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Effect of Insulin on Farnesyltransferase Gene Transcription and mRNA Stability

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Recently, we have shown that hyperinsulinemia increases the activity of farnesyltransferase (FTase) in vitro (1) and in hyperinsulinemic animals (2), stimulates the phosphorylation of the FTase α-subunit (3), increases the amounts of cellular farnesylated p21Ras (4), and potentiates the nuclear effects of other peptide growth factors, such as EGF, IGF-1 and PDGF (5). To further investigate the mechanism by which insulin stimulates FTase activity we tested the effect of insulin on the rate of FTase transcription, the rate of FTase mRNA degradation, and the amounts of FTase protein. Insulin increased the amounts of FTase α- and β-subunit mRNA in 3T3-L1 fibroblasts 2.5-fold to 4-fold after 6 h and 24 h incubation, respectively, but did not increase the rate of FTase transcription over a 24 h period. Insulin did, however, increase the stability of both α- and β-subunit mRNA. The half-life for both FTase α- and β-subunit mRNA was approximately 3 h and 6h in the absence and in the presence of insulin, respectively. Although insulin stabilized the α- and β-subunit mRNA of FTase, there was no increase in amounts of protein of either subunit. These data suggest that although insulin increases the stability of the FTase mRNA, it stimulates FTase enzymatic activity only at the post-translational level.

The proto-oncogene *ras* codes for the small GTP-binding protein, p21Ras, which plays a major role in cell signaling (6) and cell cycle regulation (7). Activation of p21Ras occurs as a result of two events: (1) post-translational modification that allows Ras proteins to anchor at the plasma membrane, and (2) the exchange of GTP for GDP.

The exchange of guanine nucleotides bound to Ras engages the "on-off" switch of p21Ras activity: Ras is inactive when bound to GDP, and active when bound to GTP (8). The exchange of GTP for GDP on p21Ras is promoted by a variety of extracellular agents, including the peptide growth factors such as EGF, IGF-1, PDGF and insulin (4), and mediated by guanine nucleotide exchange factors such as Sos (9).

Guanine nucleotide exchange on p21Ras, however, is dependent upon post-translational modification and subsequent translocation of Ras proteins to the plasma membrane (10). Post-translational modification of p21Ras appears to be a pre-requisite for its subsequent activation, and is mediated by the ubiquitous enzyme, farnesyltransferase (FTase).

We have recently shown that insulin increases the activity of FTase, promotes the phosphorylation of its αa-subunit (3), and augments the amounts of cellular farnesylated p21Ras in fibroblasts, adipocytes and vascular smooth muscle cells (1, 11). Furthermore, tissues of hyperinsulinemic animals, such as ob/ob mice and Zucker rats, exhibit increased FTase activity and increased amounts of farnesylated p21Ras (2).

Although we have previously shown that insulin promotes the phosphorylation of the α-subunit of FTase and stimulates the activity of FTase, we now describe the effects of insulin on the transcription, mRNA and protein products of the FTase alpha and beta genes.

MATERIALS AND METHODS

Materials. Tissue culture media and gentamicin were from Life Technologies, Inc. (Grand Island, NY). Fetal Bovine serum and fungizone were from Gemini Bio-Products, Inc. (Calabasas, CA). Bovine serum albumin and other biochemicals were from Sigma (St. Louis, MO). The cDNA for the α- and β-subunit of FTase were a gift from Dr. Jackson Gibbs (Merck Research). Anti-digoxigenin Fab antibodies and RNA hybridization biochemicals were from Boehringer Mannheim, Inc. (Indianapolis, IN). CDP-Star was from Tropix (Bedford, MA). [αa-^{32}P]UTP was from NEN Life Sciences Products (Boston, MA). RNA isolation products were from Qiagen (Valencia, CA). Hybond-N+ membrane was from Amersham Life Sciences Products, Inc. (Arlington Heights, IL). Monoclonal antibodies to FTase α- and β-subunits were from Transduction Laboratories (Lexington, KY). Type HA filters are from Millipore (Bedford, MA), and Bio-Dot SF filtration apparatus was from Bio-Rad (Hercules, CA).

