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Cellular Function of Rac1 in the Insulin Signal Transduction Pathway

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We examined a functional role of Rac1 protein in the signal transduction pathway of insulin leading to cell cycle progression in Rat-1 fibroblasts expressing human insulin receptors (HIRc-B). Following microinjection with dominant-negative and oncogenic mutants of Rac1 (Rac1N17) and Ras (RasN17), DNA synthesis in the injected cells was quantitatively analyzed. Rac1N17 and RasN17 inhibited DNA synthesis and membrane ruffling induced by insulin. Maximum inhibition of DNA synthesis by Rac1N17 was less than RasN17. This differential effect of Rac1 and Ras in the mitogenic signaling pathway was confirmed by microinjection of oncogenic Rac1V12 and RasV12. MEK inhibitor, PD98095, inhibited RasV12-induced DNA synthesis, but not Rac1V12-induced DNA synthesis. The co-injection of inhibitory Rac1N17 with RasV12 inhibited RasV12-induced DNA synthesis, but co-injection of oncogenic Rac1V12 with Rac1N17 rescued Rac1N17 blockade. These results suggest that Rac1 protein plays an important role in mitogenic signal transduction pathway of insulin, and that Rac1 and Ras are differentially involved in the pathway.

Key Words: Rac1, Ras, Mitogenesis, Insulin, Microinjection

INTRODUCTION

Insulin binding to its cognate receptor elicits a spectrum of metabolic and mitogenic responses.[1,2] The principal function of insulin is to regulate the uptake, synthesis, and storage of cellular energy. But it is quite evident that through mitogenic signaling insulin plays a substantial role in cellular homeostasis by coordinating protein synthesis, cell growth and division. One of the most well characterized mitogenic pathways which insulin regulates is the Ras/Mitogen Activating Protein (MAP) kinase pathway. Several approaches have been taken to study the role of the Ras/MAP kinase pathway in the mitogenic effect of insulin. Cells have been transfected or microinjected with Ras mutants,[3~7] thereby blocking Ras activation by insulin. Also a specific chemical inhibitor of MAP kinase kinase (MEK), PD98095, was studied in muscle and adipose cell lines.[8] Collectively, our studies and others support a role for activation of the Ras/ MAP kinase pathway in

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nuclear effects of insulin on DNA synthesis and gene expression.

Rac1 is a member of the Rho subfamily of Ras-related small GTPase and originally identified as an intermediate controlling cytoskeletal reorganization.[9] In addition, Rac1 regulates G1 cell cycle progression and cell transformation.[10,11] While Ras function in insulin signaling is well studied, only two studies are reported showing a functional role of Rac1 protein in the signal transduction pathway of insulin. Microinjection of dominant-negative mutant Rac1N17 blocks induction of membrane ruffling by insulin,[12] indicating that Rac1 functions downstream of insulin receptors in the pathway leading to actin rearrangement. Expression of dominant-negative Rac1 in the insulin-responsive 3T3-L1 adipocytes did not inhibit glucose uptake,[13] indicating that Rac1 protein does not couple to the signaling pathway to insulin-stimulated glucose uptake. But a functional role of Rac1 in the insulin-mediated mitogenesis is yet to be studied. In the present study, we examined a functional role of Ras and Rac1 proteins in the signaling pathway of insulin leading to DNA synthesis through microinjection of dominant-negative and oncogenic Ras and Rac1. We found that Ras and Rac1 proteins are involved in the insulin- induced DNA synthesis with a differential potency.

MATERIALS AND METHODS

1) Materials

Glutathione-sepharose bead was from Pharmacia. Insulin was purchased from Sigma. 3-Bromo-5'- deoxyuridine (BrdU) and monoclonal anti-BrdU antibody were purchased from Amersham. Human insuln and rat immunoglobulin G (IgG) was obtained from Sigma. Fluorescein isothiocyanate (FITC) anti-rat IgG or tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG antibodies were purchased from Jackson Laboratories. Monoclonal anti-c-Jun antibody was obtained from Transduction Laboratory. Anti-ERK antibody was from Santa Cruz. Anti-phospho-ERK antibody was obtained from NEB. Electrophoresis reagents were from Bio-Rad. All other chemical reagents were purchased from Sigma.

