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Evidence for the Ras-Independent Signaling Pathway Regulating Insulin-Induced DNA Synthesis

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The existence of the Ras-independent signal transduction pathway of insulin leading to DNA synthesis was investigated in Rat-1 fibroblasts overexpressing human insulin receptor (HIRc-B) using the single-cell microinjection technique. Microinjection of a dominant-negative mutant Ras^{N17} protein into quiescent HIRc-B cells inhibited the DNA synthesis stimulated by insulin. Microinjection of oncogenic H-Ras^{V12} protein (H-Ras^{V12}) (0.1 mg/ml) induced DNA synthesis by 35%, whereas that of control-injected IgG was induced by 20%. When the marginal amount of oncogenic H-Ras^{V12} protein was coinjected with a dominant-negative mutant of the H-Ras protein (H-Ras^{N17}), DNA synthesis was 35% and 74% in the absence and presence of insulin, respectively. This full recovery of DNA synthesis by insulin suggests the existence of the Ras-independent pathway. The same recovery was observed in the cells coinjected with either H-Ras^{V12} plus H-Ras^{N17} plus SH2 domain of the p85 subunit of PI3-kinase ($p85^{SH2\text{-}N}$) or H-Ras^{V12} plus H-Ras^{N17} plus interfering anti-Shc antibody. When co-injected with a dominant-negative $Rac1^{N17}$, the DNA synthesis induced by the Ras-independent pathway was blocked. These results indicate that the Ras-independent pathway of insulin leading to DNA synthesis exists, bypassing the p85 of PI3-kinase and Shc protein, and requires Rac1 protein.

Keywords: DNA synthesis, Insulin, Microinjection, Ras, Signal transduction.

Introduction

Insulin transduces cellular signals by binding the cell surface insulin receptor and activating the insulin receptor tyrosine kinase (Olefsky, 1990; Myers *et al.*, 1996). The cytosolic β-subunit of the insulin receptor possesses an intrinsic tyrosine kinase activity, which is activated in response to insulin binding. The activated tyrosine kinase transmits downstream signals largely through the action of a class of intermediary docking proteins, including GAB-1, Shc, and a set of the insulin receptor substrate (IRS) proteins, IRS-1, IRS-2, IRS-3, and IRS-4 (Sun *et al.*, 1995; Lavan *et al.*, 1997a; 1997b; Yenush & White, 1997). Phosphorylation of IRSs upon multiple tyrosines results in its association with Src homology 2 (SH2) domain-containing proteins including Grb2, Syp, Nck, and the p85 subunit of phosphatidylinositol 3-kinase, thus activating various signaling cascades (Backer *et al.*, 1993; Skolnik *et al.*, 1993; Jhun *et al.*, 1994a).

Ras is the protein product of the *c-ras* proto-oncogene and a key regulator of cell growth in all eukaryotic cells. Genetic, biochemical, and molecular studies in *Caenorhabditis elegans*, *Drosophila*, and mammalian cells have positioned Ras centrally in signal transduction pathways that respond to diverse extracellular stimuli, including peptide growth factors, cytokines, and hormones. The biological activity of Ras is controlled by a regulated GDP/GTP cycle. Guanine nucleotide exchange factors (GEFs; RasGRF1/2 and Sos1/2) promote the formation of the active GTP-bound form of Ras (reviewed in Bourne *et al.*, 1991). Insulin activates Ras by increasing the GTP-bound form of Ras, stimulating GEF protein activity (Draznin *et al.*, 1993; Koh *et al.*, 1997). The GTP-bound activated Ras mediates its effects on cellular proliferation, such as DNA synthesis, in part by directly interacting with and recruiting the Raf kinase to the plasma membrane, thereby permitting its activation of a cascade of kinases: Raf (c-Raf-1, A-Raf, and B-Raf), MEK (MAPK/ERK

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kinases 1 and 2), and ERK1/2 (Egan & Weinberg, 1993). Upon activation, the ERKs phosphorylate cytoplasmic targets such as Rsk (Sturgill *et al.*, 1988) and Mnk (Waskiewicz *et al.*, 1997; Wang *et al.*, 1998) and translocate to the nucleus, where they stimulate the activity of various transcription factors that include the Elk-1 transcription factor, leading to immediate-early gene induction such as *c-fos* (Hill and Treisman, 1995). This so-called Ras-Raf-MAP kinase pathway is essential to growth factor-mediated G1 cell cycle progression leading to DNA synthesis.