RNA analysis by Northern hybridization. 3T3-L1 fibroblasts were grown to 80% confluence in fibroblast growth medium (FGM) (Dulbecco's modified Eagle's medium containing 5.5 mM glucose, 10% fetal bovine serum, 50 μg/ml gentamicin, 0.5 mM glutamine),

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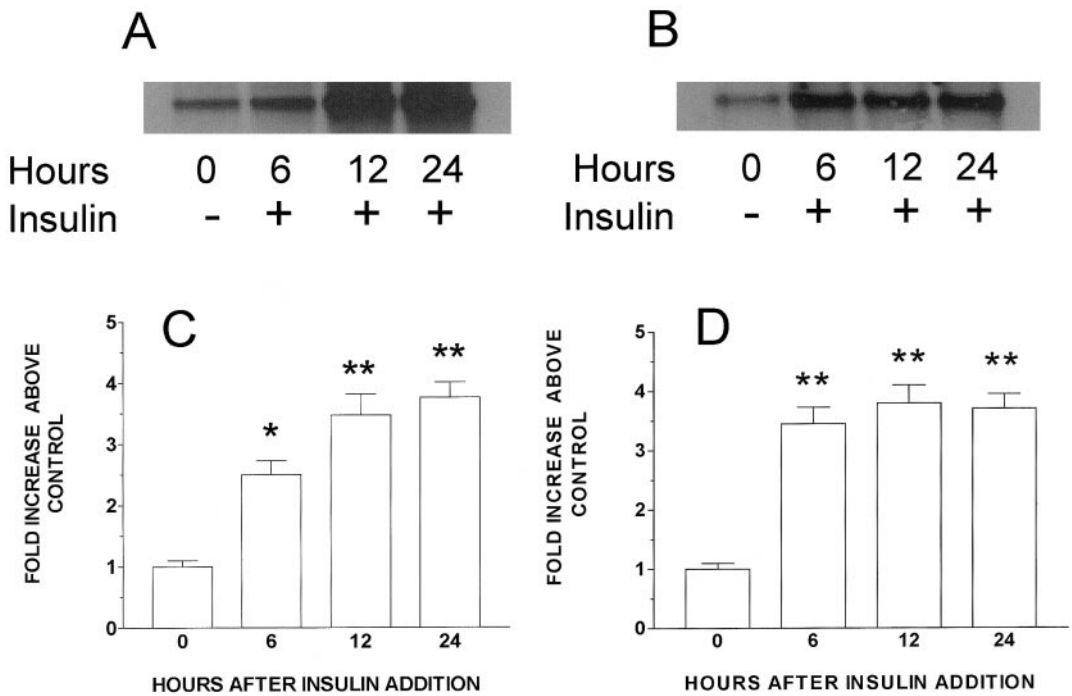

FIG. 1. Effect of insulin on levels of FTase α- and β-subunit mRNA. 3T3-L1 fibroblasts were incubated without or with insulin (100 nM) for indicated times. Amounts of FTase α- and β-subunit mRNA from cell lysates were determined by Northern blotting as described in Methods. Representative blots of FTase α-subunit (A) and β-subunit (B) mRNA are shown above the summary of 2-4 experiments for FTase α-subunit (C) and β-subunit (D). Results represent fold increase above controls, and are expressed as the mean ± SEM. *, $P < 0.05$; **, $P < 0.01$.

and subsequently placed in 2 ml of serum-free medium (Dulbecco's modified Eagle's medium, D-MEM/F-12). After 24 h, cells were incubated in the absence or presence of insulin (100 nM) for 0, 6, 12 and 24 h. RNA was isolated from crude lysates and fractionated on 4.9% formaldehyde 1.2% agarose gels, transferred to Hybond-N+ by capillary action. RNA was linked to the membrane by UV and probed with digoxigenin-labeled FTase α- or β-subunit cDNA. Anti-digoxigenin-AP Fab antibodies and CDP-Star substrate were used to determine FTase mRNA. Densitometry was used to quantitate the amounts of mRNA present in each sample.

RNA analyis by nuclear runoff. Cells were incubated in fibroblast growth medium until 80% confluent followed by a 24 h incubation in serum-free medium. Cells were then incubated for 0, 15, 30 and 60 min without or with insulin (100 nM), lysed with 1 ml NP-40 lysis buffer (10 mM Tris-HCl, 3 mM $CaCl_2$, 3 mM $MgCl_2$, 1% NP-40, pH 7.4), and nuclei were isolated. Nuclei were resuspended in 200 μl of 2X reaction buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, and 300 mM KCl) containing unlabeled nucleotides (10 μl of 100 mM ATP, 10 μl of 100 mM CTP, 10 μl of 100 mM GTP) and 10 μl of [α-^{32}P]UTP (760 Ci/mmol, 10 mCi/ml) and incubated for 30 min at 30°C. After transcription, nuclei were incubated for 30 min at 42°C with RNase-free DNase-I and proteinase-K. Transcripts were extracted from nuclei with 1 ml of phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v), and normalized. RNA samples were hybridized to linearized cDNA plasmids containing α- or β-subunits or blank cassettes vacuum filtered onto Millipore type HA (0.45 μM) membranes. Hybridized transcripts were visualized by autoradiography and quantified by densitometry.

