We have shown previously that insulin promotes phosphorylation and activation of farnesyltransferase and geranylgeranyltransferase (GGTase) II. We have now examined the effect of insulin on geranylgeranyltransferase I in MCF-7 breast cancer cells. Insulin increased GGTase I activity 3-fold and augmented the amounts of geranylgeranylated Rho-A by 18%. Both effects of the insulin were blocked by an inhibitor of GGTase I, GGTI-286. The insulin-induced increases in the amounts of geranylgeranylated Rho-A resulted in potentiation of the Rho-A-mediated effects of lysophosphatidic acid (LPA) on a serum response element-luciferase construct. Preincubation of cells with insulin augmented the LPA-stimulated serum response element-luciferase activation to 12-fold, compared with just 6-fold for LPA alone (p < 0.05). The potentiating effect of insulin was dose-dependent, inhibited by GGTI-286 and not mimicked by insulin-like growth factor-1. We conclude that insulin activates GGTase I, increases the amounts of geranylgeranylated Rho-A protein, and potentiates the Rho-A-dependent nuclear effects of LPA in MCF-7 breast cancer cells.

Small molecular weight GTPases of the Ras superfamily play an important role in cell proliferation, differentiation, structural organization, and vesicular trafficking (1–4). Members of this superfamily (including Ras, Rho, and Rab proteins) are activated by GTP loading in response to guanine nucleotide exchange factors (5). Post-translational modification of these proteins by prenylation appears to be a prerequisite for their subsequent activation (6, 7).

Prenylation of these GTPases is catalyzed by farnesyltransferase (FTase) or geranylgeranyltransferases I or II (GGTase I and II), which promote the attachment of either a farnesyl or a geranylgeranyl moiety, respectively, to conserved cysteine residues located at the C termini of Ras, Rho, and Rab proteins (8–10). Whether a protein is farnesylated or geranylgeranylated is determined by its specific C-terminal sequence. Ras proteins contain serine, methionine, or glutamine at the X position of the terminal CAAX box and are farnesylated, whereas Rho proteins have leucine at this position and are geranylgeranylated. The C terminus of the Rab proteins contains either a CC or CXC sequence, which is double geranylgeranylated by GGTase II.

We have recently demonstrated that insulin promotes the phosphorylation of the α-subunit of FTase, increases its enzymatic activity, and augments the amounts of farnesylated p21<sup>ras</sup> in 3T3-L1 fibroblasts, 3T3-L1 adipocytes, and vascular smooth muscle cells (11–13). Furthermore, tissues of hyperinsulinemic animals display both increased activity of FTase and increased amounts of farnesylated p21<sup>ras</sup> (14). Because FTase and GGTase I share the same α-subunit of the heterodimeric structure (15), we decided to evaluate the potential effect of insulin on GGTase I and the amount of geranylgeranylated Rho proteins. This has not been tested experimentally to date.

Furthermore, because Rho-A mediates transcriptional activation of the serum response element (SRE)-dependent genes by lysophosphatidic acid (LPA), we also examined whether the insulin-induced increases in the amounts of geranylgeranylated Rho-A potentiate the transcriptional activity of LPA in MCF-7 breast cancer cells. These cells possess insulin receptors and represent a good model to study insulin’s mitogenic influence.

**MATERIALS AND METHODS**

Tissue culture media, gentamicin, and the LipofectAMINE/Plus Reagent transfection kit were from Life Technologies, Inc. Fetal calf serum was from Gemini Bio-Products, Inc. (Calabasas, CA). Bovine serum albumin and other biochemicals were from Sigma. Insulin was from Eli Lilly (Indianapolis, IN). IGF-1, 1α-lysophosphatidic acid and acid moieties of reagent spray were from Sigma. Anti-Rho-A mouse monoclonal antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Protein G-Plus/Protein A-agarose immunoprecipitation reagents were from Oncogene Science, Inc. (Cambridge, MA). Bicinchoninic acid (BCA) protein assay kit was from Pierce. [3H]Geranylgeranylyl pyrophosphate and [32P]orthophosphate were from PerkinElmer Life Sciences.

