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INSULIN STIMULATES THE PHOSPHORYLATION LEVEL OF v-Ha-ras PROTEIN IN MEMBRANE FRACTION

Tohru Kamata[+], Satish Kathuria[*], and Yoko Fujita-Yamaguchi[*]

[+]Cold Spring Harbor Laboratory,
Cold Spring Harbor, New York 11724
[*]Department of Molecular Genetics,
Beckman Research Institute of the City of Hope,
Duarte, California 91010

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SUMMARY: Insulin was found to stimulate the phosphorylation of the 21,000-dalton protein encoded by the ras oncogene of Harvey murine sarcoma virus in membrane fraction both in vivo and in vitro. When the human ras proteins expressed in E. coli were reconstituted with purified human insulin receptor, GTPase activity of normal or its mutated oncogenic ras protein was not stimulated by the addition of insulin. Likewise, tyrosine kinase activity or insulin binding capacity of the receptor was not influenced when assayed in the presence of the ras proteins. These results suggest that ras proteins may be coupled with the insulin receptor system through some unidentified membrane factors.

The Ha-ras gene family encodes membrane-associated proteins of molecular weight 21,000 (p21) which bind GTP and GDP (1). While the human protooncogenic H-ras protein has an intrinsic GTPase activity, its mutated counterpart, the oncogenic (T24) ras protein (Gly->Val, at position 12), shows a reduced GTPase activity (2,3). A GTP-dependent autophosphorylation (on threonine 59) activity has been detected in the case of the 21,000-dalton protein encoded by the ras oncogene of Harvey murine sarcoma virus(v-Ha-ras) (4). Although the biological significance of the apparent autophosphorylation of v-Ha-ras protein on Thr-59 is not as yet known, this phosphorylation activity is probably a consequence of the GTPase activity (3). The deficiency in the GTPase activity has been postulated to be associated with the transforming ability of T24 (2,3).

[+]Current address: National Cancer Institute, Frederick Cancer Research Facility, Frederick, Maryland 21701.

Abbreviations: v-Ha, Harvey sarcoma virus; T24, T24 transforming (Gly->Val at position 12) Ha-ras protein; IR, Insulin receptor.

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The biochemical interactions of ras proteins with other cellular components, however, remain to be established. While it has been demonstrated that in yeast the ras proteins can stimulate the adenylate cyclase activity (5), such data in mammalian cells are not yet available. In intact cells, we have indirect evidence for some type of communication between certain growth factors and the ras proteins; EGF treatment of serum-starved cells transformed by the v-Ha-ras gene causes an increase in the phosphorylation level of the v-Ha-ras protein (6). Experiments using isolated membranes from these cells indicated that the addition of EGF causes a GTP-specific increase in the phosphorylation of the v-Ha-ras protein on the threonine residue. In this report, we describe experiments similar to the previous studies using insulin in order to study the possible interaction of ras proteins with the insulin receptor system.

MATERIALS AND METHODS

The effect of insulin on the phosphorylation state of the v-Ha-ras protein in broken cell system. All the procedures were essentially the same as described previously (6). Briefly HaNRK cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. After the cells reached confluence, they were maintained in serum-free DMEM for 28-48 hrs. The cells were harvested and homogenized in buffer A (10mM Tris-HCl pH 7.4, 10mM NaCl, 0.1mM EDTA, 0.1mM 2-mercaptoethanol, 1mM phenylmethylsulphonyl fluoride). After removal of nuclei by centrifugation at 5,000xg for 5 min, the membrane fractions were prepared by centrifugation at 100,000xg for 60 min at 4°C. Aliquots of the membranes ($2x10^6$ cell equivalent) were solubilized in 100µl of buffer A containing 1% Triton X-100 and preincubated with varying concentrations of insulin (0-200 ng/ml). Phosphorylation reactions were carried out by the addition of 10 µCi of [γ-^{32}P]GTP (2500 Ci.mmol^{-1}, NEN) and 5mM $MgCl_2$ at 30°C for 10 min. The v-Ha-ras protein was immunoprecipitated by 4 µl of a rat anti-p21 monoclonal antibody (Y-259) (7) and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), followed by autoradiography.

