BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **239,** 42–45 (1997)
ARTICLE NO. RC977413

GTP Loading of Farnesylated p21Ras by Insulin at the Plasma Membrane

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Received August 22, 1997

Insulin promotes the phosphorylation and activation of farnesyltransferase (FTase) in a time- and a dose-dependent manner. Increased FTase activity results in a larger pool of farnesylated p21Ras and allows for enhanced GTP loading. Insulin significantly increases the pool of farnesylated p21Ras from 20–25% in quiescent 3T3-L1 fibroblasts to approximately 70%, most of which is targeted to the plasma membrane. Furthermore, insulin promotes GTP loading of plasma membrane and not cytosolic p21Ras. The half-life of plasma membrane-associated farnesylated p21Ras is approximately 6 hours, and is identical in control and insulin-treated cells. We have also observed a direct correlation between the amounts of farnesylated p21Ras at the plasma membrane and the magnitude of insulin-induced GTP loading of p21Ras.

We have recently demonstrated (1,2) that insulin promotes the phosphorylation and activation of farnesyltransferase (FTase), a ubiquitous enzyme that catalyzes the farnesylation of p21Ras, nuclear lamins A and B, and the γ subunit of the rhodopsin kinase (transducin) (Reviewed in 3). Newly farnesylated Ras protein is further processed: proteolytically cleaved to remove the three C-terminal amino acids (aaX), carboxymethylated at the nascent C-terminal cysteine residue, and translocated to the plasma membrane (3), where it can be activated by various growth factors. Activation of p21Ras is accomplished by loading the protein with GTP (4,5). The exchange of GTP for GDP is stimulated by the guanine nucleotide exchange activity of Sos, a mammalian homolog of the Drosophila Son of sevenless gene product (6-8). Subsequent hydrolysis of the p21Ras-bound GTP into GDP is promoted by the GTPase activity of p21Ras itself and by GAP, a GTPase activating protein (9,10). Sos and GAP interact only with farnesylated p21Ras, following its anchoring at the plasma membrane (3,8,11,12). Therefore, post-translational modification of p21Ras, resulting in its association with the plasma membrane, is critical for Ras activation.

If only farnesylated and membrane-associated p21Ras can be activated by Sos, then activation of membrane-associated p21Ras should be proportional to the amount of p21Ras at the plasma membrane. Since insulin is a potent activator of Ras (4,5,13), this study was designed to evaluate whether insulin's effect on GTP loading of p21Ras is dependent upon the amount of farnesylated p21Ras located at the plasma membrane. In the process, we have also assessed the rate of turnover of farnesylated p21Ras at the plasma membrane of 3T3-L1 fibroblasts. The results of these studies demonstrate that insulin indeed activates only plasma membrane-associated p21Ras and that this activation is proportional to the amount of farnesylated and membrane-associated Ras.

MATERIALS AND METHODS

Materials. Tissue culture media, gentamicin, methotrexate, and phosphate-free Dulbecco's modified Eagle medium were from Life Technologies, Inc. (Gaithersburg, MD). Fetal calf serum (FCS) was from Gemini Bio-Products, Inc. (Calabasas, CA). Bovine serum albumin and other biochemicals were from Sigma (St. Louis, MO). The anti-p21Ras rat monoclonal antibody (Y13-259) and Protein G-PLUS/Protein A-agarose immunoprecipitation reagents were from Oncogene Research Products (Cambridge, MA).

[^{32}P]Orthophosphate was from DuPont New England Nuclear (Boston, MA). SDS-PAGE supplies and reagents were from Bio-Rad (Hercules, CA); the enhanced chemiluminescence (ECL) kit was a product of Amersham (Arlington Heights. IL); α-hyrdroxyfarnesylphosphonic acid (α-HFPA) was from Biomol (Plymouth Meeting, PA); and thin layer chromatography (TLC) plates were cellulose PEI 20x20 cm (100μm) from Fisher (Pittsburgh, PA).

