Hormonal Control of Reversible Translocation of Perilipin B to the Plasma Membrane in Primary Human Adipocytes

In adipocytes, perilipin coats and protects the central lipid droplet, which stores triacylglycerol. Alternative mRNA splicing gives rise to perilipin A and B. Hormones such as catecholamines and insulin regulate triacylglycerol metabolism through reversible serine phosphorylation of perilipin A. It was recently shown that perilipin was also located in triacylglycerol-synthesizing caveolae of the plasma membrane. We now report that perilipin at the plasma membrane of primary human adipocytes was phosphorylated on a cluster of threonine residues (299, 301, and 306) within an acidic domain that forms part of the lipid targeting domain. Perilipin B comprised <10% of total perilipin but was the major isofrom associated with the plasma membrane of human adipocytes. This association was controlled by insulin and catecholamine: perilipin B was specifically depleted from the plasma membrane in response to the catecholamine isoproterenol, while insulin increased the amount of threonine phosphorylated perilipin at the plasma membrane. The reversible translocation of perilipin B to and from the plasma membrane in response to insulin and isoproterenol, respectively, suggests a specific function for perilipin B to protect newly synthesized triacylglycerol in the plasma membrane.

Adipose tissue is the major site in the body for storage of fatty acids as triacylglycerols. When energy is needed, hormones, such as the catecholamine noradrenaline, stimulate hydrolysis of triacylglycerol (lipolysis) to mobilize the stored fatty acids, which are the primary source of energy for different tissues. The storage of triacylglycerol in adipocytes, on the other hand, is favored by hormones such as catecholamines and insulin regulating triacylglycerol-synthesizing caveolae at the plasma membrane (11, 12). Moreover, at the plasma membrane perilipin may also be located to perilipin B. It has recently been shown that perilipin may also be located at the plasma membrane (11, 12). Moreover, at the plasma membrane perilipin was found associated with caveolae membrane that contained triacylglycerol synthesized from exogenous fatty acids (12).

In the present study we identified phosphorylated proteins in the plasma membrane of primary human adipocytes by applying our vectorial proteomics approach and mass spectrometry (13). We found that perilipin, predominantly perilipin B, at the plasma membrane was phosphorylated at a cluster of three threonine residues (299, 301, and 306). Insulin and β-adrenergic treatment of adipocytes induced a reversible translocation of perilipin B to and from the plasma membrane, suggesting that the hormone-controlled association of perilipin B with the plasma membrane may act to protect the newly formed triacylglycerol in the plasma membrane from hydrolysis.

EXPERIMENTAL PROCEDURES

Isolation of Human Adipocytes and Preparation of Plasma Membrane Fractions—Subcutaneous fat tissue was obtained during elective abdominal surgery on female patients. All participants gave their informed approval, and the Local Ethics Committee approved our study. Adipocytes were isolated by collagenase digestion (14) and the cells preincubated in Krebs-Ringer solution (0.12 M NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4) containing 20 mM Hepes, pH 7.4, 3.5% (w/v) fatty acid-free bovine serum albumin, 100 mM phenylisopropyladenosine, 0.5 unit/ml adenosine deaminase with 2 mM glucose, at 37 °C and then incubated for 10 min with or without 100 nM insulin or with 100 nM isoproterenol. Plasma membrane fractions were obtained by homogenization of the cells at room temperature (22 °C) in 10 mM NaH2PO4, pH 7.4, 1 mM EDTA, 0.25 M sucrose, 25 mM NaF, 1 mM Na3P2O7, 2 mM NaN3, 0.5 mM EGTA, 4 mM iodoacetate. Plasma membrane-containing pellets obtained by centrifugation at 16,000 × g for 20 min were resuspended in 10 mM Tris/HCl, 1 mM EDTA, and 2 mM NaN3. Plasma membranes were then purified by sucrose density centrifugation (15) a cluster of three

2 The abbreviations used are: IMAC, immobilized metal affinity chromatography; SRBC, a protein kinase C-binding protein; SDPR, a protein kinase C-binding protein; PTRF, polymersase I and transcript release factor.

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**TABLE 1**
Identification of phosphoproteins in the plasma membrane of human adipocytes