2) Cell culture

Rat 1 fibroblasts overexpressing wild type human insulin receptors (HIRc-B) were maintained in Dulbecco's modified Eagle's/F12 medium containing 10% fetal bovine serum as previously described.[14] Cells were starved with serum-free Dulbecco's modified Eagle's Medium containing penicillin (100 units/ml) and stereptomycin (100 ug/ml) for 24 hr.

3) GST-fusion proteins preperation

cDNAs of H-RasV12 and H-RasN17 were kindly provided by Dr. Feramisco (University of Califonia, San Diego, USA). And cDNAs of Rac1N17 and Rac1V12 were generously provided by Dr. J. Kim (KJIST, Korea). The full length of oncogenic and dominant-negative Ras and Rac1 were subcloned as glutathione S-transferase (GST)-fusion proteins and prepared as described previously.[14] PCR primer sequences used here were following.
H-RasN17 and H-RasV12:
sense:
5'-GCCGGCGGATCCACCGAATACAAACTGGTTGTAGTT-3'
antisense:
5'-GCCGGCGCGGCCGCTCAAGACAGAACGCATTTGCAGGA-3'
Rac1N17 and Rac1V12:
sense:
5'-GCCGGCGGATCCGAGCAGAAGCTGATCTCCGAGGAG-3'
antisense:
5'-GCCGGCGAATTCCTTACAACAGCAGGCATTTTCTCTT-3'

cDNAs were amplified by PCR, using oligonucleotides with BamHI (5'-3') EcoRI linkers. The purified BamHI-EcoRI DNA fragments from PCR products were ligated into BamHI/EcoRI-digested pGEX-2T expression vector. GST-fusion proteins were produced in BL21 (DE3) by isopropyl-b-D-

thiogalactopyranoside induction and purified by affinity chromatography on glutathione-agarose beads. The proteins were eluted with 5 mM glutathione and then concentrated with a microinjection buffer containing 20 mM Tris-acetate, pH 7.4, 20 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 5 mM 2- mercaptoethanol. The purified recombinant Ras, Rac and Rho proteins did not contain any contaminants when analyzed in SDS-PAGE and then stored at 70°C until use.

4) Single cell microinjection and DNA synthesis

The microinjection into HIRc-B cells and DNA synthesis has been previously described.[3,14] Briefly, HIRc-B cells were plated on scored 12-mm glass coverslips and incubated for 24 h. The cells were rendered quiescent by starvation for 24 h in serum-free Dulbecco's modified Eagle's medium (DMEM). The covereslips were transferred to 35-mm tissue culture dishes. Cells were microinjected by using glass capillary needles made with a vertical pipette puller (David Kopf Instruments). To identify the microinjected cells, Rat IgG (5 mg/ml) was coinjected with various GST-fusion proteins. After 2 h stabilization, BrdU (1 : 1,000 dilution, Amersham) was added to the medium, and the cells were incubated in the presence or absence of insulin for 16 h at 37°C. After fixed with acidic alcohol (90% ethanol, 5% acetic acid, 5% dH_2O) for 20 min at 25°C, the cells were permeabilized with TPBS (phosphate-buffered saline containing 0.1% Tween 20) for 10 min. Then the cells were sequentially incubated with mouse anti-BrdU antibody for 1h at 37°C, with TRITC-conjugated anti-mouse antibody for 1 h at 37°C and then with FITC-conjugated anti-rat IgG antibody for 1h at 37°C. The coverslips were washed with PBS, rinsed with water, and mounted in PBS containing 15% Gelvatol (polyvinyl alcohol), 33% glycerol, and 0.1% sodium azide. The immunostained cells were examined under the Zeiss Axioplan 2 microscope and photographed. DNA synthesis in the injected and uninjected cells on the same coverslips was counted and compared.

