Insulin Stimulates the Tyrosine Phosphorylation of Caveolin

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Abstract. The specialized plasma membrane structures termed caveolae and the caveolar-coat protein caveolin are highly expressed in insulin-sensitive cells such as adipocytes and muscle. Stimulation of 3T3-L1 adipocytes with insulin significantly increased the tyrosine phosphorylation of caveolin and a 29-kD caveolin-associated protein in caveolin-enriched Triton-insoluble complexes. Maximal phosphorylation occurred within 5 min, and the levels of phosphorylation remained elevated for at least 30 min. The insulin-dose responses for the tyrosine phosphorylation of caveolin and the 29-kD caveolin-associated protein paralleled those for the phosphorylation of the insulin receptor. The stimulation of caveolin tyrosine phosphorylation was specific for insulin and was not observed with PDGF or EGF, although PDGF stimulated the tyrosine phosphorylation of the 29-kD caveolin-associated protein. Increased tyrosine phosphorylation of caveolin, its associated 29-kD protein, and a 60-kD protein was observed in an in vitro kinase assay after incubation of the caveolin-enriched Triton-insoluble complexes with Mg-ATP, suggesting the presence of an intrinsic tyrosine kinase in these complexes. These fractions contain only trace amounts of the activated insulin receptor. In addition, these complexes contain a 60-kD kinase detected in an in situ gel kinase assay and an ~60 kD protein that cross-reacts with an antibody against the Src-family kinase $p59^{Fyn}$. Thus, the insulin-dependent tyrosine phosphorylation of caveolin represents a novel, insulin-specific signal transduction pathway that may involve activation of a tyrosine kinase downstream of the insulin receptor.

CAVEOLAE are small, flask-shaped invaginations of the plasma membrane ~50–100 nanometers in diameter (reviewed in 1, 2, 36). They are dynamic structures, cycling between an "open" state in which the interior is accessible to the extracellular media and a "closed" or vesicular conformation which remains associated with the plasma membrane. They can be readily distinguished from clathrin-coated pits by their size, morphology, and characteristic striated coat. This coat is composed in part of the protein caveolin, a 22-kD integral membrane protein, with an unusual 30–40 amino acid hydrophobic domain (7, 11, 13, 27). Caveolin is localized at the plasma membrane and associated with the trans-Golgi region (7). It was originally identified as a major tyrosine-phosphorylated protein in Rous sarcoma virus and v-Src–transformed fibroblast cells (10, 12), suggesting it plays a role in the transformed phenotype.

Caveolae are abundant in cells from many tissues, including lung, muscle, and adipose tissue (5, 18, 31). Freeze-fracture electron micrographs of the inner surface of the adipocyte plasma membrane reveal a high prevalence of small invaginations with the characteristic striated coat of caveolae (23). The coat protein caveolin is most abundantly expressed in white adipose tissue, followed by lung and muscle tissue (31). Caveolin expression is greatly increased during the differentiation of 3T3-L1 fibroblasts into adipocytes, suggesting an important role in adipocyte physiology, perhaps in the regulation of glucose transport (31) or glucose metabolism.

Caveolin is enriched 150- to 200-fold in low-density Triton-insoluble complexes that can be purified by flotation in sucrose density gradients (5, 18, 30). These protein/lipid complexes are also enriched in sphingolipids, cholesterol, and glycolipids (3, 17), and are over 150-fold enriched in glycosylphosphatidylinositol (GPI)[1]–anchored membrane proteins as well (5, 18, 30). However, the extent to which caveolin and GPI-anchored proteins are colocalized in the same strutures remains controversial (9, 19, 26, 40). GPI-linked signaling proteins, such as the T-cell receptor, are coupled to and bind cytoplasmic tyrosine kinases, many of which are Src-family kinases. Lck, Fyn, Lyn, c-Fgr, and c-Yes have all been shown to localize to Triton-insoluble complexes (18, 25, 34).

Caveolin-enriched Triton-insoluble complexes are highly enriched in GPI-linked proteins and possibly in free GPI;

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1. *Abbreviations used in this paper*: GPI, glycosylphosphatidylinositol; IPG, inositol phosphoglycan.

they may be derived from membrane microdomains involved in insulin-dependent GPI hydrolysis (4, 16, 21) and subsequent generation of inositol phosphoglycan (IPG) second messengers. Purified IPGs structurally related to GPI-membrane anchors can mimic the activity of insulin on such metabolic processes as glucose utilization and oxidation as well as anti-lipolysis in isolated adipocyte cells (29). Because many of insulin's actions are initiated by tyrosine phosphorylation, we isolated caveolin-enriched Triton-insoluble complexes from 3T3-L1 adipocytes and looked for changes in phosphorylation induced by insulin. We report here that insulin specifically stimulates the tyrosine phosphorylation of caveolin and a 29-kD caveolin-associated protein in a time- and dose-dependent manner. This phosphorylation may involve activation of a second tyrosine kinase downstream of the insulin receptor.

Materials and Methods

Materials

All tissue culture reagents were purchased from GIBCO-BRL (Gaithersburg, MD). Insulin (porcine) was purchased from Sigma (St. Louis, MO); platelet-derived growth factor–BB (PDGF; human, recombinant) was purchased from R&D Systems, Inc. (Minneapolis, MN); epidermal growth factor (EGF; mouse) was purchased from Becton Dickinson (Bedford, MA). BSA (fraction V) was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). Monoclonal and polyclonal anti-caveolin antibodies were purchased from Transduction Laboratories (Lexington, KY). Monoclonal anti-phosphotyrosine antibody 4G10 was purchased from UBI (Lake Placid, NY). Polyclonal rabbit anti-fyn antiserum was prepared against a fyn/TrpE fusion protein (amino acid 268–389; numbered according to reference 33). Prestained molecular weight-markers (high range) and horseradish peroxidase–conjugated goat anti–mouse IgG antibodies were purchased from GIBCO BRL (Gaithersburg, MD). Octylthioglucoside and Triton X-100 were purchased from Boehringer Mannheim (Indianapolis, IN). [γ-^{32}P]ATP (3,000 Ci/mmol) was purchased from New England Nuclear Corp. (Wilmington, DE). All other reagents were purchased from Sigma. Protein concentrations were determined using the BCA protein determination kit from Pierce (Rockford, IL).

Cell Culture

3T3-L1 fibroblast cells (CCL 92.1; American Type Culture Collection, Rockville, MD) were grown in DME (high glucose) supplemented with 10% calf serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50 mg/ml streptomycin. They were differentiated into adipocytes on 150-mm culture plates as described (28). Cells were used for experiments 5 or 6 d after initiation of differentiation. At this point in the differentiation protocol, most of the cells (>90%) contained several small fat droplets. These cells showed large increases in glucose uptake and metabolism in response to nanomolar concentrations of insulin when serum deprived as described below (38).