The effects of insulin or EGF on the phosphorylation state of the v-Ha-ras protein in intact cell system. After serum deprivation described above, Ha-NRK cells were labelled with ^{32}P-orthophosphate (0.5mCi/ml) in serum-free DMEM for 1 hr and further incubated with or without insulin (10 µg/ml) and with or without EGF (500 ng/ml; BRL) for 15 min, respectively. After the cells (~10^6) were lysed in 100 µl of Dulbecco's phosphate buffered saline containing 1% Triton X-100 and sodium deoxycholate, the cell lysate was centrifuged at 100,000xg for 60 min at 4°C. The supernatants were immunoprecipitated by 4 µl of anti-p21 monoclonal antibody (Y-259) or rat non-immune antibody. The immunoprecipitates were analyzed by SDS-PAGE and autoradiography.

Reconstitution assay. Human ras proteins expressed in E. coli as described by Gross *et al* (8) were purified by using DE52, Sephadex G-75, and phenyl-Sepharose column chromatography (9). Insulin receptor (IR) was purified 2400-fold from human placental membranes by sequential affinity chromatography on wheat germ agglutinin- and insulin-Sepharose as described previously (10). The receptor was apparently pure and retained both the insulin binding activity and the

tyrosine kinase activity (10,11). Normal or T24 ras protein (0.25μg) was incubated with 0.2 μg of native IR in 20 μl of a reaction buffer containing 50mM Tris-HCl, pH 7.4, 0.05% Triton X-100, 2.5 mM $MgCl_2$, 0.5 μCi of [γ-^{32}P]GTP (1005 Ci.mmol^{-1} ICN), and 500 ng/ml of insulin (Cal Biochem) at 30°C for 30 min. As control experiments, BSA (BRL) was incubated with IR or ras proteins in place of ras proteins or IR respectively. After the reaction was terminated by the addition of cold GTP (1mM), 1 μl of each sample was loaded on a PEI-cellulose plate (J.T. Baker) and a released Pi was analyzed as described (6).

Protein kinase and insulin binding assay. Phosphorylation of the purified receptor was performed as described before (11). Insulin binding activity was determined as described previously (10).

RESULTS AND DISCUSSION

We have examined the effect of insulin on the phosphorylation state of the v-Ha-ras protein in broken cell and intact cell systems. Membranes were isolated from serum-starved Harvey murine sarcoma virus-transformed cells (HaNRK) and incubated with varying amounts of insulin in the presence of [γ-^{32}P] $MgGTP^{2-}$. Immunoprecipitation of v-Ha-ras protein (pp21) indicated that the phosphorylation level of v-Ha-ras protein was enhanced in an insulin dose-dependent manner (Fig. 1a). As mentioned in the previous report (6), the use of membranes from serum-deprived HaNRK cells was crucial in these experiments since the serum deprivation of the cells led to a lower basal phosphorylation activity of v-Ha-ras protein. For intact cell experiments, serum-deprived HaNRK cells were incubated with ^{32}P-orthophosphate with or without 10 μg/ml of insulin to determine whether or not insulin would affect the phosphorylation of the protein. The results indicated approximately a two-fold increase in the phosphorylation level of v-Ha-ras protein (Fig. 1b). The degree of the insulin-stimulated phosphorylation appeared to be similar to that of EGF-stimulated phosphorylation. While the intact cell experiment could be complicated by changes in the specific activities of the radiolabelled nucleotide pools during the course of the experiment, growth factors in general do not seem to change this for at least the ATP pool of cells (12). The phosphoaminoacid analysis of the v-Ha-ras proteins in the presence or absence of insulin indicated the presence of phosphothreonine and a trace of phosphoserine but no phosphotyrosine (data not shown).