Measurements of GDP- and GTP-bound p21Ras. 3T3-L1 fibroblasts were grown to confluence in fibroblast growth medium (Dul-

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0006-291X/97 $25.00

becco's modified Eagle medium containing 5.5 mM glucose, 10% FCS, 50 μg/ml gentamicin, and 0.5 mM glutamine). Cells were serum starved for 24 hours in serum- and phosphate-free medium (phosphate-free Dulbecco's modified Eagle medium, D-MEM/F-12) followed by the addition of 0.5 mCi of [^{32}P]orthophosphate for 12 h at 37°C. Cells were challenged with 100 nM insulin for designated times in the presence or absence of 1 μM α-HFPA and lysed in detergent-free lysis buffer (150 mM NaCl, 15 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMFS), 1 mM Na_2HPO_4, 1 mM dithiothreitol (DTT), 1 mM sodium vanadate, 10 μg/ml aprotinin and 10 μg/ml leupeptin, pH 7.5). Cell lysates were fractionated by ultra-centrifugation at 100,000 x g for 30 minutes and pellets were resuspended in lysis buffer with 1% Triton X-100 and 0.05% sodium dodecyl sulfate (SDS). Protein was determined in both cytosolic (supernatant) and plasma membrane (pellet) fractions by the bicinchoninic acid assay. GTP- and GDP-bound p21Ras was immunoprecipitated from each fraction and nucleotides were eluted as previously described (4,13). GTP and GDP were separated by TLC and labeled nucleotides were visualized by autoradiography or molybdate spray reagent, and quantified by densitometry. Results are expressed as the percentage of GTP relative to total GTP plus GDP detected.

Extraction of Farnesylated p21Ras from the plasma membrane. Confluent cells were serum-starved for 24 h, pre-incubated in the presence of α-HFPA and incubated with or without 100 nM insulin for 1 h. Cells were lysed in 1 ml of detergent-free lysis buffer. Total protein in crude lysates was diluted to 0.2 mg/ml per sample. Lysates were fractionated by ultra-centrifugation into plasma membrane and cytosolic fractions. Farnesylated p21Ras was extracted from the plasma membrane fraction by Triton X-114 phase separation and immunoprecipitated using the monoclonal antibody, Y13-259, as previously described (1). Relative amounts of p21Ras were determined by Western blotting followed by densitometry.

Statistical analysis. Statistics were analyzed by Student's "t" test or paired "t" test with a p value of < 0.05 considered significant.

RESULTS AND DISCUSSION

Insulin promotes the phosphorylation and activation of FTase in a time- and a dose-dependent manner (1,2). Increased activity of FTase results in a greater cellular pool of farnesylated p21Ras (1,2). Previously, we have shown that the majority of newly farnesylated Ras proteins that translocate to the plasma membrane becomes a target for GTP loading as a result of stimulation with insulin or other growth factors (1).

To determine whether insulin activates the membrane-associated or cytosolic pools of p21Ras, we compared the amounts of p21Ras·GTP located in the plasma membrane with those in the cytosolic fractions of control and insulin-treated cells. Subcellular fractionation of 3T3-L1 fibroblasts was performed after the cells were exposed to insulin (100 nM) for various times. Insulin activated p21Ras at the plasma membrane (Fig. 1A), but failed to enhance GTP loading of cytosolic p21Ras (Fig. 1B). The effect of insulin on the activation of membrane-associated Ras was biphasic, with an early peak (10 min), a decline (30 min), and a subsequent rise (1, 24 and 48 h). Conceivably, a temporary decline in the insulin action is related to a negative feedback exerted on the guanine nucleotide activity of Sos (15-17). Subsequent increases in the activity of FTase and the enhancement of the cellular pool of farnesylated Ras provide a positive feedback stimulus for the second peak of activation of p21Ras as a result of a longer exposure to insulin. We recently demonstrated that the insulin signal that resulted in the phosphorylation and activation of FTase appeared to travel via the Ras-MAP kinase pathway (2). In cells transfected with the dominant negative mutant of Ras (N17), insulin was unable to phosphorylate or activate FTase (2). Similarly, PD98059, an inhibitor of the MAP kinase kinase (MEK), blocked the insulin effect on FTase, again indicating the presence of a positive feedback influence of p21Ras activation on subsequent farnesylation of other Ras molecules (2).