Beside this well-known Ras-dependent pathway, there are evidences which indicate a Ras-independent pathway in the signaling pathway leading to Raf activation (Hou *et al.*, 1995), Ral activation (Hofer *et al.*, 1998), *c-fos* and *Egr*-1 gene expression (Hipskind *et al.*, 1994), and SAP kinase activation (Bird *et al.*, 1994). Transformation of Rat-2 fibroblasts by v-Src is mediated through the Ras-independent pathway, although v-Src activates Ras (Aftab *et al.*, 1997). Sakaue *et al.* (1995) suggested that Ras activation is not required for insulin-induced stimulation of glycogen synthase and S6 kinase. But the characteristics of this Ras-independent pathway are not well understood. In the present study, we examined the existence of a Ras-independent pathway of insulin leading to DNA synthesis by applying a single cell microinjection. We have previously reported that microinjection of dominant-negative H-Ras^{N17} protein, N-terminal SH2 domain of the p85 subunit of PI3-kinase (p85$^{SH2\text{-}N}$), and anti-Shc antibody suppressed insulin-induced mitogenesis (Jhun *et al.*, 1994a; 1994b; Sasaoka *et al.*, 1994). In this study, we coinjected these inhibitory proteins with oncogenic H-Ras^{V12} and examined the insulin-induced DNA synthesis. We found the existence of the Ras-independent mitogenic pathway of insulin.

Materials and Methods

Cell lines and materials Rat-1 fibroblasts overexpressing wild-type human insulin receptors (HIRc-B) were kindly provided by Dr. J. M. Olefsky (University of Califonia, San Diego) and were maintained in Dulbecco's modified Eagle's/F12 medium containing 10% fetal bovine serum as previously described (Jhun *et al.*, 1995a). Glutathione-sepharose bead was from Pharmacia (Uppsala, Sweden). Insulin was purchased from Sigma. EGF were purchased from Gibco BRL. 3-Bromo-5'-deoxyuridine (BrdU) and monoclonal anti-BrdU antibody were purchased from Amersham. 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. Affinity purified polyclonal anti-Shc antibody was obtained from Transduction Laboratory. Electrophoresis reagents were from Bio-Rad (Richmond, USA). All other reagents were purchased from Sigma (St. Louis, USA).

GST-fusion proteins preperation cDNAs of H-Ras^{V12} and H-Ras^{N17} were kindly provided by Dr. Feramisco (University of Califonia, San Diego). cDNA of Rac1^{N17} was generously provided by Dr. J. Kim (Hallym Univerisity). The p85 fusion protein contained residues 321 to 440 from p85 of PI3-kinase, including the N-terminal SH2 domain, and was subcloned as glutathione S-transferase (GST)-fusion proteins and prepared as described previously (Jhun *et al.*, 1995b; Jin *et al.*, 1997; Oh *et al.*, 1997). GST-H-Ras^{N17} and GST-Rac1^{N17} were newly sucloned into pGEX-2T. The PCR primer sequences were as follows. H-Ras^{N17}: Sense: 5'-GCCGGCGGATCCACCGAATAC-AAACTG-GTTGTAGTT-3'. Antisense: 5'-GCCGGCGCGGCC-GCTCAAGACAGAACGCATTTGCAGGA-3'. Rac1^{N17}: Sense: 5'-GCCGGCGGATCCGAGCAGAAGCTGATCTCCGAGGAG-3'. Antisense: 5'-GCCGGCGAATTCCTTACAACAGCAG-GCATTTTCTCTT-3'

cDNAs of H-Ras^{N17} and Rac1^{N17} were amplified by PCR, using oligonucleotides with *Bam*HI(5'-3')/*Eco*RI linkers. The purified *Bam*HI-*Eco*RI DNA fragments from PCR products were ligated into the *Bam*HI/*Eco*RI-digested pGEX-2T expression vector. GST-fusion proteins including H-Ras^{V12}, H-Ras^{N17}, and Rac1^{N17} were produced in *E. coli* by isopropyl-β-D-thio-galactopyranoside induction and were purified by affinity chromatography on glutathione-agarose beads. The proteins were eluted with 5mM glutathione as describe elsewhere 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.

