Effect of Insulin on Farnesyltransferase Activity in 3T3-L1 Adipocytes*

(Received for publication, March 28, 1996, and in revised form, August 8, 1996)

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Activation of p21ras by GTP loading is a critical step in a cascade of intracellular insulin signaling. Farnesylation of p21ras protein is an obligatory event that facilitates Ras migration to the plasma membrane and subsequent activation. Farnesyltransferase (FTase) is a ubiquitous enzyme that catalyzes the lipid modification of p21ras by the addition of farnesyl to the C-terminal “CAAX” motif. In vitro and in vivo FTase activities were studied in 3T3-L1 adipocytes in response to insulin challenge. Insulin exerted a biphasic stimulatory effect on FTase activity measured in vitro with a 21% increase at 5 min and a 130% increase at 60 min. Insulin-stimulated farnesylation of p21ras pools in vivo correlated with FTase activity seen in vitro by displaying an increase in farnesylated p21ras from 40% of total cellular Ras in control cells to 63% by 5 min and 80% by 60 min (p < 0.05) in insulin-treated cells. Insulin challenge of 3T3-L1 adipocytes increased incorporation of tritiated mevalonic acid in p21ras in a dose-dependent manner and stimulated a 2-fold increase in phosphorylation of the α-subunit of FTase at 5 min and a 4-fold increase at 60 min.

Activation of p21ras is a central event in the mechanism of action of many growth factors, including insulin (1). Cycling of p21ras proteins from the inactive, GDP-bound to the active, GTP-bound state and back is regulated by the guanine nucleotide exchange proteins and GTPase activating proteins (2). A prerequisite for this regulation is an association of p21ras with the plasma membrane (3, 4).

Membrane association of p21ras is promoted by lipid modification of the C terminus of p21ras by the protein prenyltransferase enzyme, farnesyltransferase (FTase) (5–7). FTase, a ubiquitous heterodimer, links the lipid moiety, farnesyl, to the conserved cysteine residue 186 of p21ras via a thioether bond (7). Subsequent proteolysis of the three C-terminal residues of p21ras and carboxyl methylation of the nascent cysteine C-terminal residue, provides a hydrophobic domain by which p21ras anchors to the inner leaflet of the plasma membrane (8).

Inhibition of FTase activity with either 3-hydroxy-3-methylglutaryl CoA-reductase inhibitors or specific inhibitors of FTase results in the inability of growth factors to activate p21ras (9–11). Thus, farnesylation is a posttranslational process that is essential for p21ras attachment to the plasma membrane and subsequent activation (3, 12).

Although it has been shown that p21ras farnesylation by FTase (3, 13–16), the regulation of FTase activity and the mechanism of such regulation are unclear. Recently, Kawabata et al. (17) have demonstrated that the transforming growth factor β1 (TGF-β1) receptor (TβR-1) interacts with and phosphorylates the α-subunit of FTase. Accordingly, we were interested in the cellular regulation of FTase activity in 3T3-L1 adipocytes in response to insulin. Here we show that insulin promotes the phosphorylation of the α-subunit of FTase and stimulates the activity of FTase in a time- and dose-dependent manner in 3T3-L1 adipocytes. As a result of the activation of FTase by insulin, the cellular pool of farnesylated p21ras doubled after 1 h of cell exposure to insulin.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture medium, gentamicin, methotrexate, and phosphate-free Dulbecco’s modified Eagle’s medium were from Life Technologies, Inc. Fetal calf serum (FCS) was from Gemini Bio-Products, Inc. (Calabasas, CA). Bovine serum albumin and other biochemicals were from Sigma. The anti-p21ras monoclonal IgG, Y13–259, and Protein G-PLUS/protein A-agarose immunoprecipitation reagents were from Oncogene Science, Inc. (Uniondale, NY). [3H]Mevalonolactone were from DuPont NEN. Bacterially expressed Ras protein with a Cys-Val-Lys-Scr C terminus and FTase α and β-subunit antibodies were kind gifts from Dr. Charles Omer (Merck and Co., West Point, PA). All supplies and reagents for SDS-PAGE were from Bio-Rad. Lovastatin was from Merck and Co. Porcine insulin was from Lilly. The enhanced chemiluminescence kit was a product of Amersham Corp.

