Desensitization of Ras Activation by a Feedback Disassociation of the SOS-Grb2 Complex

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Activation of Ras by the exchange of bound GDP for GTP is predominantly catalyzed by the guanylnucleotide exchange factor SOS. Receptor tyrosine kinases increase Ras-GTP loading by targeting SOS to the plasma membrane location of Ras through the small adapter protein Grb2. However, despite the continuous stimulation of receptor tyrosine kinase activity, Ras activation is transient and, in the case of insulin, begins returning to the GDP-bound state within 5 min. We report here that the cascade of serine kinases activated directly by Ras results in a mitogen-activated protein kinase (MEK)-dependent phosphorylation of SOS and subsequent disassociation of the Grb2-SOS complex, thereby interrupting the ability of SOS to catalyze nucleotide exchange on Ras. These data demonstrate a molecular feedback mechanism accounting for the desensitization of Ras-GTP loading following insulin stimulation.

Previous studies have demonstrated that insulin stimulation of the insulin receptor tyrosine kinase results in Ras activation and subsequent downstream stimulation of the Raf/MEK/ERK pathway (1–3). The activation of Ras occurs predominately through the tyrosine phosphorylation of Shc followed by the association with the Grb2-SOS complex (4, 5). However, Ras activation is transient and rapidly returns to the inactive state despite continuous activation of the insulin receptor tyrosine kinase and prolonged Shc tyrosine phosphorylation (6, 7). Since insulin does not affect Ras-GTPase activating protein activity and/or targeting (8, 9), the mechanism responsible for the desensitization of Ras has remained obscure.

Recently it has been reported that stimulation of several cell types with growth factors and other mitogenic agents results in the serine/threonine phosphorylation of SOS (10, 11). In addition, SOS phosphorylation precedes an insulin-dependent disassociation of the Grb2-SOS complex (7, 12). The insulin time dependence of the SOS phosphorylation and uncoupling of Grb2 from SOS was consistent with the desensitization phase of Ras inactivation. To determine whether the ERK pathway is involved in this event, we used two independent approaches to inhibit MEK activity and, hence, ERK activation. In this study we demonstrate that prevention of insulin-stimulated SOS phosphorylation and subsequent dissociation of the Grb2-SOS complex results in a prolongation of Ras activation.

EXPERIMENTAL PROCEDURES

Cell Culture—Chinese hamster ovary cells expressing the human insulin receptor (CHO/IR) and 3T3L1 adipocytes were isolated and cultured as described previously (7). Cells were incubated for 16 h in serum-free media and then pretreated for 1 h with vehicle (0.5% dimethyl sulfoxide) or 100 μM PD98059. The cells were then incubated with and without 100 nM insulin for various times, followed by lysis in 50 mM Hepes, pH 7.8, 1% Triton X-100, 2.5 mM EDTA, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium vanadate, 2 μM pepstatin, 0.5 μg/ml aprotinin, 1 mM phenylmethylsulfonate fluoride, and 10 μM leupeptin.

Immunoprecipitation and Immunoblotting—Grb2 was immunoprecipitated from the whole cell lysates by incubation with a Grb2 polyclonal antibody (Santa Cruz Biotechnology) for 2 h at 4°C. The resultant immune complexes were precipitated by incubation with protein A-agarose for 1 h at 4°C. The pellets were washed three times with Tris-buffered saline (20 mM Tris, pH 7.6, 150 mM NaCl), resuspended in SDS-sample buffer (125 mM Tris-HCl, pH 6.8, 20% [v/v] glycerol, 4% [w/v] SDS, 100 mM dithiothreitol, 0.1% [w/v] bromophenol blue) and heated at 100°C for 5 min. Whole cell lysates or immunoprecipitates were separated on reducing 5–10% SDS-polyacrylamide gradient gels and transferred to polyvinylidene difluoride membranes using 1 A for 2 h at 4°C. Immunoblotting of the whole cell lysates or Grb2 immunoprecipitates was performed using an ERK polyclonal antibody (Zymed), a pp90(RSK) polyclonal antibody, or a Raf polyclonal antibody (Santa Cruz Biotechnology) and a SOS polyclonal antibody (Upstate Biotechnology Inc.).