5) Activation of ERK-1/-2 and c-Jun protein expresson

Activation of Erk-1 and 2 upon insulin stimulation and the effect of PD98095, MEK inhibitor, was examined by immunoblotting. Serum-starved HIRc-B cells in 12 well plates were pretreated with 50 uM PD98095 for 30 min and then stimulated with insulin (100 ng/ml) for 15 min. The cells were solubilized with 100 ul of SDS-PAGE lysis buffer and 15 ul of the lysed samples were separated by 10%-PAGE. ERK-1/-2 were detected with anti-ERK antibodies and phosphorylated ERK-1/-2 with anti-phospho-ERK antibodies. To detect the induction of c-Jun protein by insulin, the serum-starved HIRc-B cells were pretreated with 50 uM PD98095 for 30 min and then stimulated with insulin for 2 hr at 37°C. The cells were solubilized with 100 ul of SDS-PAGE buffer and 10 ul of the lysed samples was separated by 10%-PAGE. The c-Jun protein expression was examined by monoclonal c-Jun antibody from Transduction Laboratories. The blots were incubated with a peroxidase-conjugated secondary antibody, and proteins were visualized by the enhanced chemiluminescence system.

RESULTS

1) Inhibition of insulin-induced DNA synthesis by dominant-negative Rac1N17 and RasN17 proteins

Previously we have shown that Ras plays an important role in the signal transuction pathway of insulin leading to DNA synthesis and c-fos gene expression.[3] In the present study, we examined a functional role of Rac1 protein in the insulin signal transduction pathway. We prepared dominant-negative Ras (RasN17) and Rac1 (Rac1N17) as GST-fusion proteins and purified over 90% homogeneity (data not shown). The proteins were microinjected into HIRc-B cells followed by examination of the effects on the insulin-induced DNA synthesis and actin rearragement. Microinjection

of RasN17 and Rac1N17 proteins inhibited the membrane ruffling (data not shown), consistent with previous reports on Rac1 inhibition of insulin-induced membrane ruffling in KB cells.[12] Next, we examined DNA synthesis after microinjection of RasN17 and Rac1N17. Microinjection of RasN17 and Rac1N17 proteins with increasing concentrations up to 7 mg/ml into quiescent HIRc-B cells inhibited DNA synthesis in a dose-dependent manner. DNA synthesis of cells injected with RasN17 and Rac1N17 at the maximal inhibition was 23% and 38%, respectively. There is a significant diference in the inhibitory potency, where the relative potency of Rac1N17 inhibition is lower than that of RasN17 inhibition. In uninjected and control IgG-injected cells, insulin stimulated about 68% of the cells to incorporate BrdU, demonstrating that the microinjection process itself was without adverse effects. These results indicate that Ras and Rac1 proteins are important intermediates in the signaling pathway of insulin leading to DNA synthesis and actin rearrangement, and differentially involved in the pathway.

2) Differential inhibition of DNA synthesis by injecting cDNAs of Rac1N17 and RasN17

In order to confirm the relative potency of inhibition, CMV-promoter driven plasmids encoding RasN17 and Rac1N17 were microinjected into the nucleus of serum-straved HIRc-B cells and examined the effects on the DNA synthesis by insulin. Similar to the result of protein injection, injection of DNA of RasN17 and Rac1N17 also inhibited the DNA synthesis (Fig. 2). Again, the potency of inhibition by Rac1N17 was lower than that by RasN17. Taken together with the above result, these results indicate the differential regulation of Ras and Rac1 in the signal transduction pathway of insulin leading to mitogenesis.