Preparation of Triton-insoluble Complexes

Caveolin-enriched Triton-insoluble complexes were prepared essentially as described (18, 31). Adipocytes were incubated 2–3 h prior to assay in Krebs-Ringer bicarbonate buffer supplemented with 30 mM Hepes, pH 7.4, 0.5% BSA, and 2.5 mM glucose (KRBH/BSA/glucose). Insulin (0.1–100 nM), PDGF (100 ng/ml), or EGF (200 ng/ml) were added, and incubation continued at 37°C for 0.5–30 min, as indicated. Cells for basal samples were treated identically, without addition of growth factors. Cells (1 or 2 150-mm plates per condition) were washed three times in ice-cold PBS and harvested by scraping into 25 mM MES, pH 6.0, 150 mM NaCl, 1% Triton X-100, 1 mM $NaVO_4$, 10 μg/ml aprotinin, and 10 μg/ml leupeptin (MES/NaCl/TX-100). After homogenization (10 strokes in glass homogenizer with a Teflon pestle), 2 ml of cell lysate were diluted 1:1 with 80% sucrose (40% sucrose final concentration), 25 mM MES, pH 6.0, 150 mM NaCl. A gradient was formed by overlaying the extract with 2 ml each of 35, 25, 15, and 5% sucrose in 25 mM MES, pH 6.0, 150 mM NaCl. The gradients were centrifuged for 20 h at 39,000 rpm in an SW40ti rotor (Beckman Instruments, Fullerton, CA). Triton-insoluble complexes enriched in caveolin were collected as a flocculent band of material just below the 15–25% interface using a 19-gauge needle and a syringe (~1–2 ml). This fraction was diluted two- to threefold with MES/NaCl/TX-100, and the complexes were collected by centrifugation at 14,000 rpm in a microfuge for 10 min at 4°C. In some experiments, samples of the 40% sucrose layer below the gradient and the material in the pellet were also collected.

Immunoblotting

Samples in Laemmli sample buffer (4% SDS, 115 mM Tris-Cl, pH 6.8, 1 mM EDTA, 10% glycerol, 4 mg/ml bromophenol blue) with 10 mM DTT were boiled for 2 min, proteins were separated by SDS-PAGE, and the proteins were transferred to nitrocellulose (0.2-m pore size; Schleicher & Schuell, Keene, NH) for 30 min at 100 mA, followed by 2 h at 400 mA in 20% methanol, 192 mM glycine, 25 mM Tris, 0.005% SDS. The membranes were blocked by incubation for 1 h in TBST (20 mM Tris, pH 7.6, 150 mM NaCl, 0.05% Tween 20, 0.01% NP-40, 0.1% Brij 58) with 1% ovalbumin and 1% BSA (anti-phosphotyrosine) or with 5% nonfat dried milk (anti-caveolin or anti-fyn). The membranes were incubated overnight with anti-phosphotyrosine antibody diluted in TBST with 1% ovalbumin and 1% BSA (1/4,000), with monoclonal anti-caveolin antibody diluted in TBST with 1% milk (1/2,000), or with polyclonal anti-fyn diluted in TBST with 1% milk (1/1,000). After washing four times in TBST, the membranes were incubated for 30 min with horseradish peroxidase–conjugated goat anti–mouse IgG antibody or goat anti–rabbit IgG antibody diluted in the appropriate blocking buffer (1/3,000), washed two times in TBST, two times in TBS (20 mM Tris, pH 7.6, 150 mM NaCl), and the labeled proteins visualized by the enhanced chemiluminescence method (Amersham Corp., Arlington Heights, IL). Different lot numbers of the monoclonal anti-caveolin antibody showed either one or two bands by western blotting. This difference was also observed by Transduction Laboratories.

Colloidal Gold Staining

Proteins were separated by SDS-PAGE and transferred to nitrocellulose as described earlier. The membranes were blocked by incubation for 1 h in TBST. After washing with dH_2O, the proteins were visualized by overnight incubation with Colloidal Gold Total Protein Stain (Bio-Rad Laboratories, Hercules, CA). The membranes were rinsed with dH_2O and air dried.

Immunoprecipitation

Caveolin-enriched Triton-insoluble complexes were prepared as described earlier, and solubilized in 10 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 60 mM octylthioglucoside, 1 mM $NaVO_4$, 10 μg/ml aprotinin, and 10 μg/ml leupeptin (1 h on ice). The samples were precleared with protein A–Sepharose and immunoprecipitated with 4 μg polyclonal rabbit anti–caveolin antibody, or 2 μl polyclonal rabbit anti–fyn antibody, and protein A–Sepharose. After washing three times with the solubilization buffer, proteins were eluted using Laemmli sample buffer with 8 M urea, without DTT.

In Vitro Kinase Assays

Caveolin-enriched Triton-insoluble complexes were prepared as described above from adipocytes (2 150-mm plates/condition) incubated with or without 100 nM insulin for 15 min. The gradient fractions enriched in caveolin were divided into three samples prior to the final centrifugation step. The caveolin-enriched pellets were resuspended in 5 μl MES/NaCl/TX-100. The reaction was initiated by the addition of 20 μl kinase assay buffer (22.5 mM Hepes, pH 7.5, 12.5 mM $MgCl_2$, 1.25 mM $MnCl_2$, 1.25 mM EGTA) with or without 1 mM ATP, and the samples were incubated for 10 min at room temperature. The reaction was terminated with 4×-concentrated Laemmli sample buffer and protein tyrosine phosphorylation determined by Western blotting.

In one set of experiments, Triton-insoluble complexes were prepared by solubilizing cells (1 150-mm dish) in 1 ml MES/NaCl/TX-100 and the insoluble material pelleted by centrifugation at 14,000 rpm in a microfuge for 15 min at 4°C. The pelleted fraction was enriched in caveolin relative to the whole cell lysate, although a significant amount of the caveolin remained in the supernatant. In vitro kinase assays were performed as described previously in 50 μl kinase assay buffer, and the reaction was terminated by the addition of 1 ml solubilization buffer (see *Immunoprecipitation*). After incubation for 1.5 h on ice, the insoluble material was re-

moved by centrifugation, and caveolin was immunoprecipitated as described previously.

In Gel Kinase Assays

Triton-insoluble complexes were isolated from basal adipocytes or adipocytes that had been treated for 15 min with 100 nM insulin (2 150-mm plates per condition). The proteins were resolved by SDS-PAGE, and in situ gel kinase assays were performed as previously described (14, as modified by 22). No exogenous substrates were incorporated into the gels.

Image Processing

Autoradiographs were quantified by computer-assisted video densitometry using the Bio Image system (Imaging Systems, Millipore Corp., Ann Arbor, MI). Figures of autoradiographs were constructed using a Microtek 600ZS ScanMaker Scanner (Microtek International, Inc., Hsinchu, Taiwan, R.O.C.) and Adobe Photoshop software (Adobe Systems Inc., Mountain View, CA).