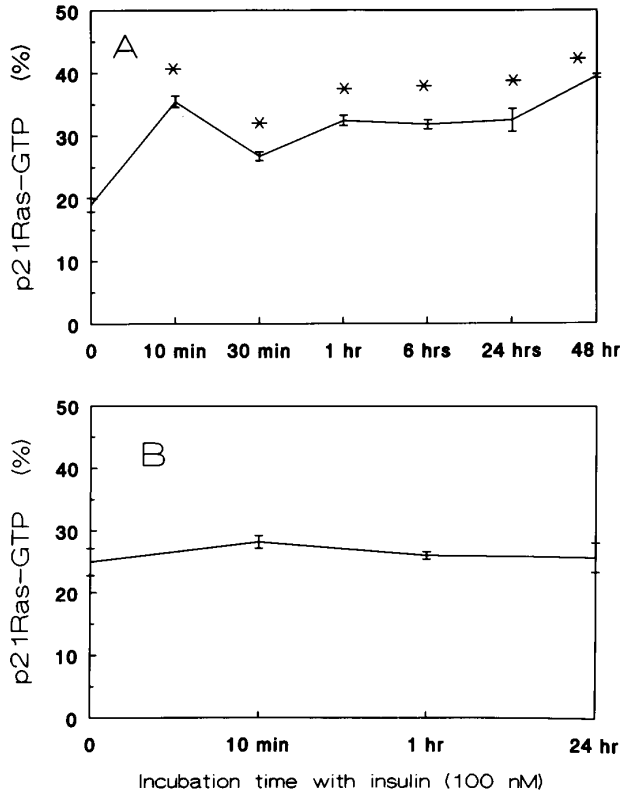

FIG. 1. Effect of insulin on GTP loading of p21Ras at the plasma membrane and in the cytosol. 3T3-L1 fibroblasts were challenged with 100 nM insulin for designated times, lysed in detergent-free lysis buffer, fractionated by ultra-centrifugation into plasma membrane (A) and cytosolic fractions (B). GTP- and GDP-bound p21Ras was immunoprecipitated from each fraction and nucleotides were separated by TLC as described under Methods. Results are expressed as the mean ± SEM of 4 experiments. * $p < 0.0001$ vs control.

Unexpectedly, we found that approximately 25% of the cytosolic p21Ras was bound to GTP, an amount greater than that found at the plasma membrane (20%) in untreated cells. Since it is commonly thought that only plasma membrane-associated p21Ras can be loaded with GTP, these findings suggest that cytosolic p21Ras·GTP resulted from the dissociation of p21Ras·GTP from the plasma membrane. The mecha-