3) Differential induction of DNA synthesis by oncogenic Rac1V12 and RasV12

Oncogenic mutants of Ras and Rac1 are known to induce cell cycle progression and transformation.[11] Next, we examined induction of DNA synthesis in the cells after microinjection of oncogenic RasV12 and Rac1V12. As can be seen in Fig. 3, microinjection of oncogenic RasV12 and Rac1V12 protein induced DNA synthesis in a dose-responsive manner. Again, we observed that the maximum induction of DNA synthesis by oncogenic RasV12 and Rac1V12 was 73% and 45%, respectively. When the Rac1V12-injected cells were subsequently stimulated with insulin, the DNA synthesis was additive reaching 74% (data not shown), indicating microinjection itself

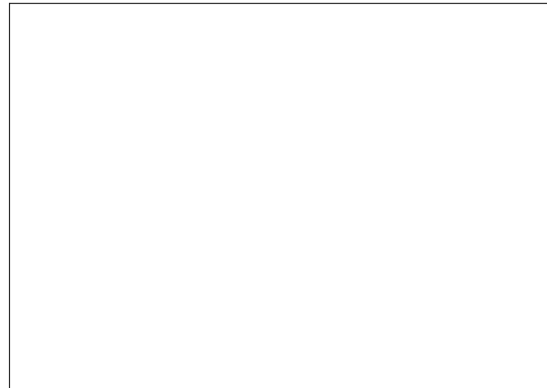

Fig. 1. Inhibition of insulin-induced DNA synthesis by dominant-negative Ras (RasN17) or Rac1 (Rac1N17) proteins in Rat-1 fibroblasts expressing human insulin receptors (HIRc-B). HIRc-B cells grown on coverslip at 40~50% confluency were serum-starved for 24 h and then injected with the indicated amount of either contol rat IgG (◦) or Rac1N17 (□) or RasN17 (■). Each condition contained rat IgG (5 mg/ml). After stabilization for 2 h, cells were stimulated with insulin (100 ng/ml) in the presence of BrdU for 16 h at 37°C. The cells were fixed and processed for double- label indirect immuno-fluorescence by sequential incubation with mouse anti-BrdU antibody, TRITC- conjugated anti-mouse IgG antibody, and then FITC-conjugated anti-rat IgG antibody. The injected cells were identified by cytoplasmic FITC staining and DNA synthesis was identified by nuclear rhodamine staining. DNA synthesis in the injected cells was expressed as stained cells per injected cells. A total of 150~200 injected cells were counted per coverslips. The results presented here are the mean of three coverslips and two independent experiments. Therefore, data shown here are the average of 1,000~1,200 injected cells.

Fig. 2. Nuclear injection of plasmids containing dominant- negative ras (RasN17) and rac1 (Rac1N17) inhibits DNA synthesis induced by insulin in HIRc-B cells. Serum- straved cells were injected with pcDNA3 plasmids (10 ng/ml) which contain either dominant-negative ras or rac1 and also rat IgG (5 mg/ml). The injected cells were stabilized for 2 hr and incubated with BrdU in the presence of insuln (100 ng/ml) for 16 h at 37°C. DNA synthesis was determined as described in the legend of Fig. 1. Data shown here are the average of 400~600 injected cells per conditions.

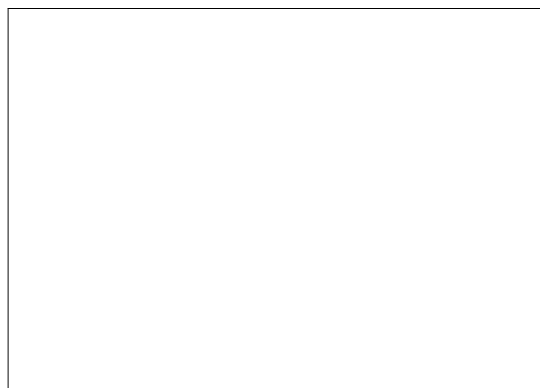

Fig. 3. Differential induction of DNA synthesis by oncogenic Ras (RasV12) or oncogenic Rac1 (Rac1V12) in HIRc-B cells. The indicated amount of oncogenic forms of RasV12 or Rac1V12 containing rat IgG (5 mg/ml) was microinjected into quiescent HIRc-B cells. After 2hr stabilization, the injected cells were incubated with BrdU in the absence of any stimulators for 16 h at 37°C. DNA synthesis was determined as described in the legend of Fig. 1. Data shown here are the average of 1,000~1,200 injected cells per conditions.

has no adverse effect. In contrast, there was no further increase in the DNA synthesis of the RasV12- injected cells by addition of insulin. In order to exclude the functional interference of GST domain, the RasV12 and Rac1V12 proteins are cleaved with thrombin. DNA synthesis induced by the purified proteins of RasV12 and Rac1V12 was 75% and 42%, respectively, indicating the GST domain has no major interference. From these results, we concluded that Ras protein is mostly involved in the signal transduction pathway of insulin leading to DNA synthesis, while Rac1 protein is partially.