This paper is available online at http://www.jbc.org
strate for GGTase I. FTase β-subunit antibodies and GGTase I/FTase α-subunit antibodies were from Transduction Laboratories (San Diego, CA).

**GGTase I Activity**—MCF-7 cells were grown to 80% confluance at 37 °C, 5% CO₂ (Eagle’s minimal essential medium (MEM) (Life Technologies, Inc.) + 5% heat-inactivated fetal bovine serum, nonessential amino acids, l-glutamine (200 μM), and insulin (60 μU). The cells were serum- and insulin-starved for 24 h and then preincubated with insulin (100 nM) for 1 h with and without 3 μM GGTI-286. The cells were washed with phosphate-buffered saline and lysed using a Triton X-100-based lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 15 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, 1% Triton X-100, 1 mM dithiothreitol, 1 mM sodium vanadate, 0.05% SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin). After pelleting the cellular debris, the supernatant was normalized for protein using the BCA protein assay. A 5-μl aliquot of the lysate was incubated with a reaction assay solution containing recombinant Ras-CVLL (a Rho analog) and [3H]geranylgeranylated pyrophosphate for 30 min at 30 °C. The reaction was stopped using 1 N HCl in ethanol and filtered through Whatman GF/C filter paper. The quantity of [3H]geranylgeranylated incorporation into recombinant human Ras-CVLL per milligram of protein was determined by liquid scintillation counting.

**32P Phosphorylation of α-Subunit of Geranylgeranyltransferase I**—MCF-7 cells were grown to confluance at 37 °C, 5% CO₂ (as described above). Cells were then incubated for 60 min at 37 °C in serum- and phosphate-free MEM + nonessential amino acids, and l-glutamine (200 mM), then preincubated overnight with 250 μCi of [32P]orthophosphate (10 μCi/mmol). Cells were then incubated for 60 min with insulin (100 nM) or IGF-1 (13 nM). Cells were lysed as described previously (11–13, 16), sonicated, centrifuged, and protein concentrations diluted to 0.5 mg/ml. FTase protein was immunoprecipitated, using antibodies to the β-subunit of farnesyltransferase. Geranylgeranyltransferase was then immunoprecipitated from the post-FTase immunoprecipitation lysates with antibodies to the α-subunit of GGTase I and analyzed by SDS-PAGE. Relative amounts of phosphorylation were determined by autoradiography. A representative experiment is shown.

**Measurements of Geranylgeranylated Rho-A**—MCF-7 cells were grown to confluance at 37 °C, 5% CO₂ (as described above). The cells were serum-starved for 24 h at 37 °C in serum- and phosphate-free MEM + nonessential amino acids, and l-glutamine (200 mM). The cells were then preincubated for 24 h with insulin (1 nM) ± 3 μM GGTI-286. 500 μCi of [32P]orthophosphate (10 μCi/mmol) was added, and the cells were incubated another 4 h. 10 μl LPA was added, and the cells were then incubated for an additional 60 min. Cells were lysed as described previously (11–13, 16), sonicated, centrifuged, and protein concentrations diluted to 0.5 mg/ml. Rho-A was immunoprecipitated and resolved by thin layer chromatography (TLC Silica gel 60 plates) using a solution containing 0.75 M ammonium formate, 0.5 M LiCl, and 0.57 M HCl. Gels were dried overnight, then sprayed with acid molybdate reagent, and allowed to dry. The position of labeled GFP and GDP on the TLC plates was visualized by autoradiography. The areas of the gel corresponding to the GDP and GDP bands of the autoradiogram were excised from the plates, and the amounts of labeled nucleotide were assessed by scintillation counting. The amounts of GDP were expressed in percentage above control of total GDP/GDP binding to Rho-A.