Analysis of RNA stability. Cells were grown to confluence in FGM and then serum starved for 24 h. The cells were incubated with insulin (100 nM) for 1 h then placed in serum-free medium containing Actinomycin D (5 μM) in the absence or presence of insulin (100 nM) for 0,1, 2, 4 and 6 h. RNA was isolated and anaylzed by agarose fractionation and determined by Northern blotting. Amounts of RNA were quantified by densitometry.

Analysis of protein by Western blotting. Cells were grown to 80% confluence in FGM, serum starved for 24 h and incubated without or with insulin (100 nM) for 0, 6, 12 and 24 h. Cells were lysed with 500 μl of lysis buffer A (150 mM NaCl, 5 mM $MgCl_2$, 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 aprotonin, 10 μg/ml leupeptin, 50 mM HEPES, pH 7.5) and normalized for protein. FTase α- and β-subunits were immunoprecipitated from cell lysates using anti-FTase α- and β-subunit monoclonal antibodies. FTase proteins were analyzed by SDS-PAGE, determined by Western blotting and 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 ± SEM of 3 independent experiments.

RESULTS AND DISCUSSION

FTase is a member of a family of protein prenyltransferases, which transfer isoprenyl groups (intermediates of the cholesterol synthesis pathway) to conserved C-terminal cysteines of the small molecular weight GTPases (12, 13). In particular, FTase transfers the 15-carbon aliphatic cholesterol intermediate farnesyl to the conserved residue, cysteine 186, of the CaaX box (C = cysteine; a = any aliphatic residue; X = serine, methionine, alanine or glutamine) C-terminal

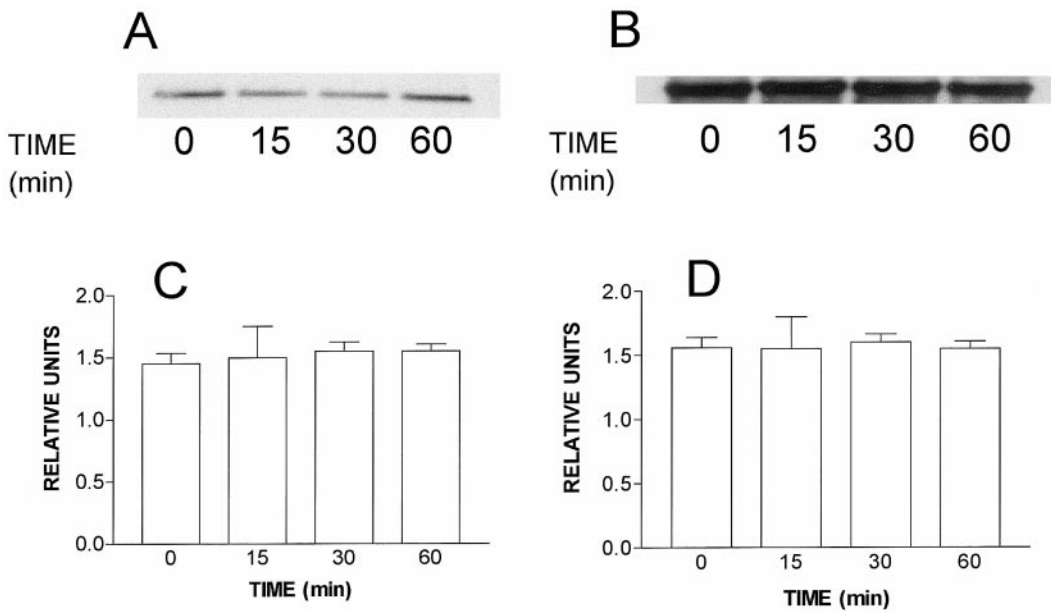

FIG. 2. Effect of insulin on the rate of FTase α- and β-subunit RNA transcription. Cells were incubated in the absence or presence of insulin (100 nM) for designated times as described in Methods. Newly formed transcripts were isolated from nuclear extracts and determined by nuclear run-off assay. Representative blots of FTase α-subunit (A) and β-subunit (B) are shown above the summary of 3 experiments for FTase α-subunit (C) and β-subunit (D) mRNA. Results represent the fold increase above controls, and are expressed as the mean ± SEM.