Results

Insulin Stimulates the Tyrosine Phosphorylation of Proteins in Caveolin-enriched Triton-insoluble Complexes

Caveolae are abundant in adipocytes (23, 31). In addition, the expression of caveolin is increased 30-fold upon differentiation of 3T3-L1 fibroblasts into adipocytes, with a time course similar to that for expression of proteins involved in the regulation of glucose uptake and metabolism by insulin. To assess the role of this plasma membrane–associated organelle in insulin signal transduction, Triton-insoluble complexes enriched in caveolin were isolated by sucrose density gradient centrifugation from basal 3T3-L1 adipocytes or cells stimulated with 100 nM insulin for 15 min. These protein/lipid complexes are less dense than the majority of proteins solubilized by Triton extraction and float up into the gradients. They are recovered as a band of flocculent material just below the 25–15% sucrose interface. The amount of caveolin in fractions from the gradients was determined by Western blotting (Fig. 1 *A*). After centrifugation, the caveolin was completely depleted from the 40% sucrose layer, which contained the original lysate (Fig. 1 *A*; compare lanes *7* and *8* with lanes *1* and *2*). None of the caveolin was recovered in the pellet (lane *9*). Approximately 70% of the caveolin was recovered in the Triton-insoluble complexes (lanes *3–6*). Insulin stimulation did not change the recovery of caveolin in these complexes. The differences observed in this experiment in the recovery of caveolin in the basal and insulin-stimulated Triton-insoluble complexes are indicative of the level of variability of recovery of caveolin in these experiments (see Figs. 2 and 3).

Phosphorylation of proteins in the caveolin-enriched complexes in response to insulin was investigated by Western blotting using an anti–phosphotyrosine-specific antibody (Fig. 1 *B*). Two major phosphotyrosine-containing proteins were detected in the Triton-insoluble fractions under basal conditions, with molecular weights of 23 and 60 kD. Treatment of adipocytes with 100 nM insulin increased the tyrosine phosphorylation of the 23-kD protein and led to the tyrosine phosphorylation of proteins migrating at 21 and 29 kD. There was no detectable change in the level of tyrosine phosphorylation of the 60-kD protein in response to insulin relative to the amount of caveolin isolated in these fractions. Loading more of the caveolin-enriched fractions on the gels revealed that the 21-, 23-, and 29-kD proteins were all tyrosine phosphorylated in the basal state, although insulin markedly increased this phosphorylation (Fig. 2). The 21- and 23-kD proteins exactly comigrated with caveolin and could be immunoprecipitated with an anti-caveolin antibody (Fig. 4 *B*). The 29-kD phosphoprotein also coimmunoprecipitated with caveolin (Fig. 4 *B*) and may represent a previously described 31-kD caveolin-associated protein which is phosphorylated in response to insulin (31). A small and variable amount of a tyrosine-phosphorylated 95-kD protein was also detected in the Triton-insoluble complexes. This protein comigrates with the major insulin-induced tyrosine-phosphorylated protein in whole-cell lysates and is likely to be the β-subunit of the insulin receptor.

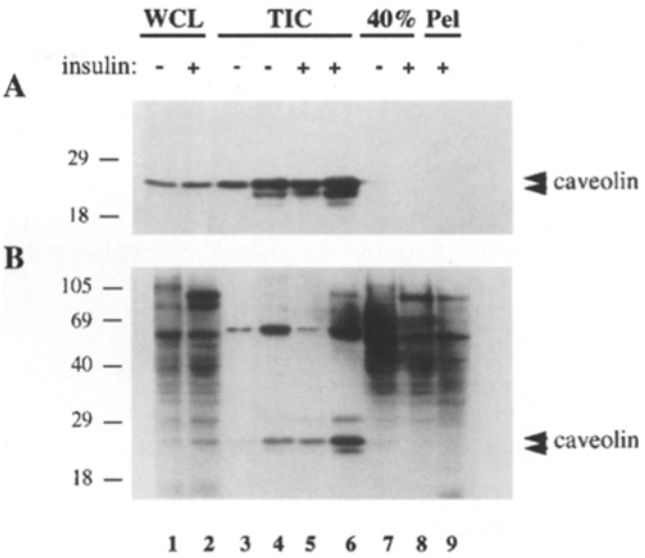

Figure 1. Insulin stimulates the tyrosine phosphorylation of several proteins in caveolin-enriched Triton-insoluble complexes. 3T3-L1 adipocytes (1 150-mm dish per condition) were incubated with or without 100 nM insulin for 15 min, and caveolin-enriched Triton-insoluble complexes (*TIC*) were isolated. Samples of the original lysate (*WCL*), the 40% sucrose layer below the gradient (*40%*), and the pelleted material (*Pel*)were also collected. The relative distribution of (*A*) caveolin and (*B*) tyrosine-phosphorylated proteins in the fractions were determined by Western blotting. Samples loaded represent 0.8% of the original lysate (50 μg), 3% (lanes *3* and *5*) or 10% (lanes *4* and *6*) of the Triton-insoluble complexes, 0.8% of the 40% sucrose fraction, and 5% of the pellet. The 21- and 23-kD tyrosine-phosphorylated proteins comigrate with caveolin and are immunoprecipitated with anti-caveolin antibodies (Fig. 4); the 29-kD protein coimmunoprecipitates with caveolin (Fig. 4). The 95-kD protein comigrates with the β-subunit of the insulin receptor. Positions of molecular weight standards are indicated (in kD).

The time course of tyrosine phosphorylation of proteins in response to 100 nM insulin was investigated by Western blotting (Fig. 2). Insulin receptor phosphorylation was measured in whole-cell lysates (Fig. 2 *A*). Phosphorylation of the major 95-kD protein reached a maximum within 0.5 min of stimulation and decreased after 15–30 min. This result is similar to the time courses previously reported for the phosphorylation of the insulin receptor and its substrates IRS-1 and SHC. Tyrosine phosphorylation of cave-