**Effect on SRE-dependent Gene Transcription**—MCF-7 cells were grown to confluance at 37 °C, 5% CO₂ (as described above). Using LipofectAMINE/Plus reagent, the cells were co-transfected with either a SRE-Luc construct or a luciferase construct minus the SRE (PGL 3), and a CMV-βGal construct, which was used as a control for transfection efficiency and processing. The cells were serum-starved for 24 h, preincubated with insulin (100 nM) in the absence or presence of GGTI-286 (3 μM) for 24 h, and then challenged with LPA (10 μM) for 16 h. Control cells were incubated with or without insulin for 40 h. The cells were washed with phosphate-buffered saline and lysed by a freeze/thaw method using a Triton X-100-based lysis buffer provided in the Enhanced Luciferase Assay kit. Luciferase activity was then measured on a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Sparks, MD) using the Enhanced Luciferase Assay kit and corrected for by β-galactosidase activity (determined by chemiluminescence). Efficiency is equal to the luciferase activity divided by the β-galactosidase activity.
Statistical Analysis—Statistics were analyzed by Student’s paired or unpaired t test, with \( p < 0.05 \) considered significant.

RESULTS

GGTase I Activity and Phosphorylation—In the initial experiments, we examined the effect of insulin on GGTase I activity in MCF-7 cells. Insulin activated GGTase I, and its effect on GGTase I activity was inhibited by a specific inhibitor of this prenyltransferase, GGTI-286 (Fig. 1). The effect of insulin was evident by 10 min of incubation. Although the time course shown in Fig. 1 is limited to 120 min, the effect of insulin on all of the prenyltransferases remains for as long as insulin is present (data not shown).

We then examined the effect of insulin on the phosphorylation of the GGTase I \( \alpha \)-subunit. Because FTase and GGTase I share a common \( \alpha \)-subunit (15), we eliminated FTase by immunoprecipitation with antibody to the \( \beta \)-subunit of FTase. We then used the anti-\( \alpha \)-subunit antibody to immunoprecipitate and analyze the \( \alpha \)-subunit remaining with GGTase I. Insulin increased phosphorylation of the GGTase I \( \alpha \)-subunit (Fig. 2) without affecting the total amount of GGTase I protein (data not shown). Insulin’s ability to activate GGTase I was consistent with its effects on the phosphorylation of the \( \alpha \)-subunit and similar to its influence on FTase (11–13) and GGTase II (16). We also tested the effect of IGF-1 on the phosphorylation of the GGTase I \( \alpha \)-subunit and found that the ability to enhance the phosphorylation of the GGTase I \( \alpha \)-subunit was specific to insulin (Fig. 2).

Since activation of the prenyltransferases result in increases in the amounts of prenylated proteins, we investigated the effect of insulin on the amounts of geranylgeranylated Rho-A, a substrate of GGTase I. The amount of geranylgeranylated Rho-A was determined by Triton X-114 extraction, as described for prenylated Ras and Rab proteins (11, 16). The detergent phase completely extracts prenylated proteins from the cell lysate, while non-prenylated proteins remain in the aqueous phase (11, 16). The amount of geranylgeranylated Rho-A is then expressed as the percent of the total cellular Rho-A. In accord with its stimulatory effect on GGTase I, insulin significantly increased the amounts of geranylgeranylated Rho-A from \( 38 \pm 3\% \) to \( 56 \pm 4\% \) by 1 h \( (p < 0.01) \). These increases were completely blocked by GGTI-286 (Fig. 3). The total amount of cellular Rho-A were not influenced by insulin and remained constant in all experiments (data not shown).

GTP Loading of Rho-A—Because insulin increased the amount of prenylated Rho-A available for activation by GTP loading, we were interested in determining the levels of Rho-
A-GTP in cells preincubated with insulin and then challenged with LPA. Insulin and LPA alone stimulated loading of Rho-A with GTP above control by 32% and 45%, respectively. Preincubation with insulin (1 nM) and subsequent challenge with LPA increased Rho-A GTP loading by 57% (Fig. 4). Because incubation of cells in the presence of GGTI-286 resulted in essentially complete inhibition of Rho-A geranylgeranylation, we observed only a negligible effect of insulin with LPA on Rho-A GTP loading in the presence of GGTI-286.