We have shown that insulin, like EGF (6), modulates the GTP-dependent phosphorylation activity as judged by autophosphorylation activity of endogenous

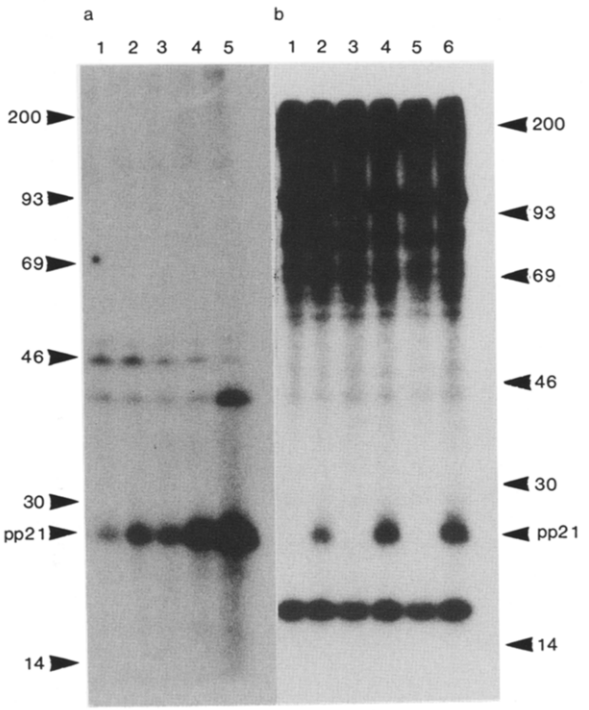

Fig. 1. **The effect of insulin (a and b) or EGF (b) on the phosphorylation level of the v-Ha-ras protein in the broken cell (a) or intact cell (b) system.** a. Immunoprecipitation of phosphorylated v-Ha-ras p21 (pp21) from the membranes treated with varying concentrations of insulin in the presence of [γ-^{32}P] GTP was performed as described in the Methods. Lanes 1-5 correspond to 0, 25, 50, 100, 200 ng/ml of insulin added, respectively. b. Immunoprecipiration of phosphorylated v-Ha-ras p21(pp21) from HaNRK cells labeled with ^{32}P-orthophosphate followed by insulin or EGF treatment was performed as described in the Methods. Lanes 1 and 2 are from cells without insulin or EGF treatment, lanes 3 and 4 are from insulin-treated cells, and lanes 5 and 6 are from EGF-treated cells. Anti-p21 antibody was used in lanes 2, 4 and 6, while a non-immune antibody was used in lanes 1, 3 and 5. Total amount of ^{32}P incorporated into cell extracts used were 2.2 x 10^6, 1.9 x 10^6, 2.5 x 10^6, 2.0 x 10^6, and 2.3 x 10^6 cpm for lanes 1-5, respectively.

v-Ha-ras proteins, in either serum-deprived HaNRK cells or isolated membranes from these cells. Attempts to determine whether insulin can enhance the GTPase activity of cellular ras proteins in normal rat kidney cell or HaNRK membrane fraction has not been successful because non-specific GTP hydrolytic activity is high in membrane fractions. These observatons led us to examine whether the insulin receptor can directly modulate the activity of the ras proteins in the reconstituted systems using purified proteins. The GTPase activity was examined for human normal or T24 ras protein purified from E. coli in the presence of the purified human insulin receptor (IR), insulin, and [γ-^{32}P] Mg GTP^{2-}. SDS-PAGE