Single cell microinjection and immunostaining The microinjection of purified fusion protein has been described (Jhun *et al.*, 1994a; 1994b). Briefly, HIRc-B cells were plated on scored 12-mm glass coverslips, and the coverslips were 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 coverslips 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 (1:100 dilution) was added to the medium, and the cells were incubated in the presence or absence of insulin for 16 h at 37°C. After fixation with acidic alcohol (90% ethanol, 5% acetic acid, 5% dH_2O) for 20 min at 37°C, the cells were permeabilized with TPBS [phosphate-buffered saline (PBS) containing 0.1% Tween 20] for 10 min. The cells were then incubated with mouse anti-BrdU antibody for 1 h at 37°C followed by incubation with TRITC-conjugated anti-mouse antibody and FITC-conjugated anti-rat IgG antibody for 1 h 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.

Results

Recovery of insulin-induced DNA synthesis by oncogenic H-Ras^{V12} in the presence of inhibitory, dominant-negative H-Ras^{N17} We have demonstrated that microinjection of dominant-negative H-Ras mutant (H-Ras^{N17}) or interfering anti-Ras antibody inhibits

insulin-induced DNA synthesis (Jhun *et al.*, 1994). In order to evaluate the existence of the Ras-independent pathway in insulin mitogenic signaling, we newly prepared the GST-fusion protein containing dominant-negative H-Ras^{N17} and microinjected it into quiescent Rat-1 fibroblasts overexpressing human insulin receptors (HIRc-B). Insulin-induced DNA synthesis was assessed. Within the cell, the microinjected H-Ras^{N17} should competitively bind to the endogenous RasGEF activity and inhibit activation of an endogenous Ras protein (Feig *et al.*, 1988).

Cells were plated on coverslips and groups of 150 to 200 quiescent HIRc-B cells were microinjected with H-Ras^{N17}. Figure 1 depicts insulin-stimulated HIRc-B cells microinjected with either dominant-negative H-Ras^{N17} (5 mg/ml) alone or dominant-negative H-Ras^{N17} (5 mg/ml) protein plus oncogenic H-Ras^{V12} protein (0.1 mg/ml) in the absence and presence of insulin (100 ng/ml). As seen in Figs. 1A and 1B, microinjection of H-Ras^{N17} protein itself did not induced DNA synthesis in the basal HIRc-B cells. Approximately 70 to 80% of the uninjected cells underwent DNA synthesis (contained labelled nuclei), and the control rabbit IgG or GST protein alone had no effect on DNA synthesis induced by insulin (Jhun, 1994a; 1994b). In contrast, microinjection of H-Ras^{N17} (Figs. 1C and 1D) markedly reduced the number of cells stimulated to undergo DNA synthesis by insulin. When co-injected with both inhibitory H-Ras^{N17} and oncogenic H-Ras^{V12} (0.1 mg/ml) into quiescent cells (Figs. 1E and 1F), 32% of injected cells exhibited DNA synthesis. When treated with insulin (Figs. 1G and 1H), DNA synthesis was fully recovered even in the presence of inhibitory H-Ras^{N17}.

The results of two independent experiments are summarized in Fig. 2. Microinjection of oncogenic H-Ras^{V12} (0.1 mg/ml) protein stimulated 35% of injected cells to incorporate BrdU, where 20% of the uninjected basal cells underwent DNA synthesis. When injected alone, microinjection of dominant-negative H-Ras^{N17} (5 mg/ml) protein inhibited insulin-induced DNA synthesis up to 20%. When coinjected, low concentrated H-Ras^{V12}