4) Co-injectional analysis of RasV12, Rac1V12 and Rac1N17

The site of action of Rac1 has been known to be downstream of the Ras. So, we co-injected inhibitory Rac1N17 with oncogenic RasV12 into quiescent HIRc-B and examined DNA synthesis (Fig. 4). In the cells co-injected with inhibitory Rac1N17, DNA synthesis induced by oncogenic RasV12 was inhibited up to 40%. Insulin stimulation had no further increase. It should be noted that Rac1 protein did not completely inhibited Ras-induced DNA synthesis. These results indicate that Rac1 lies downstream of Ras and partially involves in the Ras-induced mitogenesis.

Next, we asked whether Rac1V12 could rescue cells from the inhibition induced by the dominant-negative Rac1N17. Since Rac1N17 blocks guanine nucleotide exchange by GEF activity, and since Rac1V12 is active independent of RacGEF activity, one could reason that the site of activity of Rac1V12 is downstream of the Rac1N17 blockade, and, therefore, Rac1V12 should restore DNA synthesis. Consequently, Rac1N17 and Rac1V12 were co-injected into quiescent HIRc-B cells and examined DNA synthesis (Fig. 4). When co-injected with inhibitory Rac1N17, oncogenic Rac1V12 stimulated DNA synthesis as the same extent of Rac1V12 alone, despite the presence of the inhibitory Rac1N17. Insulin stimulation combined with microinjection of active RacV12 completely restored the DNA synthesis even in the

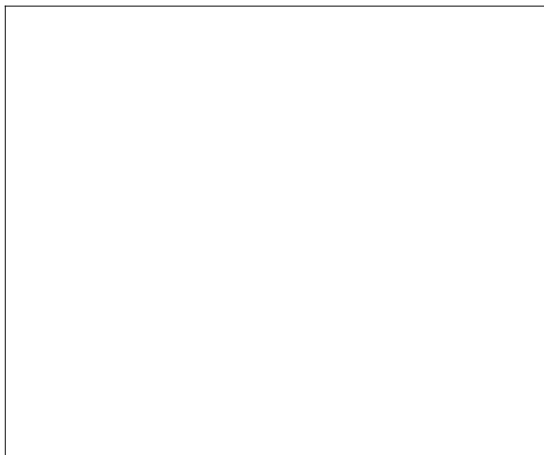

Fig. 4. Co-injection of dominant-negative Rac1N17, oncogenic RasV12 and oncogenic Rac1V12 into HIRc- B cells. Serum-starved HIRc-B cells were microinjected with various combination of oncogenic RasV12 (2 mg/ml), inhibitory Rac1N17 (8 mg/ml), oncogenic Rac1V12 (6 mg/ml). All microinjection mixtures included control rat IgG (5 mg/ml). After stabilization for 2 h, the cells were incubated with BrdU in the absence or presence of insulin (100 ng/ml) for 16 h at 37°C. DNA synthesis was examined as described in the legend of Fig. 1. Data shown are the average of 1,000~1,200 injected cells.

continuous presence of inhibitory RacN17, demonstrating rescue of the RacN17 blockade, consistent with the formulation that the site of action of Rac1V12 is distal to Rac1N17 and independent of Rac1GEF activity.