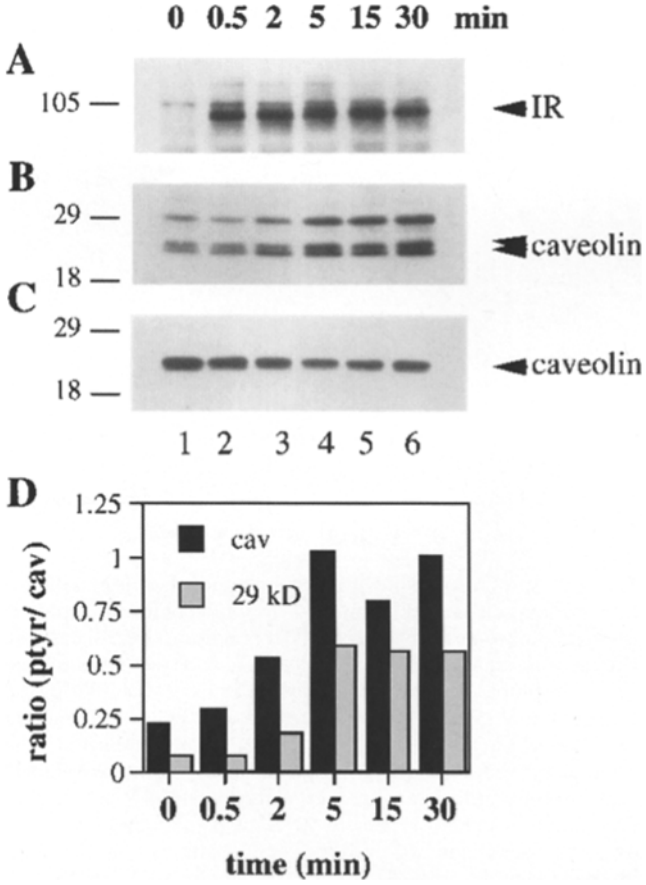

Figure 2. Time course for the tyrosine phosphorylation of caveolin and the 29-kD caveolin-associated protein in response to insulin. 3T3-L1 adipocytes (1 150-mm dish per condition) were incubated with 100 nM insulin for 0–30 min, and caveolin-enriched Triton-insoluble complexes were isolated. (*A*) Tyrosine phosphorylation of the insulin receptor (*IR*) was measured as the tyrosine phosphorylation of the major 95-kD protein in whole-cell lysates (25 μg). (*B*) Tyrosine phosphorylation of caveolin and the 29-kD caveolin-associated protein in the Triton-insoluble complexes (7.5% of fraction) were determined by Western blotting. (*C*) Caveolin in the Triton-insoluble complexes (7.5% of fraction) was determined by Western blotting. Note that different preparations of anti-caveolin antibodies recognized either one or two forms of the protein (see Materials and Methods). (*D*) Integrated optical densities were determined for the 21- and 23-kD tyrosine-phosphorylated proteins (*cav*) and the 29-kD protein (*29kD*) shown in panel (*B*). These values were normalized by the integrated optical densities for caveolin in each sample as shown in panel (*C*) and expressed as the ratio of tyrosine-phosphorylated proteins to caveolin (*ratio [ptyr/cav]*), in arbitrary units. These data are representative of three separate experiments.

olin and the 29-kD caveolin-associated protein were measured in the Triton-insoluble complexes (Fig. 2 *B*). The relative levels of caveolin in each preparation were determined by western blotting (Fig 2 *C*), and the levels of tyrosine-phosphorylation of caveolin and the 29-kD protein were expressed as a ratio of tyrosine phosphorylated protein to caveolin (ptyr/cav). The increased tyrosine phosphorylation of caveolin (21 and 23 kD) and the 29-kD protein in the Triton-insoluble complexes (4.7-fold and 8-fold, respectively, in this experiment) reached a maximum within 5 min and remained elevated for 30 min (Fig. 2 *D*).

The dose dependencies for the insulin-stimulated phos-

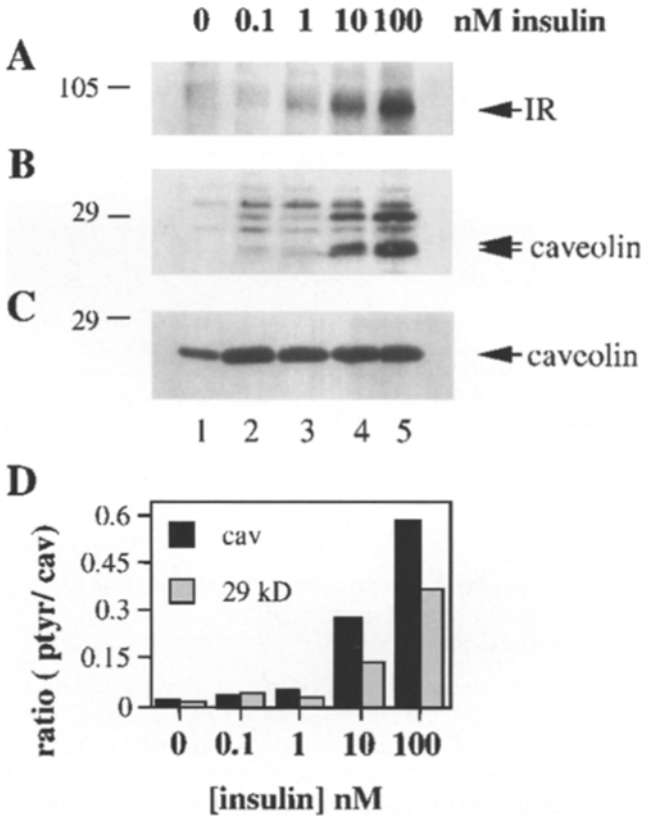

Figure 3. Insulin dose response of the tyrosine phosphorylation of caveolin and the 29-kD caveolin-associated protein. 3T3-L1 adipocytes (1 150-mm dish per condition) were incubated with 0 to 100 nM insulin for 15 min, and caveolin-enriched Triton-insoluble complexes were isolated. (*A*) Tyrosine phosphorylation of the insulin receptor (*IR*) in whole-cell lysates was determined as in Fig. 2 (25 μg). (*B*) Tyrosine phosphorylation of caveolin and the 29-kD caveolin-associated protein in the Triton-insoluble complexes (30% of fraction) were determined by Western blotting. (*C*) Caveolin in the Triton-insoluble complexes (10% of fraction) was determined by Western blotting. (*D*) Tyrosine phosphorylation of caveolin (*cav*) and the 29-kD protein (*29kD*) were quantified as described in the legend for Fig. 2 and expressed as the ratio of tyrosine-phosphorylated protein to caveolin. These data are representative of two separate experiments.

phorylation of the insulin receptor, caveolin, and the 29-kD caveolin-associated protein were determined (Fig. 3). Phosphorylation of the insulin receptor β-subunit in whole-cell lysates was detectable at 1 nM insulin and continued to increase up to 100 nM insulin (Fig. 3 *A*). Phosphorylation of caveolin (21 and 23 kD) and the 29-kD protein in the Triton-insoluble complexes occurred over a similar concentration range (Fig. 3, *B–D*). Increased tyrosine phosphorylation of caveolin and the 29-kD protein was observed at 10 nM, and in this experiment reached 17-fold and 14-fold over the basal level at 100 nM insulin. The stimulation of glucose uptake and lipogenesis measured in parallel samples of cells increased similarly over a concentration range of 0.1 to 100 nM insulin (data not shown).