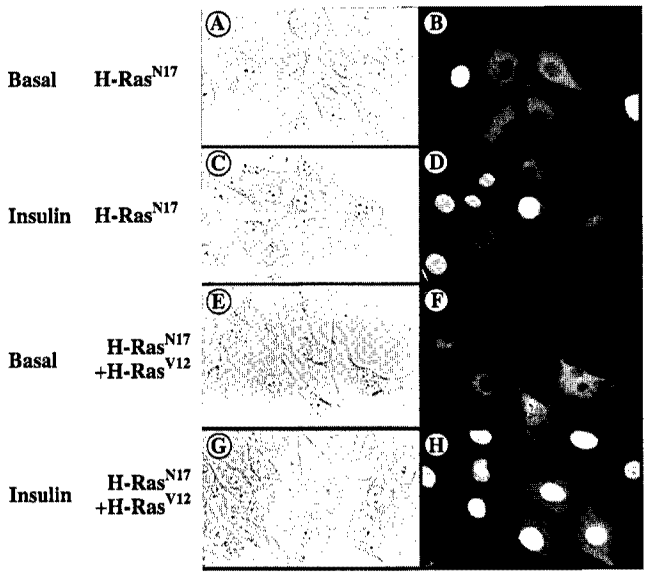

Fig. 1. Effects of dominant-negative H-Ras^{N17} alone or H-Ras^{N17} plus oncogenic H-Ras^{V12} on insulin-induced DNA synthesis. HIRc-B cells were grown on coverslips at 40–50% confluency and serum-starved for 16 h. The cells were then microinjected with either dominant-negative H-Ras^{N17} (5 mg/ml) alone (A–D) or oncogenic H-Ras^{V12} (0.1 mg/ml) plus H-Ras^{N17} (5 mg/ml) (E–H). All microinjection mixtures included rat IgG (5 mg/ml). After stabilization for 2 h, cells were incubated with BrdU in the presence or absence of insulin (100 ng/ml) for 16 h at 37°C. They were then fixed and processed for double-label indirect immunofluorescence by sequential incubation with mouse anti-BrdU antibody and then FITC-conjugated anti-rat antibody mixed with TRITC-conjugated anti-mouse IgG antibody. The injected cells were identified by cytoplasm FITC staining (showing as cytoplasmic white color) and DNA synthesis was identified by nuclear TRITC staining (showing as nuclear white).

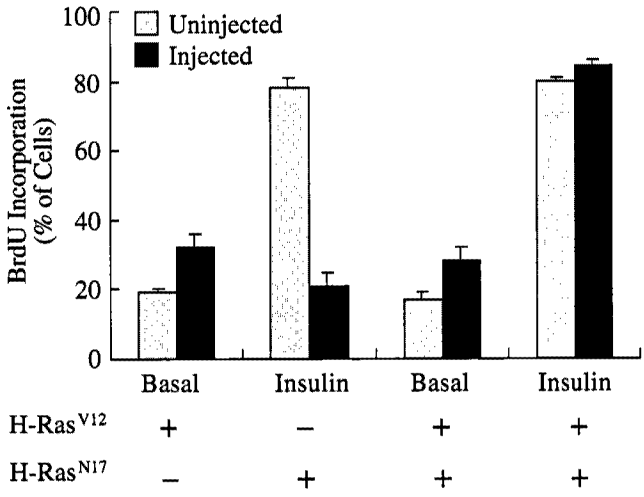

Fig. 2. Evidence of a Ras-independent pathway of insulin leading to DNA synthesis. Serum-starved HIRc-B cells were microinjected with either dominant-negative H-Ras^{N17} (5 mg/ml) alone or oncogenic H-Ras^{V12} (0.1 mg/ml) alone or oncogenic H-Ras^{V12} (0.1 mg/ml) plus H-Ras^{N17} (5 mg/ml). All microinjection mixtures included rat IgG (5 mg/ml). After stabilization for 2 h, cells were incubated with BrdU in the presence or absence of insulin (100 ng/ml) for 16 h at 37°C. They were then fixed and processed for double-label indirect immunofluorescence by sequential incubation with mouse anti-BrdU antibody and then FITC-conjugated anti-rat antibody mixed with TRITC-conjugated anti-mouse IgG antibody. The injected cells were identified by cytoplasm FITC staining and DNA synthesis were identified by nuclear TRITC staining. On the same coverslip, DNA synthesis in the uninjected cells counted as a control. A total of 150–200 injected cells were counted. The results presented are the means of three coverslips and two independent experiments. Data shown are the average of 1000–1200 injected cells.

induced DNA synthesis by 32% in the basal HIRc-B cells, even in the continuous presence of dominant-negative H-Ras^{N17} protein, indicating that the action site of H-Ras^{V12} protein lies downstream of inhibitory H-Ras^{N17}.