5) Rac1 and MEK/ERK proteins involve in separated mitogenic signaling pathways of insulin

We examined whether Rac1 and MEK/ERK proteins are responsible for the separate, individual mitogenic signaling pathway of insulin. First, we examined the effects of MEK inhibitor, PD98095, on the expression of c-Fos and c-Jun by insulin. Previously, we have shown that insulin rapidly and transiently induces the expression of c-fos using RT-PCR and immunostaining assay.[15] In this study, we examined the induction of c-Fos and c-Jun protein using imunoblot

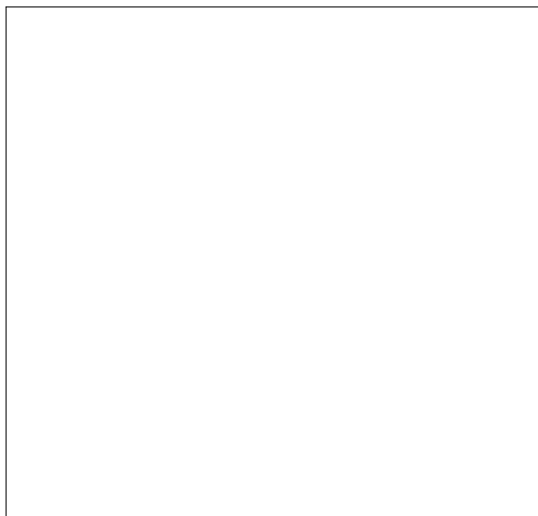

Fig. 5. MEK inhibitor, PD98095, inhibits activation of ERK-1/2 and induction of c-Jun protein by insulin in HIRc-B cells. Serum-starved HIRc-B cells in 12 well plates were pretreated with 50 uM PD98095, MEK-inhibitor, for 30 min and then stimulated with insulin (100 ng/ml) for 2 hr. The cells were lysed with 100 ul of SDS-PAGE lysis buffer and 15 ul of the samples was applied to 14% gel. Expression of c-Fos protein (first panel) and c-Jun protein (second panel) was detected with anti- c-Fos and c-Jun antibody. ERK activation was examined in the cells pretreated with 50 uM PD98095 for 30 min and then with insulin for 15 min. The cells were lysed with 100 uul of SDS- PAGE lysis buffer and 15 ul of each samples was used for immunoblotting. Proteins were determined by immunoblotting using either anti-ERK antibody or anti-phospho-ERK antibody. Molecular weights are c-Fos (62 kDa), c-Jun (39 kDa), ERK-1 (44 kDa) and ERK-2 (42 kDa).

assay and the effects of MEK inhibitor, PD98095 (Fig. 5). Induction of c-Fos and c-Jun protein by insulin was inhibited by PD98095. Interestingly, PD98095 partially inhibited the induction of c-Jun protein by insulin, suggesting the existence of MEK-independent pathway, probably JNK pathway. The phosphorylation of ERK-1 and 2 by insulin was completely abolished, while protein level of ERK-1 and 2 is similar. These results indicate the involvement of MEK and ERK in the signaling pathways of insulin leading to c-Fos and c-Jun protein.

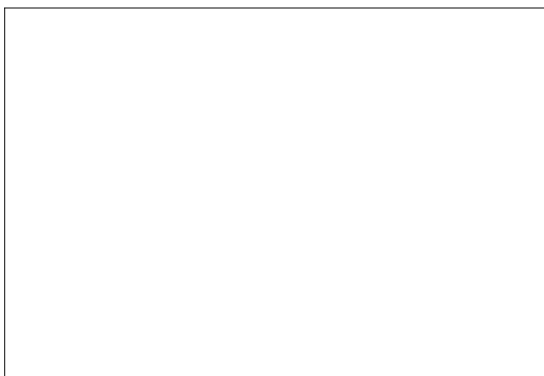

Fig. 6. MEK inhibitor, PD98095, inhibits DNA synthesis by oncogenic RasV12, but not by oncogenic Rac1V12. Serum-starved HIRc-B cells were pretreated with 50 uM of PD98095, MEK-inhibitor, for 30 min and then microinjected with either RasV12 (2 mg/ml) or Rac1V12 (6 mg/ml) or Rac1N17 (8 mg/ml) containing rat IgG (5 mg/ml). After stabilization for 2 h, the injected cells were incubated with BrdU in the presence and absence of insulin (100 ng/ml) for 16h at 37°C. DNA synthesis was examined as described in the legend of Fig. 1. Data shown are the average of 1,000~1,200 injected cells.