Insulin and PDGF Stimulate Different Patterns of Protein Tyrosine Phosphorylation in the Triton-insoluble Complexes

Several early phosphorylation pathways stimulated by in-

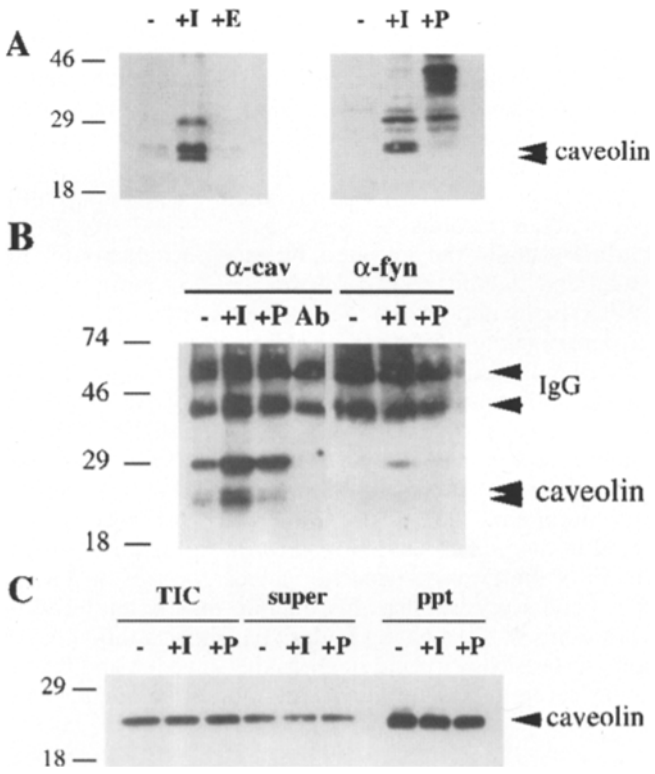

Figure 4. Growth factors induce distinct patterns of tyrosine phosphorylation in caveolin-enriched Triton-insoluble complexes. Caveolin-enriched Triton-insoluble complexes were isolated from basal 3T3-L1 adipocytes (−) or adipocytes that had been incubated with 100 nM insulin (+*I*), 200 ng/ml EGF (+*E*), or 100 ng/ml PDGF (+*P*) for 15 min. (*A*) Tyrosine phosphorylation of proteins in the Triton-insoluble complexes was determined by Western blotting (30% of each fraction was used). The relative effects of insulin and EGF, and of insulin and PDGF were determined in separate experiments. The data shown are representative of three separate experiments for each comparison. (*B*) Caveolin was immunoprecipitated from solubilized complexes isolated from basal cells (−) or cells treated with 100 nM insulin (+*I*) or 100 ng/ml PDGF (+*P*). The tyrosine-phosphorylated proteins in the immunoprecipitates were determined by Western blotting. Very little caveolin or the 29-kD tyrosine-phosphorylated protein was precipitated using an anti-fyn antibody. Samples represent 16% of the protein precipitated with anti-caveolin antibody (*α-cav*) and 62% of the protein precipitated with anti-fyn antibody (*α-fyn*) from complexes from 2 150-mm dishes. A control sample with antibody but no lysate is also shown (*Ab*). (*C*) Distribution of caveolin in the original complexes (*TIC*), supernatants (*super*), and pellets (*ppt*) from the anti-caveolin immunoprecipitations. Equal amounts of caveolin were immunoprecipitated in each sample. Samples represent 5% of the original complexes, 5% of the supernatants, and 25% of the pellets.

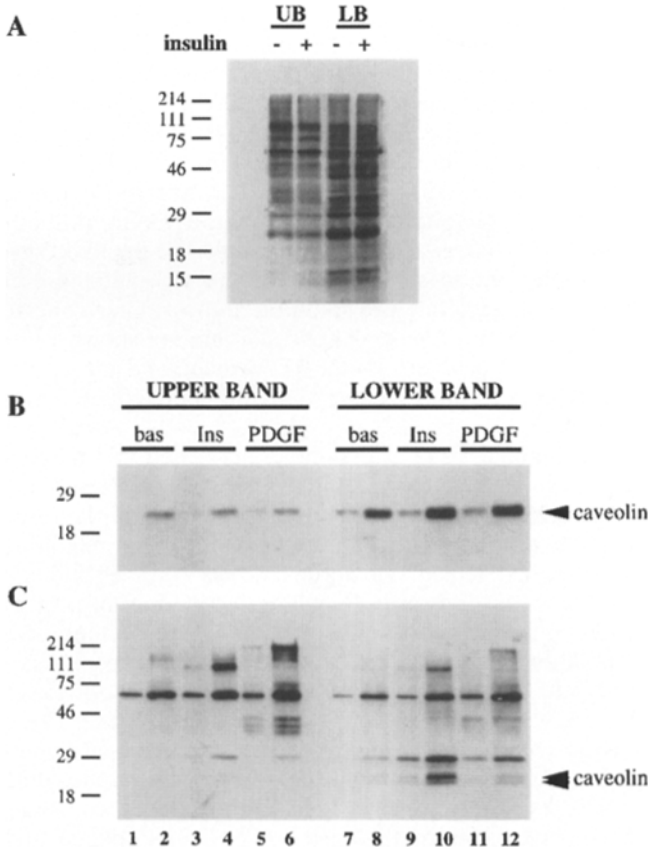

Figure 5. The caveolin-enriched fraction can be resolved into two different populations of Triton-insoluble complexes. Cell lysates were prepared from untreated (*bas*) adipocytes (2 150-mm plates per condition) or from cells treated for 15 min with 100 nM insulin (*Ins*) or 100 ng/ml PDGF (*PDGF*), and Triton-insoluble complexes were isolated by density gradient centrifugation as described in Materials and Methods. Two bands of flocculent material were observed in the gradients, one within the 15% sucrose layer (*UPPER BAND*), and one just below the 25–15% interface (*LOWER BAND*). Triton-insoluble complexes were prepared from both. (*A*) Colloidal gold protein staining of the Triton-insoluble complexes prepared from the upper band (*UB*) and the lower band (*LB*) from basal cells (−) or cells treated with insulin (+) (2.5% of each fraction). (*B*) The distribution of caveolin in the two Triton-insoluble complexes was determined by Western blotting. Samples represent either 1.7% (lanes *1, 3, 5, 7, 9, 11*) or 5% (lanes *2, 4, 6, 8, 10, 12*) of each fraction. (*C*) The distribution of tyrosine-phosphorylated proteins in the two Triton-insoluble complexes was determined by Western blotting. Samples represent either 1.7% (lanes *1, 3, 5, 7, 9, 11*) or 5% (lanes *2, 4, 6, 8, 10, 12*) of each fraction.