Next, cells were coinjected with oncogenic H-Ras^{V12} (0.1 mg/ml) and dominant-negative H-Ras^{N17}, and insulin-stimulated DNA synthesis was assessed. The oncogenic H-Ras^{V12} (0.1 mg/ml) cooperating with insulin fully stimulated DNA synthesis, despite the presence of inhibitory H-Ras^{N17}, demonstrating rescue of the H-Ras^{N17} blockade. These results suggest that there is the Ras-independent pathway of insulin leading to DNA synthesis. This Ras-independent pathway alone is not enough to induce DNA synthesis by insulin, but when potentiated with an additional mitogenic signal, it can fully induce the DNA synthesis and presumably cell proliferation.

Ras-independent pathway bypassing p85^{SH2-N} and Shc protein Several experiments were performed to examine whether the p85 subunit of PI3-kinase and Shc protein are involved in the Ras-independent pathway. The oncogenic H-Ras^{V12} (0.1 mg/ml) plus inhibitory H-Ras^{N17} and p85^{SH2-N} was microinjected into quiescent HIRc-B cells and DNA synthesis was assessed (Fig. 3). In the basal cells, oncogenic H-Ras$^{V12[0.1]}$ induced 35% cells of injected cells to incorporate BrdU, in the presence of both of the inhibitory proteins. In the insulin-stimulated cells, DNA synthesis was fully activated, despite the presence of inhibitory H-Ras^{N17} and p85^{SH2-N}, demonstrating rescue of both of the H-Ras^{N17} and p85^{SH2-N} blockade.

Next, the oncogenic H-Ras^{V12} plus inhibitory H-Ras^{N17} and anti-Shc antibody were microinjected into quiescent HIRc-B cells, and DNA synthesis was assessed. It has been shown that microinjection of anti-Shc antibody blocked insulin-induced DNA synthesis, suggesting that Shc protein is a critical link in the insulin signal transduction pathway to DNA synthesis (Sasaoka *et al.*, 1994). In the basal cells, oncogenic H-Ras^{V12} induced 35% of injected cells to incorporate BrdU, in the presence of both inhibitory proteins. In the insulin-stimulated cells, DNA synthesis was fully activated, despite the presence of inhibitory H-Ras^{N17} and anti-Shc antibody. These results suggest that the Ras-independent pathway of insulin leading to DNA synthesis bypasses both p85 of PI3-kinase and Shc protein.

Functional role of Rac1 protein in the Ras-independent pathway To investigate a functional role of Rac1 protein in the Ras-independent pathway, oncogenic H-Ras^{V12} plus inhibitory H-Ras^{N17} and dominant-negative Rac1^{N17} was microinjected into quiescent HIRc-B cells, and DNA synthesis was assessed (Fig. 4). Microinjection of Rac1^{N17}