Effect of Insulin on LPA-mediated SRE-dependent Gene Transcription—Geranylgeranylated Rho-A plays an important role in mediating the transcriptional activation of SRE-dependent genes by LPA (17). If insulin increases the amounts of geranylgeranylated Rho-A available for activation, conceivably this effect might enhance the transcriptional activity of LPA. To test this hypothesis, we co-transfected MCF-7 cells with either an SRE-Luc construct or a luciferase construct without the SRE (PGL3), and a constitutively active CMV-βGal construct. The cells were then incubated with insulin alone, LPA alone, or insulin for 24 h followed by LPA. After appropriate corrections for the transfection efficiency (using β-galactosidase) and background (using PGL3) we found that 100 nM insulin increased the luciferase reporter activity 3-fold, whereas LPA increased the luciferase reporter activity 6-fold (Fig. 5A). Preincubation of the cells with insulin increased LPA effect to 12-fold. This “priming” effect of insulin was blocked by GGTI-286 and was not mimicked by IGF-1 (Fig. 5A). The effect of GGTI-286 was specific for insulin because no effect was noted in the presence of LPA alone (data not shown). Furthermore, the ability of insulin to potentiate the effect of LPA was dose-dependent (Fig. 5B). Preincubation of MCF-7 cells with 1, 10, or 100 nM insulin increased LPA’s effect by 5%, 40%, and 102%, respectively. Although the data in Fig. 5B show that the LPA effect is significant only in the presence of 100 nM insulin, potentiation by 10 nM insulin approached significance in the number of experiments run.

DISCUSSION

The salient feature of this investigation is that insulin activates GGTase I and increases the cellular amounts of ger-
nylgeranylated Rho-A. Physiologically, this action of insulin results in the augmentation of the Ras-mediated transcriptional activation of SRE-dependent genes by LPA. Cells preincubated with insulin and then followed by LPA exhibited a 12-fold increase in luciferase activity, which was greater than stimulation by insulin alone (3-fold), LPA alone (6-fold), or the addition of these two results. Thus, the response to insulin plus LPA was synergistic and gave a greater than additive effect in luciferase activity, clearly showing the ability of insulin to potentiate LPA’s action on SRE-dependent genes. Synergy is defined as a result greater than that of either A or B alone and greater than could be expected from simple addition of the individual effects (18). The effect of insulin alone was not significantly different from the control, but the dose response to insulin showed a greater “priming” effect with increasing doses of insulin (Fig. 5B).

We have previously shown that insulin is a potent activator of FTase and GGTase II in a variety of cell types and tissues (11, 13, 16, 19). Insulin-stimulated FTase activity results in significant increases in the amounts of farnesylated p21 Ras and augmentation of DNA synthesis in response to other growth factors, as measured by incorporation of bromodeoxyuridine (13, 20). Thus, IGF-1, epidermal growth factor, and platelet-derived growth factor elicit greater incorporation of bromodeoxyuridine into DNA of 3T3-L1 fibroblasts preincubated with insulin than in its absence. In vascular smooth muscle cells, insulin potentiates the platelet-derived growth factor-induced vascular endothelial growth factor gene expression and thymidine incorporation (12). These “priming” effects of insulin are blocked by an inhibitor of FTase, α-hydroxyfarnesylphosphonic acid, suggesting that the effects of insulin are mediated by increased activity of FTase (12, 20). In 3T3-L1 adipocytes, insulin also promotes increases in the activity of GGTase II and the amounts of geranylgeranylated Rab-3 and Rab-4 (16). The physiological relevance of the effect of insulin on geranylgeranylation of Rab proteins remains to be determined.