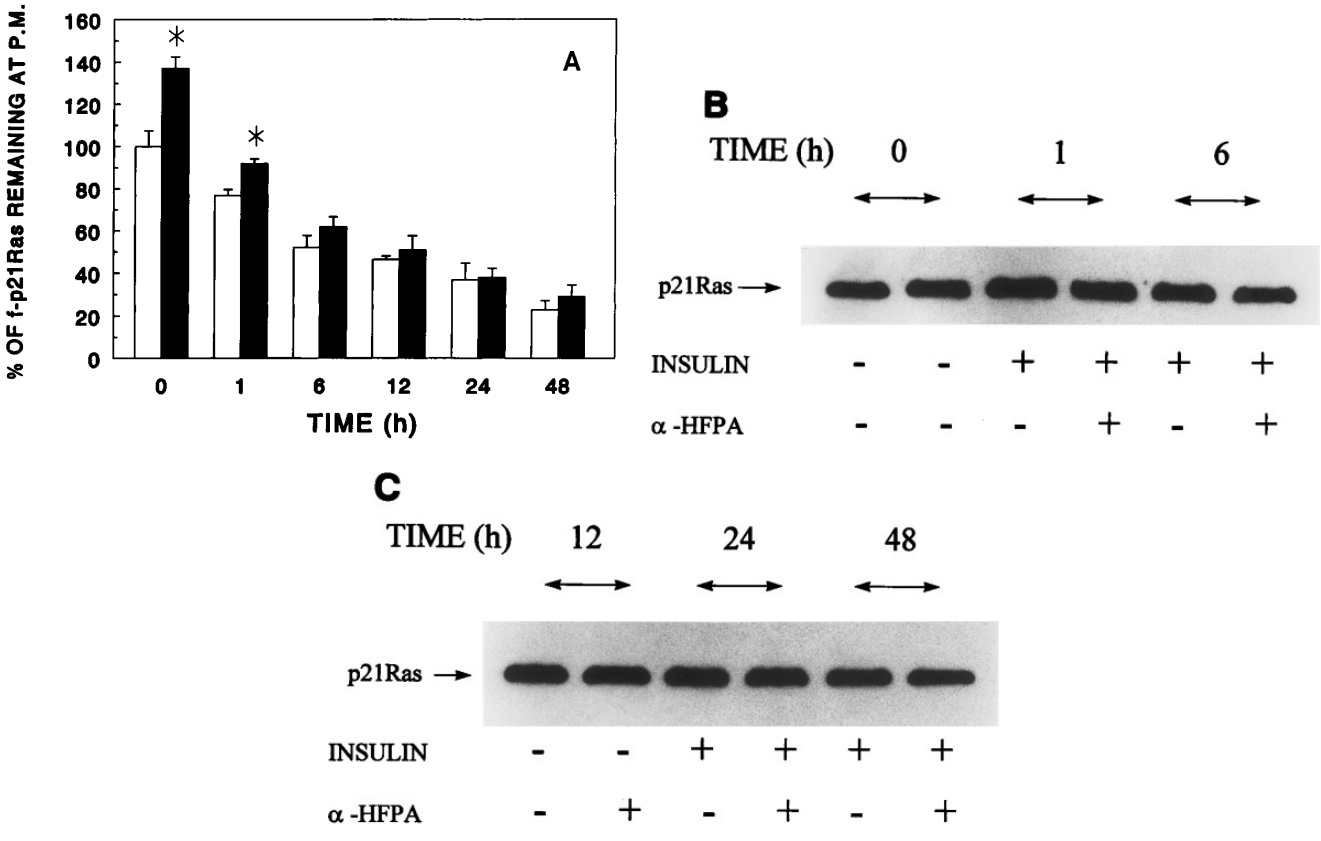

FIG. 2. Half-life of plasma membrane-associated farnesylated p21Ras. (A) Confluent cells were pre-incubated in the presence of α-HFPA with (closed bars) or without (open bars) 100 nM insulin for 1 h. Insulin was removed at time zero and incubation continued with only α-HFPA for the indicated times. Farnesylated p21Ras was extracted from the plasma membrane fraction by Triton X-114 phase separation and immunoprecipitated using the monoclonal antibody, Y13-259, as previously described (1). The amount of farnesylated p21Ras in the plasma membrane fraction of the control cells is expressed as 100%. Results represent the mean ± SEM of 4 experiments. * $p < 0.05$ (B and C) Representative experiments with relative amounts of farnesylated p21Ras determined by Western blotting.

nism of the separation of GTP-bound Ras from the plasm membrane is unknown. The isoprenyl connection of Ras with the plasma membrane may be broken, releasing farnesylated p21Ras·GTP intracellularly. Alternatively, a domain of the plasma membrane with the attached p21Ras may be internalized in an endocytotic vesicle and subsequently released from the putative vesicle after certain degradative steps occur. Also, the isoprenyl group may be cleaved from the Ras protein at the plasma membrane, releasing an unfarnesylated GTP-bound p21Ras into the cytosol. Furthermore, since GAP does not hydrolyze the GTP on cytosolic p21Ras (17), the process of converting cytosolic p21Ras·GTP into p21Ras·GDP must be mediated only by the intrinsic GTPase activity of p21Ras itself. The rate of this conversion is much slower than that with GAP and may explain the relatively high amounts of cytosolic p21Ras·GTP.