Cell Culture and Differentiation—3T3-L1 fibroblasts were grown to confluence in fibroblast growth medium (Dulbecco’s modified Eagle’s medium containing 5.5 mM glucose, 10% FCS, 50 μg/ml gentamicin, 0.5 mM glutamine). Two days after confluence, fibroblasts were fed differentiation medium (Dulbecco’s modified Eagle’s medium containing 25 mM glucose, 10% FCS, 50 μg/ml gentamicin, 0.5 mM glutamine plus differentiation mix (2.5 mL of 10 mM phosphate-buffered saline, 55 mM dexamethasone, 2.5 mg of insulin)). On day 4, adipocytes were fed adipocyte growth medium (Dulbecco’s modified Eagle’s medium with 25 mM glucose, 10% FCS, 50 μg/ml gentamicin, 0.5 mM glutamine) plus 100 nM insulin. Cells were refed every 2 days with the same adipocyte growth medium and used on days 10–12.

In Vitro Farnesyltransferase Assay—FTase activity was assayed in vitro using a modified method of Moores et al. (33). 3T3-L1 fibroblasts were grown to confluence and differentiated into adipocytes. On days 10–12, cells were lysed in 500 μl of buffer (150 mM NaCl, 5 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 mM sodium vanadate, 1 mM sodium phosphate, 1% Triton X-100, 0.05% SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 50 mM HEPES, pH 7.5). Crude lysates were sonicated and centrifuged at 10,000 ∗ g. Total protein was determined by bicinchoninic acid assay (Pierce) and diluted to 0.5 mg/ml/sample. The in vitro filtration assay was initiated by adding a 5-μl aliquot of diluted extract to 45 μl of reaction assay solution (5 mM
Insulin Stimulates FTase in 3T3-L1 Adipocytes

MgCl₂, 5 mM dithiothreitol, 100 mM Ras protein, 100 mM tritiated farnesyl pyrophosphate (15 mCi/mmol), 50 mM HEPES, pH 7.5) and incubated at 37°C. At the indicated times, the assay was stopped with 1 ml of ice-cold 1 M HCl in ethanol, and the samples were placed on ice for 15 min. Reaction solutions were transferred to borosilicate glass tubes (12 x 75 mm), and 2 ml of ice-cold ethanol were added to each tube. Solutions were filtered through Whatman GF/C glass-fiber filters. Each filter was air dried, placed in a scintillation vial with 10 ml of scintillation fluid, and quantified by liquid scintillation spectrometry. The in vitro FTase assay was linear with respect to time and extract protein.

Separation of Farnesylated and Unfarnesylated p21ras—10-day-old adipocytes were serum-starved overnight and incubated with or without 100 nM insulin for the indicated times. Cells were lysed and centrifuged as described above. Equal volumes of lysate and 2% Triton X-114 (18) were combined in a borosilicate glass tube, vortexed, and incubated at 37°C for 3 min. Solutions were kept at room temperature until phases had separated. Equal samples from each phase were placed in separate 1.5-ml Eppendorf tubes, and p21ras was immunoprecipitated using a monoclonal antibody, Y13–259. Relative amounts of p21ras were determined by Western blotting followed by densitometry.

In Vivo [3H]Mevalonic Acid Incorporation—10-day-old adipocytes were placed in serum-free medium and incubated at 37°C for 3 h with 2 µg/ml lovastatin. At the end of this time, adipocytes were labeled overnight with 25 µCi of [3H]mevalonic acid (33 Ci/mmol) in the presence of lovastatin. The following day, adipocytes in medium containing lovastatin and [3H]mevalonic acid were incubated at 37°C for 5 or 60 min with decreasing concentrations of insulin (0–100 nM). A monoclonal antibody, Y13–259, was used to immunoprecipitate p21ras. [3H]Mevalonic acid that was incorporated into the immunoprecipitates was quantified by liquid scintillation.

