGE (Fig. 7). Thus, the changes in lipin distribution produced by insulin and epinephrine directly correlated with changes in lipin phosphorylation. This correlation suggests that phosphorylation controls the intracellular localization of the protein. However, the results would also be consistent with a model in which changes in phosphorylation occur secondarily to the change in intracellular localization. Such appears to be the case with CTP:phosphocholine cytidylyltransferase, which is dephosphorylated after becoming bound to membranes (33).

It is clear that Ser$^{106}$ is not required for the effect of insulin on lipin localization, since rapamycin abolished phosphorylation of Ser$^{106}$ (Fig. 5) but did not attenuate the effect of insulin on increasing soluble lipin (Fig. 7). However, these findings with rapamycin do not eliminate a role of phosphorylation in controlling the localization of lipin, since rapamycin did not fully inhibit insulin-stimulated phosphorylation of the protein (Fig. 5C). Indeed, rapamycin was without effect on the insulin-stimulated loss of the highest mobility band that was associated with membrane binding (Fig. 7A). Defining the role of phosphorylation will require identification of other hormonally responsive sites, an objective complicated by the large number of phosphorylation sites in lipin. Mass spectrometric analyses indicated that at least 19 sites contained some phosphate (Fig. 4). An almost equal number were found in the NH$_2$- and COOH-terminal halves of lipin, although some regions were enriched in sites, such as the stretch between Ser$^{291}$ and Thr$^{299}$, which contained six sites. Many of the sites identified in mouse lipin are present in other mammalian lipin proteins, and three (Ser$^{106}$, Ser$^{634}$, and Ser$^{720}$) appear to have been conserved from yeast to humans.

None of the highly conserved sites were found to be phosphorylated in a recent analysis of phosphorylation sites of lipin (Pah1p) in S. cerevisiae (34). In this study, the PAP activity of Pah1p harboring Ala mutations in seven (Ser/Thr)-Pro phosphorylation sites exhibited enhanced PAP activity (34). Curiously, the large majority of phosphorylation sites in yeast Pah1p were found in a region of the protein lacking homology with lipins from other species (Fig. 4C). Consequently, how the role of lipin phosphorylation in yeast relates to that in mammals is unclear.

In view of the findings that both yeast (34) and mammalian lipins (Fig. 4) are phosphorylated in several (Ser/Thr)-Pro phosphorylation sites, it is interesting to speculate that one or more of the protein kinases and/or phosphatases acting on lipin proteins in yeasts and mammals have been conserved. Santos-Rosa et al. (10) have presented evidence that the yeast Pah1p is dephosphorylated by a membrane-localized CPD phosphatase complex, Nem1/Spo7. Thus, it is interesting to speculate that Dullard (35), the mammalian homolog of yeast Nem1, might be a lipin phosphatase. Yeast Pah1p is believed to be phosphorylated by the cyclin-dependent kinases Cdc28p and/or Pho85p (10, 34). Although there may be instances in which mammalian homologs of Cdc28, such as Cdc2, Cdk2, and Cdk3, phosphorylate lipin, the activities of most cyclin-dependent kinases are relatively low in terminally differentiated adipocytes. Therefore, other kinases, such as mitogen-activated protein kinases (36) or mTOR (37), that have been shown to phosphorylate (Ser/Thr)-Pro sites would seem to be better candidates for phosphorylating lipin in fat cells. In any event, considering the diverse nature of the sites in lipin, the phosphorylation of the protein almost certainly involves multiple protein kinases. Several of the sites in lipin did not appear to conform to any defined consensus motif for phosphorylation, but four sites, including the highly conserved Ser$^{634}$, are good candidates for phosphorylation by protein kinase CK2, and three sites fit the consensus for phosphorylation by GSK3.

Lipin is very highly expressed in adipose tissue (4, 5), consistent with its important role in TAG synthesis. However, as discussed elsewhere (32), it is paradoxical that insulin increases TAG synthesis but decreases the membrane-bound phosphatase, which has been proposed to be the more active form in TAG synthesis (16). Perhaps changes in lipin localization have roles related to functions of phosphatidic acid not involving TAG synthesis. Decreasing microsomal lipin would favor accumulation of membrane phosphatidic acid, which has been shown to increase in response to insulin in adipose tissue (38). Phosphatidic acid is important in membrane fusion events, and it has been implicated in controlling the subcellular distribution of many proteins (39). It has also been reported to bind and modify the activity of several important protein kinases and phosphatases, including mTOR, Raf-1, SHP-1, and PP1C (39). Although rapamycin did not affect lipin localization or PAP activity, the connection between mTOR and lipin phosphorylation is intriguing in view of the potential role of phosphatidic acid in controlling mTOR activity in cells (40, 41). Future experiments will be needed to investigate this connection and the possible role of phosphorylation in controlling the function of lipin as a regulator of PPAR$\alpha$ and other transcription factors (29).

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Activity and Phosphorylation of Lipin
Activity and Phosphorylation of Lipin

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