If insulin activates only farnesylated and plasma membrane-associated p21Ras, then the amount of GTP-loaded Ras after an insulin challenge should be proportional to the amount of farnesylated Ras available for this stimulation at the plasma membrane. To investigate this question, we first assessed the turnover rate of farnesylated p21Ras at the plasma membrane. To this end, we incubated control and insulin-treated cells with α-HFPA (1 μM), a potent inhibitor of FTase (18). α-HFPA inhibits the farnesylation of p21Ras and thereby blocks the translocation of p21Ras to the plasma membrane. We then isolated the plasma membrane fractions at different time points and determined the amounts of farnesylated p21Ras that were associated with these membranes. In other words, we followed the disappearance of farnesylated p21Ras from the plasma membrane (Figure 2). The half-life of farnesylated p21Ras at the plasma membrane of control cells was approximately six hours. Although insulin (100 nM for 60 min) significantly increased the amount of farnesylated p21Ras at the plasma membrane, the disappearance rate of this Ras from the plasma membrane was identical to that found in control cells: six hours after the insulin-treated cells were exposed to α-HFPA only about 50% of the farnesylated p21Ras remained at the plasma membrane.

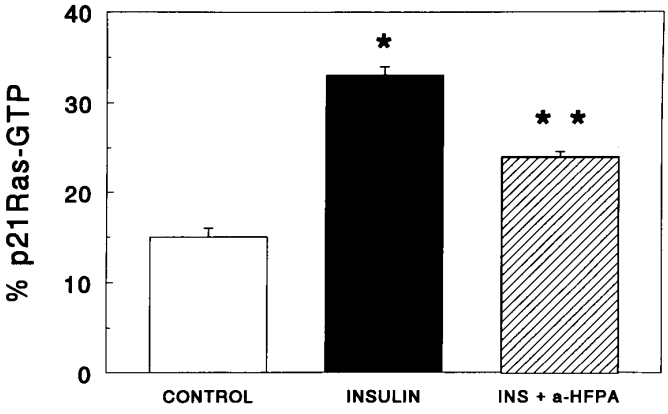

FIG. 3. Effect of insulin on GTP loading of the plasma membrane-associated p21Ras. GTP loading of p21Ras was determined in the plasma membrane fraction of control, insulin-treated, and insulin-treated cells preincubated with α-HFPA for 6 h. The results are expressed as the mean ± SEM of 3 experiments. * $p < 0.0001$ vs control, and ** $p < 0.0002$ vs insulin-treated cells.

In a subsequent set of experiments we preincubated the cells with α-HFPA for six hours and then challenged these cells with insulin (10 min). We observed a direct correlation between the amounts of farnesylated membrane-associated p21Ras and insulin-induced GTP loading. Thus, after six hours of incubation of cells with α-HFPA (when only 50% of the farnesylated Ras remained anchored at the plasma membrane) the insulin-induced activation of Ras was only 50% of the maximal stimulation observed in the control cells (Figure 3).

Farnesylation of p21Ras is a prerequisite for its anchoring at the plasma membrane. Our data suggest that in quiescent cells, those incubated in serum-free medium, only approximately 20-25% of the total cellular p21Ras exists in the farnesylated form. Insulin significantly increases the pool of farnesylated p21Ras to approximately 60-70%, with most of the newly farnesylated Ras being targeted to the plasma membrane (1). We have previously hypothesized (19) that this action of insulin may potentiate cellular mitogenic responses to other growth factors. Precise physiological significance of this potentiation will be examined experimentally in future studies.

In summary, plasma membrane-associated p21Ras can be bound to either GDP or GTP. Insulin activates plasma membrane-associated, but not cytosolic, p21Ras by loading with GTP. The magnitude of activation of the plasma membrane-associated p21Ras is proportional to the quantity of p21Ras that is anchored to the plasma membrane. The half-life of farnesylated p21Ras at the plasma membrane appears to be 6 hrs and is not affected by the acute stimulation of insulin. Finally, although isoprenylation of p21Ras is required for its association with the plasma membrane and subsequent loading with GTP, the mechanism of p21Ras dissociation from the plasma membrane still remains an enigma.

ACKNOWLEDGMENTS

This work was supported by the Medical Research Service of the Department of Veterans Affairs, Colorado Diabetes Research Foundation, and by the Foundation for Biomedical Education and Research.

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