The mechanism of insulin’s effect on the prenyltransferases involves the phosphorylation of the α-subunit of FTase (19) and GGTase II (16). We now demonstrate that insulin also promotes the phosphorylation of the α-subunit of FTase I in MCF-7 cells. Inhibition of the phosphorylation of the α-subunit of FTase results in diminution of FTase activity (19). The ability of insulin to promote the phosphorylation of the α-subunit and activate these prenyltransferases is insulin-specific and is not mimicked by IGF-1, epidermal growth factor, or platelet-derived growth factor (13). This effect requires the presence of the intact insulin receptor, as cells derived from insulin receptor knock-out mice did not respond to insulin in terms of FTase activation (13). Furthermore, cells with a chimeric insulin-IGF-1 receptor also fail to activate FTase in response to insulin (13). Even though IGF-1 is a more potent mitogen than insulin, IGF-1 alone showed no significant increase in the amounts of farnesylated p21 Ras, indicating no effect on the activity of farnesyltransferase (13). The present data are in agreement and demonstrate that IGF-1 alone exerted a greater influence on SRE-Luc than insulin, yet had no effect on either phosphorylation of the GGTase I α-subunit (Fig. 2) or potentiation of the nuclear effect of LPA (Fig. 5A). Because GGTase I shares the α-subunit with FTase (15) and IGF-1 has no effect on its phosphorylation (Fig. 2), it would not, therefore, be expected to have an effect on GGTase I activity. Furthermore, the inability of IGF-1 to prime the effect of LPA is consistent with the lack of IGF-1 effect on prenylation.

Investigation of insulin signaling to GGTase I was beyond the scope of this study. Previously, we have shown that insulin’s effect on the phosphorylation of the α-subunit of FTase and GGTase II was blocked by an inhibitor of the mitogen-activated protein kinase kinase (16, 19) and by transfection with a dominant negative mutant of Ras (19). These data suggest that insulin activates FTase in a “positive feedback” fashion using the Ras-mitogen-activated protein kinase pathway to promote the phosphorylation and activation of the enzyme. Interestingly, however, other growth factors, which activate the Ras-mitogen-activated protein kinase pathway, fail to influence FTase (13), indicating the specificity of insulin action and that other signaling intermediates may be involved for insulin to activate prenyltransferases.

The α-subunit of FTase also belongs to GGTase I (15). Because this subunit is phosphorylated in response to insulin, one could propose that insulin might activate GGTase I similarly to FTase. We have demonstrated that insulin increases the activity of GGTase I and consequently, the amounts of geranylgeranylated Rho-A. The question that presents itself is, what could be the physiological or pathophysiological consequences of this aspect of insulin action? Rho-A is a member of the Ras superfamily of small (20–25 kDa) GTPases. Currently at least 14 mammalian Ras family proteins have been identified and have been shown to share 50–90% amino acid sequence homology (2). Similar to Ras, Rho proteins function as GTP/GDP-regulated binary switches, which control signaling pathways responsible for numerous important cellular functions. Rho proteins are active when they are in the GTP-bound state. Guanine nucleotide exchange factors (of the Dbl oncogene family) catalyze the release of bound GDP, thus allowing GTP to bind and activate these GTPases (21). GTPase-activating proteins, on the other hand, stimulate the low intrinsic GTPase activity of the Rho proteins, hence acting as negative regulators of Rho protein function (22).

Rho-A is an important cellular GTPase that appears to mediate the effects of LPA on the phosphorylation of myosin light chain (23), multiple nuclear responses including cell cycle progression (2, 3, 24), and possibly malignant transformation (21). At least five Rho family proteins have been implicated as critical regulators of Ras-mediated oncogenic transformation (22), and recent observations indicate that aberrant function of Rho family proteins may also contribute to malignant transformation (21). An important aspect of the transformed phenotype is the ability of Rho-A to promote cell motility (25, 26) and invasion. Thus, Rho protein function was found to be required for the LPA-induced invasion of hepatoma cells through a me-
skeletal cell monolayer (24). Rho proteins are known to play an important role in the cytoskeletal processes that involve filamentous actin (27, 28), including focal adhesion assembly and integrin-mediated signaling. In addition, Rho proteins play a role analogous to Ras in activating gene transcription. Rho, along with Rac and Cdc42, has been shown to activate SRE-dependent transcription (17), activate the transcription factor NFκB (29, 30), and facilitate expression of cytokinas D-1 and E (31). The mechanism whereby extracellular stimuli, including LPA, activate Rho proteins remains largely unknown and may involve an interaction of the heterotrimeric G proteins with Rho guanine nucleotide exchange factors (32). Rho proteins presumably signal downstream via a family of Rho kinases (33, 34), and, like the Ras proteins, they must be prenylated in order to activate their effectors (35).