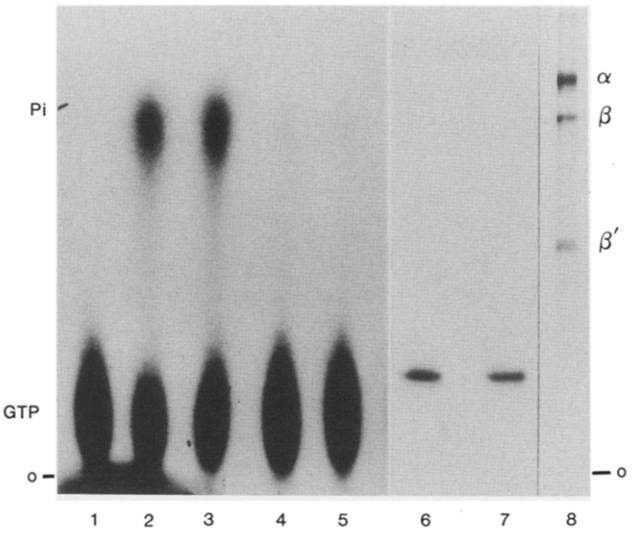

Fig. 2. GTPase activity of normal ras protein or oncogenic T24 ras protein purified from E. coli in the presence or absence of the purified insulin receptor and insulin. Reconstitution experiments using purified ras proteins and insulin receptor (IR) were performed as described in the Methods. The molar ratio of ras to IR = 17:1. GTPase activities of the receptor-ras protein mixtures are shown in lanes 1-5; (1) IR + insulin + BSA, (2) BSA + normal ras protein, (3) IR + insulin + normal ras protein, (4) BSA + T24 ras protein, and (5) IR + insulin + T24 ras protein. There was no difference in the GTPase activity between insulin-treated IR and the receptor without insulin treatment (data not shown). Quantified GTPase activities of lanes 1-5 are 0.74, 4.0, 4.6, 0.80, and 0.94 mmol phosphate $min^{-1}.mol^{-1}$ of ras. Lanes 6-8 show SDS-polyacrylamide (10%) gel electrophoresis analyses of the purified preparations of T24 ras, normal ras, and IR protein.

analyses of the purified ras proteins and insulin receptor are shown in Fig. 2 lane 6-8.

As shown in Fig. 2, lanes 2 and 3, the GTPase activity of normal ras protein was not affected by the addition of IR in the presence of insulin at least up to a molar ratio of IR/ras = 1/17 and up to 30 min incubation. Likewise, for the T24 ras protein, which has a very low basal GTPase activity, the addition of IR did not change its GTP hydrolytic activity under the same condition as described above (Fig. 2, lanes 4 and 5). The GTPase activity of IR was very low as compared to that of the ras proteins and showed no change in this activity when BSA as a control for the ras proteins was added. In a preliminary experiment, the addition of phospholipid (phosphatidylcholine 0.2 mg/ml) did not alter the GTP hydrolytic activity of normal or T24 ras protein in the presence of IR and insulin (data not shown).

The insulin receptor β subunit (90k) is a tyrosine-specific protein kinase (11,13). Insulin-stimulates phosphorylation of the β subunit of the receptor by binding to the α subunit (125K). To look for any possible effects of the ras proteins on IR activities, we tested whether the ras proteins influence the tyrosine kinase activity and insulin binding activity of the insulin receptor. Using a synthetic peptide derived from the tyrosine phosphorylation site of $pp60^{src}$ as an exogenous substrate, the kinase activity of IR was determined in the presence of $GTPMg^{2-}$, its nonhydrolyzable analogue GMPPNP, and ras proteins. Neither the normal nor the T24 ras protein changed the ability of IR to phosphorylate the peptide. The insulin binding capacity of IR was not significantly affected by the presence of either normal or T24 ras proteins (data not shown).

These results suggest that the ras proteins do not appear to couple directly with IR because the purified IR did not affect the GTPase activity of the purified normal or T24 ras protein and that the ras proteins did not alter the tyrosine kinase activity or insulin binding activity of IR. Since the reconstitution experiments shown here have utilized ras proteins produced in a bacterial expression system, the possibility that an "unprocessed form" may behave differently from "processed" ras proteins (e.g. lipidation) remains to be investigated. We are currently attempting to purify processed ras proteins from mammalian sources to test this possibility. With this limitation in mind, our present studies implicate that ras proteins may indirectly interact with the insulin receptor system through some membrane factors. This is of interest since an unidentified guanine nucleotide binding protein, which is different from the Gα protein of adenyl-cyclase system or Tα protein of transducin, has been suggested to mediate some action of insulin on cells (14). Since the enzymatic activity of the ras proteins in membranes can be regulated by EGF as well as insulin, it would be interesting to determine which membrane-derived factors are involved in the interaction between the receptor (for EGF or insulin) and the ras proteins. The reconstitution assays using purified proteins as described in this article should facilitate screening of such membrane-derived factors.

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