Quantitative Transfer and Electrophoresis—We have recently demonstrated that electrophoresis can be used to express various cDNAs in CHO/IR with 85–100% transfection efficiency (13). Briefly, CHO/IR cells were electroporated with a total of 40 μg of the dominant-interfering MEK mutant (MEK/K97R) or the empty parent vector (CMV5) at 340 V and 960 μF in 500 μl of phosphate-buffered saline. Following electroporation, the cells were plated in α-minimal essential medium containing 10% serum. Cell debris was removed by replacing media with fresh media 12 h later.

Determination of GTP-bound Ras—CHO/IR cells were incubated in serum- and phosphate-free media for 2 h, followed by the addition of 0.2 mM carrier-free 32P for 3 h. The cells were pretreated with either vehicle (0.5% dimethyl sulfoxide) or 100 μM PD98059 during the last hour of the labeling period. Cells were then either left untreated or stimulated with 100 nM insulin for the indicated times. Cells were solubilized in 50 mM Hepes, 1 mM sodium phosphate, pH 7.4, 1% Triton X-100, 100 mM NaCl, 20 mM MgCl2, 1 mg/ml bovine serum albumin, 0.1 mM GTP, 0.1 mM GDP, 1 mM ATP, 0.4 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, and 10 mM benzamide. The extract was immunoprecipitated with the Ras antibody (Y13-259, Oncogene Science) for 60 min, and the immune complexes were washed 5 times with lysis buffer and 5 times with wash buffer (50 mM Hepes, pH 7.4, 20 mM MgCl2, 150 mM NaCl, and 0.005% SDS). Ras-associated guanylnucleotides were eluted in 20 μl of 2 mM EDTA, pH 8.0, 2 mM dithiothreitol, 0.2% SDS, 0.5 mM GTP, and 0.5 mM GDP) at 65°C for 20 min. Eluted GDP and GTP were
MEK-dependent SOS Phosphorylation

![Diagram](diagram.png)

**Fig. 1. Blockade of insulin-stimulated ERK, Raf, and SOS phosphorylation and dissociation of the Grb2-SOS complex by inhibition of MEK activity.** CHO/IR cells were incubated in the absence (left, lanes 1–6) or presence of the MEK inhibitor PD98059 (right, lanes 1–6). Following 5 min of insulin stimulation, insulin-stimulated SOS phosphorylation was measured using an ERK antibody (a), a Raf antibody (b), or a SOS antibody (c) as described under "Experimental Procedures." The whole cell lysates obtained from CHO/IR cells preincubated in the absence (left, lanes 1–4) or presence of PD98059 (right, lanes 1–4) followed by a second incubation without (lane 1) or with 100 nM insulin for 3 min (lane 2), 10 min (lane 3), and 30 (lane 6) min. Whole cell lysates were separated by polyethyleneimine cellulose plates (Baker) by thin layer chromatography using 1 M KH₂PO₄ (pH 3.4) as the solvent. Labelled nucleotides were visualized by autoradiography and counted using an AMBIS β detector.

**RESULTS AND DISCUSSION**

To investigate the role of the ERK pathway mediating SOS phosphorylation and dissociation of the Grb2-SOS complex, we initially took advantage of the recently identified specific inhibitor of MEK activity, PD98059 (14, 15). This reagent is noncompetitive for ATP and does not affect the enzyme activities of over 30 tyrosine and serine/threonine kinases examined, including the highly related Jnk kinase kinase, JNKK. Since MEK is the immediate upstream kinase responsible for ERK phosphorylation, we examined the effect of PD98059 on insulin-stimulated ERK phosphorylation (Fig. 1a). Insulin treatment of Chinese hamster ovary cells expressing high levels of the insulin receptor (CHO/IR) resulted in a time-dependent phosphorylation of ERK1 and ERK2 characterized by decreased electrophoretic mobility (1). The phosphorylation of ERK was transient, with a maximal decrease in mobility following 5 min of insulin stimulation and a return to the basal state by 30 min (Fig. 1a, left, lanes 1–6). Pretreatment of cells with PD98059 prior to insulin stimulation markedly decreased the extent of ERK phosphorylation, as indicated by the near-complete absence of the slower migrating isoforms of ERK (Fig. 1a, right, lanes 1–6).