motif of p21Ras (12). FTase is a heterodimer that consists of a 48 kDa α-subunit and a 46 kDa β-subunit (14) that are stoichiometrically maintained at a ratio of 1:1 where the half-life of one is dependent on the expression of the other (15). Although the α-subunit of FTase is shared with gerylgeranyltransferase I (GGTase I) (13), the substrate specificity of these two enzymes is not. FTase attaches the prenyl group farnesyl to Ras proteins, whereas GGTase I attaches the geranylgeranyl moiety (a 20-carbon cholesterol intermediate) to proteins such as Rho that contain the C-terminal CaaX residue leucine (16).

We have previously demonstrated that insulin increases FTase activity and phosphorylation of its α-subunit in a dose- and time-dependent manner (1). These effects correlated well with insulin-stimulated increases in the amounts of farnesylated p21Ras (1, 4). In addition to the post-translational regulation of FTase, insulin might also affect the rate of transcription and translation of the FTase subunits. To examine this possibility, we first addressed the effect of insulin on the amounts of FTase mRNA for both α- and β-subunits (Fig. 1). Cells incubated with insulin (100 nM) for 6 and 24 h exhibited a 2.5 to 3-fold and 4-fold increase, respectively, in the amount of FTase α-subunit mRNA (Fig. 1A and C), and 3.5-fold and 4-fold increase, respectively, in the β-subunit mRNA over the same time period (Fig. 1B and D).

We then determined whether insulin-stimulated increases in FTase α- and β-subunit mRNA represented an increased rate of transcription rate or diminished rate of degradation of FTase mRNA. To address the first issue, we performed nuclear run-off assays for both FTase subunits. Cells were incubated in the absence or presence of insulin (100 nM) for designated times and transcripts for each subunit were isolated from nuclear extracts. Cells incubated with insulin over a 60 min period did not show an increase in the amount of transcripts for either FTase subunit as compared to time zero (Fig. 2), suggesting that insulin does not affect the rate of transcription of either FTase subunit.

To address whether insulin diminishes the degradation of FTase mRNA, we incubated cells for 1 h with insulin (100 nM), washed the cells, and re-incubated the cells for designated times with 5 μM Actinomycin D in the absence or presence of insulin (100 nM). The half-life of FTase α- and β-subunits mRNA doubled in the presence of insulin (Fig. 3). The half-life for α-subunit mRNA (Fig. 3A and C) was 3 h in the absence of insulin, and 6 h in its presence. The half-life for the β-subunit (Fig. 3B and D) in the absence or presence of insulin was 4 h and 5.5 h, respectively.

We then determined whether increases in the amounts of FTase mRNA are translated into more FTase protein in cells treated with insulin. To this end, we incubated cells in the absence or presence of insulin for designated times and determined the amounts of FTase α- and β-subunit protein by Western blotting. Western blots demonstrated that cells incubated with