sulin are shared by other growth factors, such as EGF and PDGF. The 3T3-L1 adipocytes express significant numbers of insulin, EGF, and PDGF receptors, all of which produce similar levels of mitogen-activated protein kinase activity, with similar time courses (8, 15, 24, 37). In addition, both insulin and PDGF (but not EGF) stimulate the association of phosphatidylinositol 3′-kinase activity with tyrosine-phosphorylated proteins (38). Despite these similarities, insulin stimulates glucose uptake and metabolism (lipogenesis and glycogen synthesis) in these cells, while EGF and PDGF do not (38). To determine whether the increases in the tyrosine phosphorylation of proteins in the Triton-insoluble complexes were specific for insulin, we compared the effects of insulin, EGF, and PDGF by Western blotting (Fig. 4). As described above, insulin stimulated the tyrosine phosphorylation of caveolin and the 29-kD protein. In contrast, EGF was completely without effect (Fig. 4 *A*, *left*), while exposure of cells to PDGF resulted in the tyrosine phosphorylation of the 29-kD protein but not caveolin itself (Fig. 4 *A*, *right*). In addition, three proteins of molecular weight 38, 41, and 44 kD were

phosphorylated in response to PDGF, although these phosphorylations were not detected in response to insulin. The 29-kD protein phosphorylated in response to PDGF was coimmunoprecipitated with caveolin (Fig. 4 *B*), suggesting that it is the same protein that is phosphorylated in response to insulin. Under these conditions, ~40% of the caveolin in the Triton-insoluble complexes was depleted by immunoprecipitation with the anti-caveolin antibody (Fig. 4 *C*). There was a similar reduction of the 29-kD tyrosine-phosphorylated protein from the supernatants after anti-caveolin immunoprecipitation and a similar concentration of the protein in the pellets (data not shown). The 38- to 44-kD proteins, the 60-kD protein, and the insulin and PDGF receptors were not coimmunoprecipitated with caveolin.

Careful analysis of the gradients suggested that the Triton-insoluble complexes described above could be subfractionated (Fig. 5). When only a single 150-mm plate was used to prepare the complexes, two closely migrating bands were detected, one at the 25–15% interface and one just below this interface. Doubling the amount of protein loaded onto the gradients allowed us to resolve these two populations. When two plates were used to prepare a single lysate, the upper band migrated at a significantly lower density, within the 15% layer, and was quite diffuse. There was no change in the migration of the more dense complex, which remained a tight band. When Triton-insoluble complexes were separately isolated from these two bands, the complexes from the upper band were opaque and white, while the complexes from the lower band were clear and slightly yellow. The complexes from the upper and lower bands also had different protein compositions, as evidenced by colloidal gold staining (Fig. 5 *A*). The lower band was ~3 times more enriched in caveolin than the upper band, indicating that the majority of the caveolae were recovered in the Triton-insoluble complexes from the lower band (Fig. 5 *B*). Treating cells with insulin or PDGF had no effect on the distribution of caveolin in these two fractions. The tyrosine-phosphorylated caveolin and the 29-kD caveolin-associated protein were both enriched in the lower band, whereas the 38-, 41-, and 44-kD proteins tyrosine phosphorylated in response to PDGF were concentrated in the upper band (Fig. 5 *C*). The 60-kD phosphoprotein was found at approximately equal concentration in both fractions.

Interestingly, the activated, tyrosine-phosphorylated insulin and PDGF receptors were ~5 times more concentrated in the upper band than in the lower band (Fig. 5 *B*). Approximately 1.5% of the insulin receptors and 2.8% of the PDGF receptors were recovered in the upper bands, 0.3% of the insulin receptors and 0.5% of the PDGF receptors were recovered in the lower bands, 32% of the insulin receptors and 22% of the PDGF receptors were recovered in the 40% sucrose layers, and 4.4% of the insulin receptors and 2.1% of the PDGF receptors were recovered in the pellets (data not shown). The relative enrichment of the tyrosine-phosphorylated receptors in the upper bands suggests that this fraction may be enriched in Triton-insoluble complexes derived from clathrin-coated pits and vesicles (6), and suggests that caveolin and the insulin receptor are found in different protein/lipid complexes.

Caveolin-enriched Triton-insoluble Complexes Contain Intrinsic Kinase Activities

The relatively slow time course of phosphorylation of caveolin and the 29-kD protein suggests that these proteins may not be direct substrates of the insulin receptor (Fig. 2). This is supported by the observation that only a very small fraction of the insulin receptor is found in the Triton-insoluble fractions most highly enriched in caveolin (Fig. 5). To explore the nature of the kinases responsible for the phosphorylation of caveolin and the 29-kD protein,

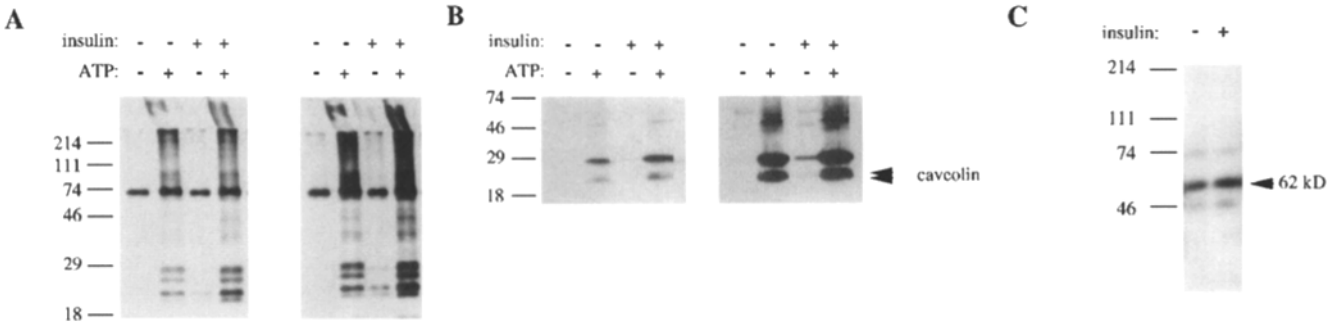

Figure 6. In vitro tyrosine phosphorylation in caveolin-enriched Triton-insoluble complexes. Caveolin-enriched membrane domains were prepared from basal cells or cells stimulated with insulin for 15 min (2 150-mm plates/condition). (*A*) Caveolin-enriched Triton-insoluble complexes (lower band only) treated with or without 1 mM ATP for 10 min at room temperature, and tyrosine phosphorylation determined by anti-phosphotyrosine Western blotting. Each sample represents 30% of the complexes isolated. *Left*: 20-s exposure; *right*: 60-s exposure. There were equal amounts of caveolin in the samples compared in each panel (data not shown). Data presented are representative of three separate experiments. (*B*) Caveolin and the 29-kD caveolin-associated protein were immunoprecipitated from less highly purified Triton-insoluble complexes (see Material and Methods) before or after incubation with 1 mM ATP for 10 min. Tyrosine phosphorylation was determined by Western blotting. The samples represent 60% of the precipitates from the Triton-insoluble pellets from 50% of 1 150-mM dish. *Left*: 10-s exposure; *right*: 60-s exposure. All samples had equal amounts of caveolin (data not shown). Data are representative of two separate experiments. (*C*) In situ gel kinase assay. Proteins in the caveolin-enriched Triton-insoluble complexes were resolved by SDS-PAGE. The proteins were renatured by incubation with a renaturation buffer, and the gels incubated with [γ-^{32}P]ATP. After extensive washing, the incorporated ^{32}P was visualized by autoradiography (15-h exposure with intensifying screen). Samples represent 100% of the complexes isolated from 2 150-mM dishes. Data are representative of three separate experiments.