| Protein name         | Accession no. | Molecular mass | Sequence determined by tandem mass spectrometry | Phosphorylation site |
|----------------------|---------------|----------------|-----------------------------------------------|----------------------|
| Caveolin 1α          | 15451856      | 20.3           | 23\(\text{AMADELESR}\)                        | Ser-37\(\text{b}\)   |
| Caveolin 1β          | 33339772      | 17.0           | 23\(\text{ADELESR}\)                        | Ser-5\(\text{a}\)    |
| Caveolin 2            | 5915890       | 18.2           | 24\(\text{MIDDYSYHHSGLEYADPEK}\)            | Ser-20, Ser-23\(\text{a}\), Ser-556 |
| Cbl-associated protein (CAP) | 51702136      | 142.5          | 25\(\text{MALAPPELDL}\)                      | Ser-209              |
| SDPR                 | 4759082       | 63.1           | 26\(\text{EELDENKSLLEELHTVIDLASDDLPHDEALEDAAEKE}\) | Thr-196, Ser-218, Ser-204, Ser-203 |
| Perilipin            | 21410323      | 56.0           | 27\(\text{NLAAAEQEDHDQIREDGEWEELEEPENK}\)    | Thr-299, Thr-301, Thr-306 |
| SRBC                 | 21450787      | 27.6           | 28\(\text{APELGLPAQSELGPEQLEAEVGESDEEPVESR}\) | Ser-165, Ser-166     |
| Spectrin IIβ         | 2493434       | 274.6          | 29\(\text{PDVHALLEIEESDAVLVDKS}\)            | Thr-1843             |
| Talin 2              | 26400725      | 271.6          | 30\(\text{LDGIPPEPKL}\)                      | Ser-26               |
| PTRF                 | 28399016      | 43.5           | 31\(\text{GAGAQAEEPSGAGSEELIK}\)             | Ser-167              |
|                      |               |                |                                               | Ser-202, Ser-365, Ser-366 |}

\(a\) Accession numbers in NCBI.  
\(b\) See Ref. 17 for published phosphorylation site.  
\(c\) See Ref. 22 for published phosphorylation site.  
\(d\) See Ref. 13 for published phosphorylation site.

µl of 20% acetonitrile in water. Phosphopeptides were eluted by four washings with 10 µl of 20 mM Na₂HPO₄ in 20% acetonitrile. The four eluted fractions were separately collected and desalted using a C₁₈ ZipTip (Millipore, Bedford, MA).

**Mass Spectrometry**—The phosphopeptides were analyzed on a hybrid mass spectrometer API-Q STAR Pulsar I (Applied Biosystems, Foster City, CA) equipped with a nano-electrospray ion source (MDS Protana, Odense, Denmark). The desalted peptides (2 µl in 50% acetonitrile in water with 1% formic acid) were loaded into the nanoelectrospray capillaries. Mass spectra of the phosphopeptides fragmented by collision-induced dissociation were acquired with instrument settings recommended by Applied Biosystems with manual control of collision energy.

**RESULTS**

**Identification of Phosphorylated Proteins in the Plasma Membrane of Human Adipocytes**—To identify phosphoproteins in the plasma membrane we subjected purified plasma membranes to proteolytic treatment with trypsin, which cleaves surface exposed domains of the membrane proteins (13, 17, 18). Generated peptides were separated from remaining membrane proteins by centrifugation and the phosphorylated peptides were then enriched by an IMAC procedure (16). The enriched phosphopeptides were sequenced by nanospray quadrupole time-of-flight mass spectrometry. We identified 26 proteins in the plasma membrane (Table 1). Serine phosphorylation at residues 5 and 37 of caveolin 1 and threonine.

When whole cell lysates were examined for effects of insulin or isoproterenol, in which we identified threonine-phosphorylated residues (Table 1), corresponded to the major phosphorylated perilipin protein bands. Immunoblotting analysis with antibodies against phosphotyrosine identified the insulin receptor β-subunit in cells treated with insulin, but insulin treatment had no detectable effect on serine phosphorylation as examined with anti-phosphothreonine antibodies (data not shown). Antibodies against phosphothreonine, on the other hand, identified four proteins that were extensively phosphorylated in the plasma membrane of cells treated with insulin (Fig. 2A).

Identification of Threonine-phosphorylated Perilipin in the Plasma Membrane—To examine the effect of insulin on the phosphorylation of the identified phosphoproteins, we isolated plasma membranes from human adipocytes incubated with or without insulin and the protein phosphorylation was examined by immunoblotting. Antibodies against phosphotyrosine identified the insulin receptor β-subunit in cells treated with insulin, but insulin treatment had no detectable effect on serine phosphorylation as examined with anti-phosphoserine antibodies (data not shown). Antibodies against phosphothreonine, on the other hand, identified four proteins that were extensively phosphorylated in the plasma membrane of cells treated with insulin (Fig. 2A).

We examined whether the phosphoproteins PTRF or perilipin, in which we identified threonine-phosphorylated residues (Table 1), corresponded to the major phosphorylated perilipin protein bands. Immunoblotting analysis with antibodies against PTRF did not match any of the phosphothreonine protein bands in Fig. 2A (data not shown), whereas immunoblotting with anti-perilipin antibodies demonstrated that perilipin-phosphorylated bands corresponded to perilipin proteins (Fig. 3). Perilipin B was the major isoform in the plasma membrane (Fig. 2B), while perilipin A was the dominant isoform (>90%) in whole cell lysates of the human adipocytes (Fig. 2B), in agreement with a previous report that more than 85% of total perilipin in rat adipocytes is perilipin A (9). This very marked enrichment of perilipin B at the plasma membrane indicates a specific function of this isoform in the plasma membrane.