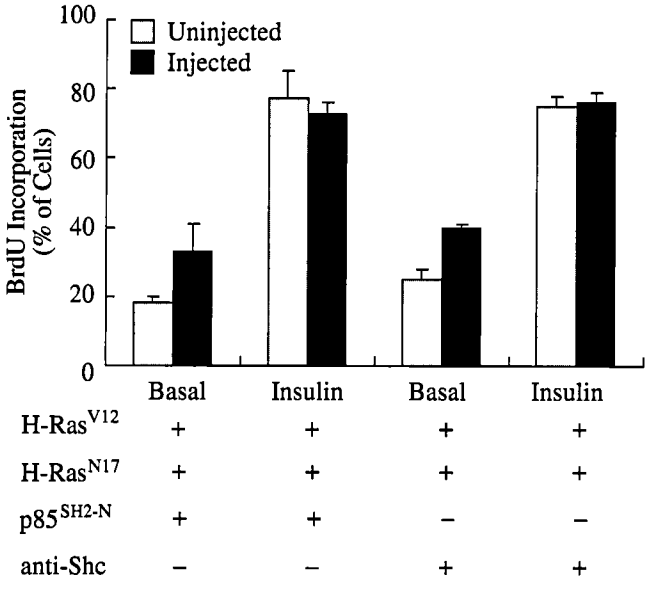

Fig. 3. Coinjection of oncogenic H-Ras^{V12} plus inhibitory H-Ras^{N17} plus p85^{SH2-N} or oncogenic H-Ras^{V12} plus inhibitory H-Ras^{N17} plus interfering anti-Shc antibody. Serum-starved HIRc-B cells were coinjected with either oncogenic H-Ras^{V12} (0.1 mg/ml) plus p85^{SH2-N} (7 mg/ml) plus dominant-negative H-Ras^{N17} (5 mg/ml) or oncogenic H-Ras^{V12} (0.1 mg/ml) plus p85^{SH2-N} (7 mg/ml) plus anti-Shc antibody. All microinjection mixtures included rat IgG (5 mg/ml). After stabilization for 2 h, cells were incubated with BrdU in the presence or abscence of insulin (100 ng/ml) for 16 h at 37°C. DNA synthesis was examined as described in the legend of Fig. 2.

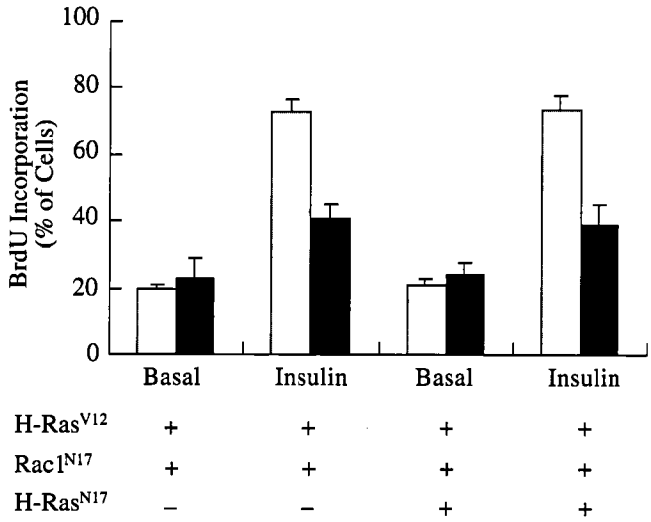

Fig. 4. Involvement of Rac1 protein in the Ras-independent pathway of insulin. Serum-starved HIRc-B cells were microinjected with either dominant-negative Rac1^{N17} (7 mg/ml) plus oncogenic H-Ras^{V12} (0.1 mg/ml) or dominant-negative Rac1^{N17} (7 mg/ml) plus oncogenic H-Ras^{V12} (0.1 mg/ml) plus dominant-negative H-Ras^{V12} (5 mg/ml). All microinjection mixtures included rat IgG (5 mg/ml). After stabilization for 2 h, cells were incubated with BrdU in the presence or absence of insulin (100 ng/ml) for 16 h at 37°C. DNA synthesis was examined as described in the legend of Fig. 2.

alone inhibited insulin-stimulated DNA synthesis. In the basal cells, oncogenic H-Ras^{V12} induced 35% of injected cells to incorporate BrdU, in the presence of both inhibitory proteins. In the insulin-stimulated cells, DNA synthesis was inhibited, demonstrating that the Rac1 protein is required in the Ras-independent pathway of insulin leading to DNA synthesis.