MEK is activated by serine phosphorylation catalyzed by members of the Raf family of protein kinases (16–18). The Raf kinase lies in a feedback pathway, in which Raf undergoes phosphorylation secondary to ERK activation (19, 20). Thus, insulin also stimulated a characteristic time-dependent gel shift of Raf which was initially detected at 5 min and was maximal by 10 min (Fig. 1b, left, lanes 1–6). In contrast to ERK, the decrease in Raf electrophoretic mobility persisted over the 30-min time period examined. However, pretreatment with the MEK inhibitor prevented the insulin-stimulated Raf gel shift (Fig. 1b, right, lanes 1–6) but did not block Raf kinase activity (data not shown). These data further demonstrate that Raf phosphorylation occurs by a MEK-dependent feedback pathway.

Previous studies have demonstrated that SOS is phosphorylated on serine/threonine residues following growth factor activation of the Ras/Raf/MEK/ERK pathway (11). Consistent with this, insulin stimulated a time-dependent reduction in the electrophoretic mobility of SOS (Fig. 1c). The decrease in SOS mobility was detected following 5 min of insulin treatment with a maximal effect reached by 10 min (Fig. 1c, left, lanes 1–6). Pretreatment of the cells with PD98059 also prevented the insulin-stimulated SOS gel shift (Fig. 1c, right, lanes 1–6), consistent with a role of MEK in the cascade leading to the phosphorylation of SOS.

In addition to the stimulation of SOS phosphorylation, various agents which activate the ERK pathway have also been observed to induce the dissociation of the Grb2-SOS complex (7, 12). Immunoprecipitation of Grb2 from unstimulated cells resulted in co-immunoprecipitation of SOS (Fig. 1d, left, lane 1). A similar amount of co-immunoprecipitated SOS was detected in the Grb2 immunoprecipitates from cells treated with insulin for 3 min (Fig. 1d, left, lane 2). However, following 10 or 30 min of insulin stimulation, there was a marked decrease in the ability of the Grb2 antibody to co-immunoprecipitate SOS (Fig. 1d, left, lanes 3 and 4). Pretreatment with PD98059 had no significant effect on the extent of Grb2-SOS association from unstimulated cells or from cells treated with insulin for 3 min (Fig. 1d, right, lanes 1 and 2). However, the MEK inhibitor completely blocked the dissociation of the Grb2-SOS complex following 10 and 30 min of insulin stimulation (Fig. 1d, right, lanes 3 and 4). Neither insulin nor PD98059 had any effect on the amount of Grb2 protein that was immunoprecipitated by the Grb2 antibody (data not shown).

To ensure that the effect of MEK inhibition on Grb2-SOS interactions was not cell type-specific, we also determined the effect of PD98059 on 3T3L1 adipocytes. As observed in the CHO/IR cells, insulin stimulation for 5 min resulted in the characteristic decrease in electrophoretic mobility characteristic of ERK phosphorylation (Fig. 2a, lanes 1 and 2). Pretreatment of the 3T3L1 adipocytes with PD98059 completely prevented the insulin stimulation of ERK phosphorylation (Fig. 2a, lanes 3 and 4). Similarly, PD98059 also inhibited the phosphorylation of SOS following 15 min of insulin stimulation (Fig. 2b) and the subsequent dissociation of the Grb2-SOS complex (Fig. 2c). Thus, the insulin-stimulated pathways leading to SOS phosphorylation and dissociation of the Grb2-SOS complex converged at the level of MEK in both CHO/IR cells and 3T3L1 adipocytes.

Since Ras desensitization and the dissociation of the Grb2-SOS complex were temporally related, we examined the effect of the MEK inhibitor on insulin-stimulated Ras-GTP loading.
dependent, several studies have observed that ERK can phosphorylate SOS both in vivo (20) and in vitro (7, 11). However, it remains possible that MEK itself or other downstream protein kinases may be involved. Further studies are necessary to determine the specific kinases and sites of SOS phosphorylation.
tion that are responsible for the disassociation of the Grb2-SOS complex leading to Ras inactivation.

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