Next, we examined the effects of MEK inhibitor in the DNA synthesis induced by RasV12, Rac1-V12 and insulin (Fig. 6). DNA synthesis in the oncogenic RasV12-injected cells was partially reduced up to 40% by MEK inhibitor, while DNA synthesis in the oncogenic Rac1V12-injected cells was unaffected. Insulin-induced DNA synthesis was also partially inhibited by PD98095. Then, after blocking ERK and Rac1-dependent pathway, we examined the effect on DNA synthesis induced by insulin. In the cells pretreated with PD98095 and microinjected with inhibitory Rac1N17, insulin-induced DNA synthesis was further reduced (Fig. 6). But the total degree of inhibition by Rac1N17 was still less than the inhibition by RasN17. These results indicate that and the action site of Rac1-V12 is independent of MEK activity, and MEK activity is partially responsible for the Ras-mediated oncogenic signaling. Also the signal transduction pathway of insulin leading to DNA synthesis is not transmitting solely through ERK- and Rac1-mediated pathways. There may exist the additional mitogenic signaling pathway of insulin other than the Rac1 and MAP kinase pathway.

DISCUSSION

In the present studies, we showed that Rac1 is involved the signaling pathway of insulin leading to DNA synthesis, like Ras. Rac1 lies downstream of Ras and involves Ras-mediated DNA synthesis. Interestingly, the extent of Rac1N17 inhibition in the insulin-induced DNA synthesis was less than that of RasN17 and also incompletely inhibited oncogenic Ras-induced DNA synthesis. This differential involvement of Ras and Rac1 in mitogenesis was confirmed by comparing DNA synthesis induced by oncogenic forms of Ras and Rac1. The extent of induction of DNA synthesis by Rac1V12 was less than that of RasV12. Therefore, Rac1 protein lies in a downstream of Ras and is responsible for partial mitogenesis induced by insulin treatment and by oncogenic Ras.

The oncogenic Rac1 mutant displays all the hallmarks of malignant transformation and also dominant-negative Rac1 (Rac1N17) inhibits focus formation by oncogenic RasV12.[11)] Rac1 also modulates transcriptional activity mediated by SRF[16)] and activates the JNK pathway, inducing changes in the pattern of gene transcription.[17~19)] Ras- induced, but not raf-induced, transformation is blocked by the expression of dominant-negative mutants of Rac1, whereas expression of constitutively active Rac1 acts synergistically with activated Raf to transform cells.[11,20)] Consistent with this, we observed that MEK inhibitor, PD98095, partially inhibited insulin- and Ras-induced DNA synthesis. Addition of Rac1 blockade further inhibited insulin-induced mitogenesis. These observations provide compelling evidence that the mitogenic signal generated through or mediated by Ras protein utilizes the well-known MAP kinase pathway and the Rac1-dependent pathway as well.

Distinct from the well characterized MAPK and Rac1/Rho pathways, the additional Ras pathway has been shown to be primarily responsible for the inhibition of myogenesis by RasV12.[21)]

Treatment of myoblasts with the MEK inhibitor, PD098095, reveals that elevated MAPK activity is not a significant contributor to RasV12-induced transformation. RalGDS pathway is another downstream of Ras and microinjection of a dominant-negative mutant Ral reduced DNA synthesis stimulated by Ras as well as mediated by cAMP. RalGDS may be an effector of Ras in cAMP-mediated growth stimulation and cell transformation.[22] Consistent with previous reports, our result shows that the MEK specific inhibitor, PD098095, partially inhibited oncogenic RasV12-induced DNA synthesis, but did not inhibit oncogenic Rac1V12-induced DNA synthesis. Therefore, RalGDS pathway would be responsible for the remaining mitogenesis. In summary, Rac1 lies in a downstream of Ras and is partially involved in the mitogenic signal transduction pathway mediated by both insulin and oncogenic Ras. MEK and ERK pathway is important to transmit the mitogenic signal generated by insulin and Ras, but does not cross-talk with the Rac1 pathway.

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