Enhanced function of Rho proteins can result either from its excessive loading with GTP by guanine nucleotide exchange factors or from increased availability of geranylgeranylated Rho. The latter step is stimulated by hyperinsulinemia, which promotes the phosphorylation and activation of GGTase I (Fig. 6). The effect of insulin on GGTase I can be blocked by the GGTase I inhibitor, GGTTI-286 (which was not due to a nonspecific effect of GGTTI-286 as shown by the lack of its effect on LPA alone), or by inhibitors of hydroxymethylglutaryl-coenzyme A reductase (statins), which block cholesterol synthesis prior to the formation of the geranylgeranyl moiety. Insulin-induced increases in the availability of geranylgeranylated Rho lead to hyperactivation of Rho in response to its usual stimuli, resulting in enhanced nuclear responses of cell and tissues exposed to hyperinsulinemia.

Our current experiments in MCF-7 breast cancer cells indicate that insulin significantly increases GGTase I activity, the amounts of prenylated Rho proteins, and augments the Rho-mediated transcriptional activity of LPA. Even though the effect of insulin on the amounts of prenylated Rho-A is modest, it translates into a 57% increase in the GTP loading of Rho-A and doubling (from 6-fold to 12-fold) of the nuclear effect of LPA (as well as nuclear effects of angiotensin II and advanced glycation end products in vascular smooth muscle cells).2

Conceivably, insulin may also augment the amounts of prenylated Rac, another GTPase that is geranylgeranylated by GGTase I. Since Rac can also activate SRE, a portion of the “priming” effect of insulin may be Rac-mediated. In any event, this would still be a prenylation-dependent influence of hyperinsulinemia. Taken together, these results suggest that hyperinsulinemia can augment the Rho-dependent proliferative responses of cancer cells and, thereby, contribute to their progression.

In summary, our present and previous observations strongly suggest that insulin is a major activator of all three prenyltransferases (11, 16, 20). There is strong evidence that hyperinsulinemia may not be an innocent bystander, but contributes significantly to the progression of atherosclerosis and certain cancers. The “priming” effect of insulin on the Ras- and Rho-dependent signaling pathways may be critical for the proliferative responses of various tissues. The ability of insulin to potentiate the action of other growth factors may provide biochemical evidence and an explanation of the association of hyperinsulinemia with an increased incidence of atherosclerosis and cancer of the breast, colon, prostate, and endometrium.

REFERENCES

1. Barbacid, M. (1987) Annu. Rev. Biochem. 56, 779–827
2. Ridley, A. J. (1996) Curr. Biol. 6, 1256–1264
3. Symons, M. (1996) Trends Biochem. Sci. 21, 178–181
4. Schimmoller, F., Simon, I., and Pfeffer, S. R. (1998) J. Biol. Chem. 273, 2216–22164
5. Quilliam, L. A., Khorosavi-Far, R., Huff, S. Y., and Der, C. J. (1995) BioEssays 17, 395–404
6. Hancock, J. F., Magee, A. I., Childs, J. E., and Marshall, C. J. (1989) Cell 57, 1167–1177
7. Willsumsen, B. M., Christensen, A., Hubert, N. L., Papageorge, A. G., and Lowy, D. R. (1984) Nature 310, 583–586
8. Zhang, F. L., and Casey, P. J. (1996) Annu. Rev. Biochem. 65, 241–269
9. Schaber, M. D., O’Hara, M. B., Garshy, V. D., Mosser, S. D., Bergstrom, J. D., Mooreo, S. L., Marshall, M. S., Friedman, P. A., Dixon, R. A. F., and Gibbs, J. B. (1990) J. Biol. Chem. 265, 14701–14704
10. Moore, S. L., Schaber, M. D., Mosser, S. D., Rands, E., O’Hara, M. B., Garshy, V. M., Marshall, M. S., Pompliano, D. L., and Gibbs, J. (1991) J. Biol. Chem. 266, 14603–14610
11. Golovchanko, M., L. Golstone, P. Watson, M. Brownlee, and B. Draznin, unpublished observation.

2 I. Golovchanko, M. L. Golstone, P. Watson, M. Brownlee, and B. Draznin, unpublished observation.