32P-Phosphorylation of FTase Subunits—Adipocytes were serum- and phosphate-starved for 6 h and then incubated at 37°C overnight with 250 µCi of [32P]orthophosphate (10 mCi/mmol). Adipocytes were then incubated for 5 min or 1 h with or without 100 nM insulin. Lysates were sonicated and centrifuged. Protein concentrations were diluted to 0.5 mg/ml. FTase α- and β-subunits were separately immunoprecipitated with FTase α- or β-subunit rabbit antisera. FTase subunits were analyzed by SDS-PAGE and visualized by autoradiography and Western blotting. The relative intensity of signal was quantified by densitometry.

Statistical Analysis—All statistics were analyzed by Student’s t test, with a p value of <0.05 considered significant. Results are expressed as the mean ± S.E. of six independent experiments.

RESULTS

In the initial experiments, the influence of insulin on adipocyte FTase activity was assessed by the ability of cell lysates to stimulate the transfer of labeled farnesyl from tritiated farnesyl pyrophosphate to p21ras in vitro. Lysates from insulin-stimulated 3T3-L1 adipocytes exhibited a significant increase (p < 0.05) in FTase activity above basal levels in a biphasic manner at 5 and 60 min (Fig. 1). FTase activity increased 31% at 5 min and 130% at 1 h, above basal levels, after insulin challenge. FTase activity remained increased through 3 h (Fig. 1) of incubation with insulin.

To confirm that insulin-induced activation of FTase resulted in enhanced farnesylation of p21ras in vivo, we used two approaches. First, we examined the amount of farnesylated p21ras in cellular extracts after insulin challenge. Lysates of the control and insulin-treated 3T3-L1 adipocytes were partitioned using Triton X-114 to separate hydrophobic from hydrophilic molecules. Farnesylated and unfarnesylated p21ras were partitioned into detergent and aqueous phases, respectively, immunoprecipitated with the Y13–259 p21ras antibody from each phase, and determined by Western blotting. The relative signal strength was quantified by densitometry. The amount of farnesylated p21ras was calculated as a percentage of total cellular p21ras. The amount of farnesylated p21ras was increased to 50 ± 5% by 60 min, the percentage increased to 63 ± 6% (p < 0.05) (Fig. 2B). Interestingly, in agreement with a biphasic increase in FTase activity, the percentage of farnesylated p21ras decreased to 24 ± 9% of the total cellular p21ras at 7 min, and it began to increase again to 46 ± 3% by 10 min (Fig. 2B), to 62 ± 4.8% by 30 min, and to 60 ± 5.1% by 60 min (p < 0.05). These results display an excellent correlation between FTase activity measured in vitro and increases in percentage of farnesylated p21ras in vivo.

Our second approach to determine the effect of insulin on FTase activity in vitro was to quantify the incorporation of
Insulin Stimulation of FTase in 3T3-L1 Adipocytes

**Fig. 3. Effect of insulin on [3H]mevalonate incorporation into p21ras.** Adipocytes were incubated with [3H]mevalonate in the presence of increasing concentrations of insulin for 5 min (panel A) or 60 min (panel B). At the end of incubation, p21ras was immunoprecipitated from cell lysates, and incorporation of [3H]mevalonate was assessed by liquid scintillation. Results represent mean ± S.E. of six experiments. *, p < 0.05 versus controls.

[3H]mevalonate, a labeled precursor of farnesyI, into endogenous p21ras. After an overnight incubation with lovastatin and [3H]mevalonate (as described under “Experimental Procedures”), the cells were incubated for 5 or 60 min in the presence of increasing concentrations of insulin (0–100 nM). Cellular p21ras was immunoprecipitated from the cell lysates using the Y13–259 monoclonal antibody, and [3H]mevalonate-labeled p21ras was quantified by scintillation spectrometry. Insulin increased the amount of labeled p21ras signal in a dose-dependent manner at 5 and 60 min (Fig. 3). At 5 min (Fig. 3A), the amount of farnesylated p21ras increased from 117 ± 27 cpm/mg of protein in the absence of insulin to 251 ± 16 cpm/mg of protein in the presence of 100 nM insulin. At 60 min (Fig. 3B), the amount of farnesylated p21ras increased to 226 ± 26 cpm/mg of protein in the presence of 100 nM insulin with a half-maximal effect seen at 0.3 nM insulin.