phosphorylation was directly assayed in the isolated Triton-insoluble complexes (Fig. 6). The intact isolated protein complexes were incubated with 1 mM Mg-ATP and tyrosine phosphorylation of proteins determined by Western blotting. Incubation with ATP produced a significant increase in the tyrosine phosphorylation of caveolin and the 29-kD protein, and a smaller increase in the phosphorylation of the 60-kD protein (Fig. 6 *A*). An additional 27-kD protein was phosphorylated after treatment with ATP, which was not detected in any of the complexes before ATP addition. Immunoprecipitation of caveolin, and its 29-kD associated protein, before and after the addition of ATP, confirmed that these two proteins are tyrosine phosphorylated in the in vitro kinase assay (Fig. 6 *B*). The caveolin and 29-kD proteins were more heavily tyrosine phosphorylated after incubation with ATP in the complexes isolated from insulin-stimulated cells than from the basal cells, even after correcting for differences in tyrosine phosphorylation before adding ATP. Therefore, the caveolin kinase activity intrinsic to the Triton-insoluble complexes is stably increased after insulin treatment. There was no difference in the level of phosphorylation of the 60-kD protein between the complexes isolated from basal and insulin-stimulated cells. The level of caveolin phosphorylation in the in vitro kinase assays did not correlate with the amount of activated insulin receptor isolated with the Triton-insoluble complexes. In the experiment shown there was no tyrosine-phosphorylated insulin receptor detected in the samples, either before or after the addition of ATP (Fig. 6 *A*), even after very long exposure (data not shown). When the kinase activity was measured in intact complexes using [γ-^{32}P]ATP and autoradiography, the tyrosine phosphorylations were masked by more prevalent serine phosphorylations (data not shown).

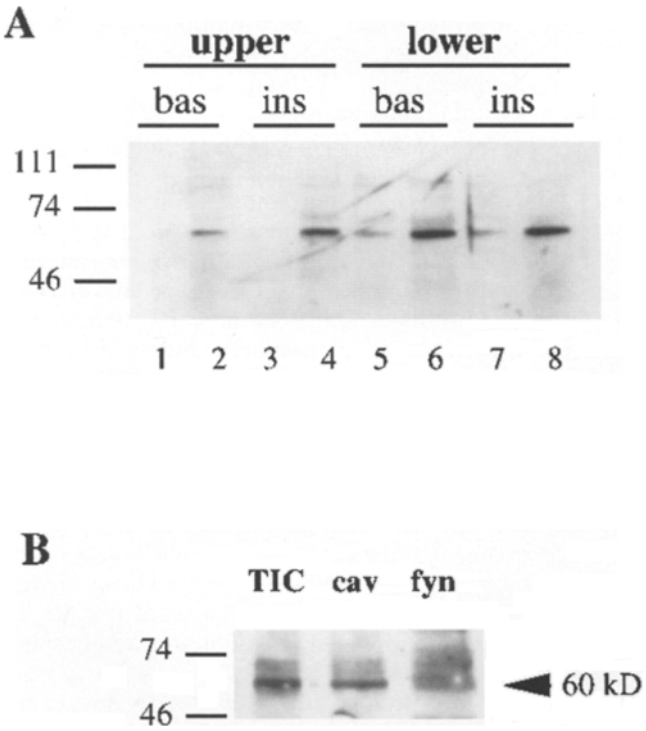

Figure 7. Caveolin-enriched Triton-insoluble complexes from 3T3-L1 adipocytes contain the Src-family kinase p59^{fyn}. Caveolin-enriched membrane domains were prepared from basal cells or cells stimulated with insulin for 15 min (2 150-mm plates/condition). (*A*) Distribution of p59^{fyn} in caveolin-enriched Triton-insoluble complexes from the upper band (*upper*) or lower band (*lower*) from basal (*bas*) or insulin-stimulated (*ins*) cells was determined by Western blotting. Samples represent 5% (lanes *1*, *3*, *5*, *7*) or 15% (lanes *2*, *4*, *6*, *8*) of the complexes isolated. (*B*) Caveolin-enriched Triton-insoluble complexes were solublized and depleted of caveolin or p59^{fyn} by immunoprecipitation. Tyrosine-phosphorylated proteins remaining in the supernatants were determined by Western blotting. Samples represent 4% of original complexes (*TIC*), or of the supernatant from the anti-caveolin immunoprecipitation (*cav*), and 12% of the supernatant from the anti-fyn immunoprecipitation (*fyn*). Data are representative of two separate experiments.

To identify which of the caveolar proteins were kinases themselves, the proteins in the Triton-insoluble complexes were resolved by SDS-PAGE before treatment with ATP, and an in situ gel kinase assay was performed (Fig. 6 *C*). The proteins in the gel were renatured by incubation in a renaturation buffer and incubated with [γ-^{32}P]ATP. A single major protein of 62 kD was labeled, and two minor proteins were also labeled. The intensity of labeling was the same for samples prepared from basal or insulin-stimulated cells. Because incorporation of ^{32}P was too low for phosphoamino acid analysis, we were unable to determine whether the 62-kD protein is a serine/threonine or tyrosine kinase. Nevertheless, these data suggest that caveolin-enriched Triton-insoluble complexes contain an intrinsic kinase with a molecular weight of ~60 kD. This kinase may be stably activated by insulin as detected in the in vitro kinase assays, if the proper substrate were provided (i.e., caveolin). However, it is possible that the activation is lost upon denaturation and renaturation of the protein, or by separation of the kinase from activating factors.

A number of Src-family tyrosine kinases have been localized to Triton-insoluble complexes. Western blotting with antibodies against a number of Src-family kinases revealed that p59^{fyn}, or a related protein, is localized to the caveolin-enriched Triton-insoluble complexes isolated from 3T3-L1 adipocytes (Fig. 7 *A*). Although it is present in both fractions, the p59^{fyn} appears enriched in the complexes from the lower band relative to the upper band. Immunoprecipitation of solubilized complexes with anti–fyn antibody significantly depleted the supernatant of the 60-kD tyrosine phosphorylated protein (Fig. 7 *B*). Note that the anti–fyn supernatant sample is three times more concentrated than the other two samples. These data show that caveolin-enriched Triton-insoluble complexes contain an intrinsic tyrosine kinase activity that can phosphorylate both caveolin and the 29-kD protein, and suggest that this kinase is not the insulin receptor but p59^{fyn} or a related protein.