Discussion

Single cell microinjection provides a direct means to determine whether an endogenous signaling molecule is required for a particular phenotype (in this case insulin bioeffects). For example, it has been shown that microinjection of dominant interfering protein or antibodies directed against IRS-1, Ras, p85 subunit of PI3-kinase, and Shc interferes insulin-stimulated DNA synthesis (Jhun *et al.*, 1994a; 1994b; Rose *et al.*, 1994; Sasaoka *et al.*, 1994), and this approach has been used to establish a role for other intracellular proteins in the actions of other mitogens. In the current studies, we have shown that microinjection of a dominant-negative H-Ras mutant (H-Ras^{N17}) abrogates the effect of insulin to promote DNA synthesis. We have also examined the existence of the Ras-independent pathway in insulin-induced DNA synthesis. Upon coinjection, the inhibitory action of H-Ras^{N17} was completely rescued by an additional existence of oncogenic H-Ras^{V12}. These results indicate the existence of the Ras-independent signal transduction pathway and suggest that the pathway alone is not sufficient to induce full DNA synthesis. This incomplete mitogenic signal generated from the Ras-independent pathway of insulin works together with an additional partial mitogenic signal from oncogenic H-Ras^{V12} and then fully stimulates the cells to incorporate BrdU. Thus, the molecular component(s) involved in the Ras-independent signaling pathway of insulin was investigated. From our previous results, microinjection of the N-terminal SH2 domain of the p85 subunit of PI3-kinase (Jhun *et al.*, 1994b) and interfering anti-Shc antibody (Sasaoka *et al.*, 1994) inhibited insulin-induced DNA synthesis, indicating that these proteins are important intermediates in the insulin's mitogenic signaling pathway leading to DNA synthesis. When coinjected with H-Ras^{V12}, p85$^{SH2\text{-}N}$ and anti-Shc antibody could not inhibit insulin-induced DNA synthesis. These results indicate that the p85 subunit and Shc protein are not involved in the Ras-independent pathway.

The SH2 domain of Grb2 recognizes and binds to specific motifs in IRS-1 and Shc (Myers *et al.*, 1996). Insulin induces the formation of both IRS-1-Grb2-Sos and Shc-Grb2-Sos complexes. To evaluate the functional role of these complexes in the Ras-independent pathway, the interfering anti-Shc antibody was utilized. Microinjection with inhibitory Ras^{N17} could not inhibit Ras-independent mitogenic signal, suggesting that Shc-Grb2-Sos complex is not required for the pathway leading to the DNA synthesis by insulin. Consistent with our result, Sakaue *et al.* (1995) has shown that in the Sos mutant lacking the guanine nucleotide exchange domain, insulin activation of Ras and MAP kinase is defective. However, insulin increased thymidine incorporation and activated glycogen synthase and S6 kinase in the Sos mutant expressing CHO-IR cells. These observations suggest that the regulatory protein of RasGEF activity such as Grb2 and Sos is not involved and that there is the Ras-independent pathway, which is bypassing the Shc-Grb2-Sos complex.

Rac is a member of the Rho family, and like Ras protein, Rac1 proteins cycle between an inactive GDP-bound form and an active GTP-bound form. Rac1 protein is implicated in inducing morphological changes associated with actin polymerization (Hall *et al.*, 1992). Insulin induces cytoskeleton reorganization such as membrane ruffling and stress fiber fromation, and microinejection of dominant-negative Rac1 protein inhibits the insulin-induced membrane ruffling (Kotani *et al.*, 1995; Nishiyama *et al.*, 1995). Rac1 protein is also involved in G1 cell cycle progression and cell transformation (Ridley *et al.*, 1992; White *et al.*, 1995). We observed that microinjection of Rac1^{N17} inhibits DNA synthesis induced by EGF (Kim *et al.*, 1998) and insulin. Therefore, the mitogenic signal of insulin utilizes the well-known Ras-MAP kinase pathway as well as the Rac-dependent pathway. Mutational analysis of Ras has revealed that Rac activation is an essential downstream signal required for Ras-induced malignant transformation (Qui *et al.*,1995).

There is evidence for the Rac activation through the Ras-independent pathway. Activation of the JNK/stress-activated kinase (SAPK) and p38 MAP kinase by UV light, osmotic shock, and inflammatory stimuli is mediated through the Rac-dependent pathway, but not the Ras-independent pathway (Bird *et al.*, 1994). However, the molecular link between these stimuli and Rac activation is unclear. Rac is a GTP binding protein and requires a guanine nucleotide exchange factor (RacGEF) activity for its activation. Tiam1 is shown to be a RacGEF (Michiels *et al.*, 1995). But there is little data on how Tiam1 is activated by extracellular agonists including insulin. It remains to be studied whether the Tiam1 is activated by insulin and involved in the Ras-independent pathway.

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