To confirm that detergent extraction separated farnesylated from unfarnesylated p21ras, we performed the following experiments. Adipocytes that were preincubated overnight with [3H]mevalonic acid were then incubated with or without 100 nM insulin for 2 or 5 min. Following the detergent extraction as described under “Experimental Procedures,” p21ras proteins were immunoprecipitated from the aqueous and detergent phases and analyzed by SDS-PAGE. Autoradiography revealed [3H]mevalonic acid incorporation only in the detergent-extracted (farnesylated) p21ras (Fig. 4A, D lanes).

In addition, it was important to verify the completeness of immunoprecipitation of p21ras from the aqueous and detergent phases (Fig. 4B). Thus, after p21ras was immunoprecipitated from the aqueous and detergent-extracted phases at 0 and 60 min, the postimmunoprecipitation supernatants of these solutions were subjected to a second p21ras immunoprecipitation. Samples from the second immunoprecipitation (lanes 2, 4, 6, and 8) were resolved by SDS-PAGE and visualized by Western blot analysis beside their respective original immunoprecipitates (lanes 1, 3, 5, and 7). p21ras proteins were only detected in the original aqueous and detergent phases and not in the postimmunoprecipitation supernatants. These experiments suggest that p21ras appears to be equally and completely immunoprecipitated from the original aqueous and detergent-extracted phases.

In the next series of experiments, we determined the cellular localization of the newly farnesylated p21ras. In these experiments, we used differential centrifugation (100,000 × g for 30 min) to prepare cytosolic and crude plasma membrane fractions from cells that were incubated with insulin for 0 or 60 min. The cytosolic and plasma membrane fractions were then detergent-extracted with Triton X-114, and p21ras was immunoprecipitated from the aqueous and detergent phases of each of these fractions (Fig. 5). In the absence of insulin (0 min), the cytosolic fraction (C) contained mainly unprocessed (unfarnesylated) p21ras detected in the aqueous phase. In contrast, 70–75% of the p21ras detected in the plasma membrane (PM) was in the farnesylated form (D). After 60 min of incubation with insulin, an increase in farnesylated p21ras was observed in both the cytosolic and plasma membrane fractions.

The mechanism of insulin-stimulated FTase activity is yet unknown. Since insulin signaling to most of its intracellular substrates includes phosphorylation/dephosphorylation cascades, it was of interest to discern whether increased FTase activity correlated to the phosphorylation of one or both FTase subunits. Thus, 3T3-L1 adipocytes were incubated with [32P]orthophosphate and challenged with 100 nM insulin for 5 min or 1 h. Lysates of control and insulin-treated cells were
The association of p21\textsuperscript{ras} with the plasma membrane appears to be a critical event for its subsequent activation (i.e. p21\textsuperscript{ras} GTP loading) (3) and is facilitated by a two-step posttranslational modification. The first step is farnesylation of the C-terminal domain of p21\textsuperscript{ras} catalyzed by the ubiquitous enzyme FTase (8). Subsequent methylation of the C-terminal domain of p21\textsuperscript{ras} completes the assembly of the hydrophobic domain that anchors p21\textsuperscript{ras} to the plasma membrane (4). Farnesylation of p21\textsuperscript{ras} can be blocked by either FTase inhibitors (19–21) or by inhibitors of 3-hydroxy-3-methylglutaryl CoA-reductase, an enzyme involved in farnesyl synthesis at a more proximal level (11, 12, 22).

Activation of farnesylated and thereby membrane-associated p21\textsuperscript{ras} is a cornerstone of the mechanism of action of many growth factors and cytokines (1, 17, 23). Insulin is among these hormones and has been shown to rapidly promote p21\textsuperscript{ras} GTP loading in a variety of tissues (2, 16). In the present study, we demonstrated that insulin also significantly increased the cellular pool of farnesylated p21\textsuperscript{ras} by stimulating FTase activity. Thus, we observed a biphasic activation of FTase by insulin with a resultant 2-fold increase in the amount of farnesylated p21\textsuperscript{ras}, measured by either \[^{3}H\]mevalonate incorporation or detergent extraction. Changes in FTase activity were accompanied by increased phosphorylation of the FTase \(\alpha\)-subunit.