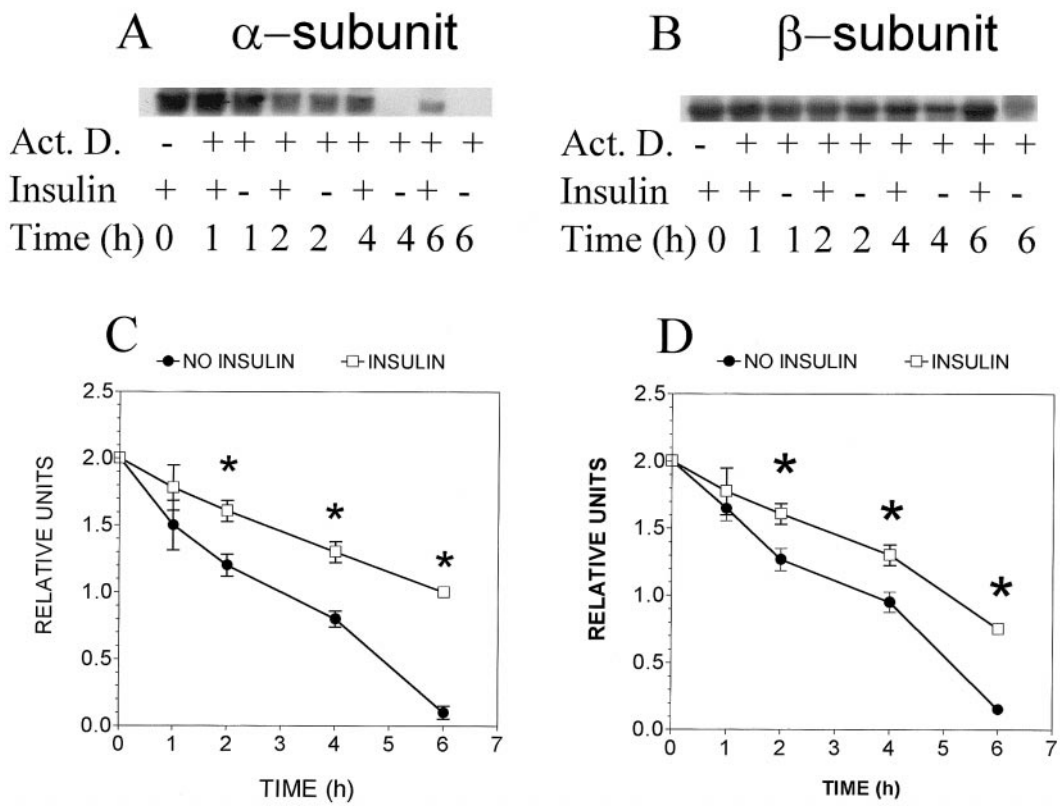

FIG. 3. Effect of insulin on the FTase α- and β-subunit RNA stability. Cells were challenged for 1 h with insulin (100 nM), washed and incubated with Actinomycin D (5 μM) in the absence or presence of insulin (100 nM) for the indicated times. RNA from cell lysates was fractionated and FTase α- and β-subunit RNA was determined by Northern blotting, and quantified by densitometry. Representative blots of FTase α-subunit mRNA (A) and β-subunit (B) are shown above summaries of 3-4 experiments for FTase α-subunit (C) β-subunits (D). Results represent the relative intensity of signal, and are expressed as the mean $\pm$ SEM. *, $P < 0.05$.

insulin did not show an increase in FTase α- or β-subunit protein over time (Fig. 4). Taken together, these data indicate that insulin decreases the degradation of both FTase α- and β-subunit mRNA, but the resulting increments in the amounts of mRNA are not translated into increased amounts of FTase protein. Thus, the previously observed insulin-stimulated increases in FTase activity (1) reflect increased phosphorylation of FTase and not increases in FTase protein.

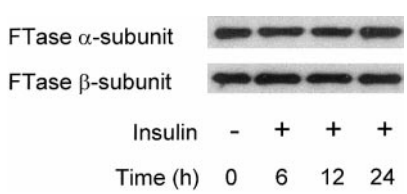

FIG. 4. The effect of insulin on the amounts of FTase α- and β-subunit protein. Cells were incubated in serum-free medium without or with insulin (100 nM) for indicated times. Cell lysates were normalized for protein and FTase α-subunit (A) and β-subunit (B) were immunoprecipitated from diluted lysates as described in Methods. FTase α- and β-subunits were determined by Western blotting.

The fact that insulin increases the amounts of FTase mRNA, but not FTase protein, is somewhat surprising, given that insulin tends to stimulate the transcriptional and translational machinery of the cell (17, 18). Translational expression of mRNAs depends on their co-localization with the appropriate ribosomes, the functioning of the cytoplasmic filaments and microtubules that propel mRNA to the sites of translation, and secondary structure of the transcript and translational product (19–23).

FTase transfers isoprenyl groups to p21Ras, allowing unprocessed Ras proteins to be associated with the inner leaflet of the plasma membrane, and to be available for activation by growth factor signals. FTase activity is stimulated specifically by insulin, but not by IGF-1, EGF and PDGF, correlating with the insulin-specific stimulation of the phosphorylation of the FTase α-subunit (5) and potentiation of growth factor-stimulated GTP-loading of farnesylated p21Ras anchored at the plasma membrane (24). Increases in the activity of an enzyme may result from increases in its transcription or translation, activation by post-translational and allosteric controls, or by increases in substrate concentration. We have

previously shown that insulin promotes the activity of FTase via the phosphorylation of the α-subunit of FTase, but does not alter the amounts of Ras protein, a substrate of FTase. We now demonstrate that insulin diminishes the degradation of FTase mRNA, but has no effect on either the FTase gene transcription or FTase protein translation. We conclude that insulin regulates the activity of FTase only by enhancing the post-translational modification (phosphorylation) of its α-subunit.

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