**Effect of Insulin or Isoproterenol**—We examined the effect of insulin or the β-adrenergic agonist isoproterenol in perilipin in the plasma membrane. Adipocytes were incubated with insulin or isoproterenol and the plasma membranes were isolated. Immunoblotting with anti-perilipin antibodies revealed that insulin recruited threonine-phosphorylated perilipin B and perilipin A to the plasma membrane (Fig. 2B). The increase of perilipin B was 11.0 ± 5.9-fold and of perilipin A 11.2 ± 4.8-fold (mean ± S.E., n = 3) in the plasma membrane in response to insulin treatment of the intact adipocytes. Isoproterenol, on the other hand, induced a very marked and specific depletion of perilipin B from the plasma membrane (Fig. 4A). The increase of perilipin B was 11.0 ± 5.9-fold and of perilipin A 11.2 ± 4.8-fold (mean ± S.E., n = 3) in the plasma membrane in response to insulin treatment of the intact adipocytes. Isoproterenol, on the other hand, induced a very marked and specific depletion of perilipin B from the plasma membrane (Fig. 4A).
In the present study we applied a proteomics approach (13) to study protein phosphorylation in the plasma membrane of human adipocytes. This allowed us to identify novel phosphorylation sites in a number of different proteins located at the plasma membrane, in particular threonine phosphorylation of perilipin. Key findings of this investigation were: first, that perilipin is triply phosphorylated on a tight cluster of threonine residues; second, that perilipin B is specifically associated with the plasma membrane of human adipocytes; and third, that this association is under insulin and β-adrenergic control.

Insulin increased the amount of perilipin A and B at the plasma membrane, while perilipin B was depleted at the plasma membrane in response to β-adrenergic stimulation. Our findings also suggest that the translocation of perilipin to the plasma membrane in response to insulin may be threonine phosphorylation-dependent. It has been shown that a central domain of perilipin consisting of amino acid residues 233–364 is important for targeting of perilipin to the central lipid droplet (19). The domain structure of perilipin is outlined in Fig. 5. Mass spectrometry analysis of protein phosphorylation in the plasma membrane revealed three phosphorylated threonine residues (299, 301, and 306) tightly clustered within this central domain of perilipin (Fig. 5). This cluster of phosphorylated threonine residues was also within an acidic domain (amino acids 292–319), which forms part of the lipid droplet targeting domain (19). The acidic character of this domain is further increased by introduction of the negative charges contributed by the threonine phosphorylation identified here. It remains to determine the function of such enhanced negative charge of the acidic domain in controlling perilipin targeting.

We have recently identified perilipin at the plasma membrane of primary rat adipocytes and demonstrated that perilipin is located to caveolae in insulin-treated adipocytes and that added long-chain fatty acids are rapidly converted to triacylglycerol in the membrane of the perilipin-containing caveolae (12). Considering the established function of perilipin A to protect the central lipid

DISCUSSION

In the present study we applied a proteomics approach (13) to study protein phosphorylation in the plasma membrane of human adipocytes. This allowed us to identify novel phosphorylation sites in a number of different proteins located at the plasma membrane, in particular threonine phosphorylation of perilipin. Key findings of this investigation were: first, that perilipin is triply phosphorylated on a tight cluster of threonine residues; second, that perilipin B is specifically associated with the plasma membrane of human adipocytes; and third, that this association is under insulin and β-adrenergic control.
The lipid-targeting domain and the acidic domain, respectively, as reported in Ref. 19. The predicted human protein sequences. Amino acids 233–364 and 292–319 comprise glycerol-synthesizing caveolae specifically.3 The finding that lipolytic stimulation causes translocation of perilipin B to the plasma membrane in response to insulin may be threonine phosphorylation-dependent. Stimulation of lipolysis with isoproterenol caused translocation of perilipin B away from the plasma membrane, presumably to allow lipolytic access to lipases such as hormone-sensitive lipase.

In conclusion, our findings suggest that perilipin B protects the triacylglycerol within the plasma membrane from being hydrolyzed during insulin-stimulated fatty acid uptake and triacylglycerol synthesis. The translocation of perilipin B to the plasma membrane in response to insulin may be threonine phosphorylation-dependent. Stimulation of lipolysis with isoproterenol caused translocation of perilipin B away from the plasma membrane, presumably to allow lipolytic access to lipases such as hormone-sensitive lipase.

Acknowledgments—We thank Drs. Preben Kjølhede and Gheorghe Andrescu for supplying biopsies of adipose tissue.

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plasma membrane was found to vary somewhat between preparations from different individuals, but perilipin B was invariably highly enriched at the plasma membrane compared with whole cells. With this in mind there is a remarkable preference for perilipin B in the plasma membrane supporting its specific function at the plasma membrane.

It is possible that the serine-phosphorylated perilipin A remaining in the plasma membrane after lipolytic activation by isoproterenol reflects a function of serine-phosphorylated perilipin A to support the hydrolysis of triacylglycerol in the plasma membrane, in agreement with a study showing that in perilipin null mice the hormone-sensitive lipase failed to translocate from the cytosol to the lipid droplet after stimulation of lipolysis but after introduction of perilipin A hormone-sensitive lipase was translocated to the lipid droplet (20). Furthermore, lipolytic stimulation of serine phosphorylation of perilipin A has been described to enhance the activity of hormone-sensitive lipase (10).

3 U. Orterguren and P. Strålfors, unpublished data.