Despite the wide distribution (24, 25) and well studied structure of FTase (26, 27), the mechanism that regulates the activity of FTase is unknown. Kawabata \textit{et al.} (17) have recently demonstrated that the TjR-1 interacts with and phosphorylates the \(\alpha\)-subunit of FTase \textit{in vitro}. Similar findings were also reported by Wang \textit{et al.} (36) who, using the yeast two-hybrid system, have demonstrated that an interaction of the ligand-free TjR-1 with FTase resulted in phosphorylation and release of the FTase enzyme upon ligand binding. Moreover, phosphorylation of FTase was dependent upon the serine/threonine kinase activity of the TGF-\(\beta\) receptor that forms heteromeric complexes with TjR-1 (28–31). Both groups have suggested that the phosphorylation of the \(\alpha\)-subunit of FTase may be important for the regulation of FTase enzymatic activity.

The \(\alpha\)-subunit is common to both FTase and geranylgeranyltransferase I (GGTase-1) (32), which transfers the isoprenoid group geranylgeranylo to specific protein substrates (33). It is believed that the role of the \(\alpha\)-subunit is to stabilize the \(\alpha\)-\(\beta\) heterodimer complex (34), a condition that is necessary for FTase and GGTase-1 activity (35), and to catalyze the transfer of a farnesyl or geranylgeranyl group, respectively, to the protein substrate (34). The \(\beta\)-subunit of FTase and GGTase-1 may also play a catalytic role in protein prenyltransferase activity, yet each specifically selects its protein substrate (33). However, in contrast to the \(\alpha\)-subunit, the \(\beta\)-subunit does not appear to be phosphorylated in response to insulin stimulation.

The mechanism of insulin-stimulated phosphorylation of the \(\alpha\)-subunit is unknown. FTase can be a direct substrate of the insulin receptor tyrosine kinase or be a substrate of one of the serine/threonine kinases in the insulin-induced phosphorylation cascade. The latter appears to be more plausible in light of the observations that FTase in \textit{Xenopus} is phosphorylated by the serine/threonine kinase of the TGF-\(\beta\) receptor (36). Future experiments are necessary to explore this mechanism of insulin signaling to FTase. At present, it appears that in 3T3-L1 adipocytes, insulin promotes the phosphorylation of the \(\alpha\)-subunit of FTase and increases the activity FTase, resulting in a 2-fold (~100%) increase in the amount of farnesylated p21\textsuperscript{ras}. It is tempting to speculate that this increased pool of farnesylated p21\textsuperscript{ras} may be used by insulin and/or other growth factors to stimulate greater mitogenic responses.

Finally, it should be noted that the time courses for p21\textsuperscript{ras} GTP loading and FTase activity are not the same. Loading of endogenous p21\textsuperscript{ras} with GTP occurs within 1–5 min of insulin challenge, peaks at approximately 5–10 min, and is followed by a decrease after 15–30 min (2, 23). In comparison, FTase activity shows a moderate increase at 5 min (31% above basal level) and is followed by a sustained increase (130%) between 1 and 3 h of incubation with insulin. It appears that insulin independently promotes two events in the Ras signaling system: 1) farnesylation of p21\textsuperscript{ras} with its subsequent translocation to the plasma membrane and 2) GTP loading of p21\textsuperscript{ras}. Additional experiments that determine the association of GDP and GTP with the newly farnesylated and plasma membrane-associated p21\textsuperscript{ras} are needed to clarify this issue. At present, our experiments suggest that insulin significantly increases the pool of farnesylated p21\textsuperscript{ras}, making more p21\textsuperscript{ras} available for subsequent GTP loading in response to various growth factors. Physiological relevance of this novel aspect of insulin action remains to be determined.
Acknowledgment—We thank Gloria Smith for help in preparing this manuscript.

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