Discussion

Caveolin-enriched Triton-insoluble complexes have unique protein and lipid compositions. They are highly enriched in sphingolipids, cholesterol, and glycolipids (3, 17) relative to the total cell membranes, accounting for their detergent insolubility and decreased fluidity. It is likely that this lipid environment plays a key role in the localization of proteins

to these complexes. GPI-anchored proteins are also highly enriched in these fractions (5, 18, 30). The GPI moiety is sufficient for localization of GPI-anchored proteins to Triton-insoluble complexes (25). Many of the GPI-anchored receptors are coupled to Src-family kinases, some of which have been found in caveolin-enriched Triton-insoluble complexes (18, 25, 34). Amino terminal tandem acylation (palmitoylation and myristoylation) is both necessary and sufficient for the association of the Src-family kinases with GPI-linked proteins and Triton-insoluble complexes (25, 34). This motif is present in G-protein α-subunits, which have also been localized to these complexes. Although the biochemical basis for the concentration of lipid-modified proteins in Triton-insoluble complexes remains unknown, both acylated and GPI-linked proteins are frequently modified with saturated fatty acids, perhaps directing these proteins to the less fluid environment in these complexes (32).

In addition to clustering GPI-linked proteins, it is likely that the caveolin-enriched Triton-insoluble complexes also concentrate free GPIs, the precursors of inositol phosphoglycans that have been proposed as second messengers for insulin. Indeed, caveolae have been proposed to act as signaling organelles (1). Since initiation of the early signaling events in insulin action involves tyrosine phosphorylation (39), we explored the possibility that these phosphorylations might target caveolar molecules. Caveolin-enriched Triton-insoluble complexes from basal or insulin-treated 3T3-L1 adipocytes were isolated and protein tyrosine phosphorylation was compared in the two fractions. Caveolin-enriched Triton-insoluble complexes contain four major tyrosine-phosphorylated proteins under basal conditions, two isoforms of the caveolar coat protein caveolin (21 and 23 kD), a 29-kD caveolin-associated protein, and a 60-kD protein (Figs. 1–5). Insulin stimulated the tyrosine phosphorylation of both forms of caveolin as well as an unidentified 29-kD caveolin-associated protein, which may be identical to a 31-kD caveolin-associated protein previously shown by Scherer et al. (31) to be phosphorylated in response to insulin. Tyrosine phosphorylation of caveolin was not detected by Scherer et al. under either basal or insulin-stimulated conditions, probably due to the relative insensitivity of the technique used to evaluate tyrosine phosphorylation. Maximal phosphorylation of caveolin and the 29-kD caveolin-associated protein occurred after 5 min of incubation, lagging behind the phosphorylation of the insulin receptor, which was observed within 30 s (Fig. 2). However, the dose dependencies for the tyrosine phosphorylation of caveolin, the 29-kD caveolin-associated protein, and the insulin receptor were all identical (Fig. 3) and closely paralleled those observed for the stimulation of glucose uptake and metabolism in these cells. Although tyrosine phosphorylation of caveolin has previously been reported (10, 12, 18), this is the first report of a change in caveolin phosphorylation in response to an extracellular stimulus.

Incubation of the caveolin-enriched Triton-insoluble complexes with 1 mM ATP resulted in an increase in tyrosine phosphorylation of both forms of caveolin, the 29-kD caveolin-associated protein, and the 60-kD phosphoprotein, indicating the presence of an intrinsic caveolin kinase (Fig. 6). It is unlikely that this kinase is the insulin receptor itself, as only a small fraction of the activated, tyrosine-phosphorylated insulin receptor (0.5%) was ever found in the caveolin-enriched Triton-insoluble complexes (Fig. 5). In addition, the kinase activities detected in the in vitro and in situ gel kinase experiments did not correlate with the relative amount of insulin receptor tyrosine kinase activity detected in these preparations (Fig. 6). There was significant caveolin kinase activity in complexes that contained no detectable activated insulin receptor, and also in complexes from basal cells in which there was little to no activated insulin receptor in the entire cell. These data, together with the slower time course of phosphorylation, suggest that the phosphorylation of caveolar proteins observed in response to insulin may involve activation of a second tyrosine kinase, downstream of the insulin receptor. Indeed, caveolin is a known substrate for v-Src (10, 12). Moreover, a number of tandemly acylated Src-family tyrosine kinases are known to be enriched in Triton-insoluble complexes, including Lck, Fyn, Lyn, c-Fgr, and c-Yes (18, 25, 34). The in situ gel kinase assays suggest that the major kinase in the caveolin-enriched Triton insoluble complexes isolated from adipocytes is a protein of ~60 kD, consistent with an Src-family kinase (Fig. 6 *C*).

Surveys of proteins by Western blotting revealed that $p59^{Fyn}$ is concentrated in the caveolin-enriched Triton-insoluble complexes isolated from adipocytes (Fig. 7 *A*). Additionally, immunoprecipitation of $p59^{Fyn}$ depleted these complexes of the 60-kD tyrosine-phosphorylated protein (Fig. 7 *B*). Although the mechanism by which Fyn or a related kinase is activated is not known, it is conceivable that the kinase may be regulated by occupancy of its *src* homology-2 (SH2) domains, perhaps involving an insulin receptor substrate. Indeed, Fyn has been shown to associate with IRS-1 via its SH2 domains (M. F. White, personal communication). Alternatively, the activation of tyrosine phosphatases such as Syp by insulin (20) may dephosphorylate the COOH-terminal tail of the kinase, resulting in its subsequent activation. We are currently investigating whether Fyn is a caveolin kinase, and the mechanism by which it might be activated by insulin. We also are currently investigating whether caveolin represents a new substrate for the insulin receptor tyrosine kinase itself, as it is possible that the caveolin-kinase activity intrinsic to the Triton-insoluble complexes is not the kinase involved in the insulin-stimulated tyrosine phosphorylation of caveolin in whole cells.

Many phosphorylation events stimulated by insulin are shared by EGF and PDGF in 3T3-L1 cells, including activation of the mitogen-activated protein kinase and phosphatidylinositol 3′-kinase pathways (8, 15, 24, 37, 38). Despite these similarities, however, insulin stimulates glucose uptake and metabolism 10- to 20-fold in these cells, while PDGF and EGF have little or no effect (38). Interestingly, the tyrosine phosphorylation of caveolin was observed only with insulin, not with EGF or PDGF (Fig. 4). However, PDGF stimulated the phosphorylation of the 29-kD caveolin-associated protein. The role of tyrosine phosphorylation of caveolin in insulin action is unknown. The apparent specificity of caveolin phosphorylation to insulin as well as the increased expression of caveolin in adipocytes during differentiation suggest an important metabolic role. The association of caveolin with the caveolar coat suggested that it might play a role in membrane invagination,

determining the "open" versus "closed" vesicular conformation (35). However, in preliminary experiments, insulin did not alter the accessibility of caveolar proteins to cell surface biotinylation (data not shown). Tyrosine-phosphorylated caveolin may serve as a docking protein for SH2 domain–containing proteins, or it may regulate associations of proteins on the extracellular leaflet of the plasma membrane with proteins in the cytosol. Phosphorylation of caveolin may also play a role in the metabolism of GPI in response to insulin. We are currently investigating these possibilities to help elucidate the role of caveolae in insulin signal transduction.

Received for publication 31 January 1995 and in revised form 6 March 1995.

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