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Insulin Stimulation of a MEK-Dependent but ERK-Independent SOS Protein Kinase

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The Ras guanylnucleotide exchange protein SOS undergoes feedback phosphorylation and dissociation from Grb2 following insulin receptor kinase activation of Ras. To determine the serine/threonine kinase(s) responsible for SOS phosphorylation in vivo, we assessed the role of mitogen-activated, extracellular-signal-regulated protein kinase kinase (MEK), extracellular-signal-regulated protein kinase (ERK), and the c-JUN protein kinase (JNK) in this phosphorylation event. Expression of a dominant-interfering MEK mutant, in which lysine 97 was replaced with arginine (MEK/K97R), resulted in an inhibition of insulin-stimulated SOS and ERK phosphorylation, whereas expression of a constitutively active MEK mutant, in which serines 218 and 222 were replaced with glutamic acid (MEK/EE), induced basal phosphorylation of both SOS and ERK. Although expression of the mitogen-activated protein kinase-specific phosphatase (MKP-1) completely inhibited the insulin stimulation of ERK activity both in vitro and in vivo, SOS phosphorylation and the dissociation of the Grb2-SOS complex were unaffected. In addition, insulin did not activate the related protein kinase JNK, demonstrating the specificity of insulin for the ERK pathway. The insulin-stimulated and MKP-1-insensitive SOS-phosphorylating activity was reconstituted in whole-cell extracts and did not bind to a MonoQ anion-exchange column. In contrast, ERK1/2 protein was retained by the MonoQ column, eluted with approximately 200 mM NaCl, and was MKP-1 sensitive. Although MEK also does not bind to MonoQ, immunodepletion analysis demonstrated that MEK is not the insulin-stimulated SOS-phosphorylating activity. Together, these data demonstrate that at least one of the kinases responsible for SOS phosphorylation and functional dissociation of the Grb2-SOS complex is an ERK-independent but MEK-dependent insulin-stimulated protein kinase.

Ras is a 21-kDa GTP-binding protein which plays a central role integrating extracellular signals for the regulation of cell development, differentiation, mitogenesis, and macromolecular biosynthesis (7, 8). Recent studies have begun to establish a complete molecular pathway by which receptor tyrosine kinase stimulation couples to Ras activation (16, 36, 43). For example, tyrosine autophosphorylation of the epidermal growth factor receptor creates docking sites for several *src* homology 2 (SH2) domain-containing effectors, including the small adapter protein Grb2 (9, 17, 29, 35). The Grb2 SH2 domain is flanked by two *src* homology 3 (SH3) domains which mediate the association with the Ras guanylnucleotide exchange factor SOS (10, 17, 27, 29, 35, 37). Since membrane-targeted forms of SOS are sufficient for Ras activation, it has been suggested that the plasma membrane receptor targeting of SOS is responsible for mediating the exchange of GDP for GTP on Ras (3, 21, 34).

Once in the activated GTP-bound state, Ras associates with the Raf family members of protein kinases and by an undefined mechanism activates Raf (32, 42, 44, 47, 50). In this manner, Ras functions as a molecular switch converting tyrosine kinase upstream signals into downstream serine/threonine kinase phosphorylation events. Activated Raf is a relatively specific activator of the family of mitogen-activated, extracellular-signal-regulated protein kinase kinases (MEK) (15, 22, 25, 51). The MEKs are unusual protein kinases in that they phosphorylate both threonine and tyrosine residues in a specific TEY motif (13). The extracellular-signal-regulated protein kinase (ERK) contains a TEY motif, and phosphorylation of both the threonine and tyrosine residues is absolutely necessary for the stimulation of ERK activity (4, 6, 13, 30). In turn, activated ERK phosphorylates a number of nuclear transcription factors and cytoplasmic proteins involved in the regulation of macromolecular synthesis and mitogenesis (5, 20, 40).

Recently, ERK has also been suggested to play a role in a negative-feedback mechanism which limits the extent of Ras activation. Stimulation of cells with various agents that activate the Ras/Raf/MEK/ERK pathway (insulin, platelet-derived growth factor, v-Ras, and v-Raf) resulted in the serine/threonine phosphorylation of SOS and dissociation of the Grb2-SOS complex (11, 12, 49). SOS contains several ERK consensus phosphorylation sites in its carboxyl-terminal proline-rich domain, which is in close proximity to the sites mediating Grb2 binding. Consistent with this model, overexpression of ERK was reported to induce a hyperphosphorylation of SOS following growth factor stimulation (41). In addition, SOS is readily phosphorylated by purified ERK in vitro (12, 49). On the basis of these data, it has been speculated that SOS may be an in vivo substrate for ERK. However, in vitro kinase assays and expression of kinases at supraphysiological levels may not necessarily reflect the appropriate functions in vivo. To evaluate the potential kinases involved in the uncoupling of the Grb2-SOS complex, we have determined the role of MEK and ERK in mediating the insulin stimulation of SOS phosphorylation in vivo. In the present study, we have identified an insulin-stimulated MEK-dependent but ERK-independent kinase activity that accounts for at least one of the kinases responsible for SOS phosphorylation and dissociation of the Grb2-SOS complex.

MATERIALS AND METHODS

Cell culture and transfection. Chinese hamster ovary cells expressing the human insulin receptors (CHO/IR cells) were isolated and maintained as previously described (48). The cells were quantitatively transfected by electroporation

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with 40 μg of the various plasmid cDNAs at 340 V and 960 μF (48). At 30 h following transfection, the cells were serum starved for 3 h and either untreated or stimulated for various times with 100 nM insulin or 50 μg of anisomycin per ml as described in the figure legends.

Immunoblotting and immunoprecipitation. Whole-cell detergent extracts were prepared by solubilization of 10^7 cells in 0.5 ml of lysis buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.8], 1% Triton X-100, 2.5 mM EDTA, 100 mM sodium fluoride, 10 mM sodium PP_i, 2 mM sodium vanadate, 2 mM pepstatin, 0.5 trypsin inhibitory unit of aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin). Immunoblotting was performed with monoclonal ERK (Zymed) or monoclonal SOS (Transduction Laboratories) antibodies as previously described (48). The Grb2-SOS complex was immunoprecipitated from 0.5 mg of total protein with a polyclonal Grb2 antibody (Santa Cruz Biotechnology) and immunoblotted with an SOS antibody as previously described (48). The primary monoclonal antibodies were detected with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G Fc (Pierce) and visualized by the enhanced chemiluminescence detection system (Amersham).

ERK in-gel protein kinase assay. ERK activity was measured by an in-gel kinase assay with myelin basic protein as the substrate, based on the method of Kameshita and Fujisawa (23) with some modifications. Briefly, cells were solubilized in lysis buffer (as above), and the cleared lysate was boiled for 5 min in Laemmli sample buffer. Protein extract (25 μg) was electrophoresed at 100 V for 1.5 h on a 12% polyacrylamide minigel (Bio-Rad) containing 0.5 mg of myelin basic protein per ml. After electrophoresis, the sodium dodecyl sulfate (SDS) was removed from the gel by two washes in 20% isopropanol–50 mM Tris-HCl (pH 8.0) for 1 h (each) at room temperature with gentle agitation. The gel was then washed in 50 mM Tris-HCl (pH 8.0)–5 mM β-mercaptoethanol for 1 h at room temperature, and the proteins were further denatured by incubating the gel in 100 ml of 50 mM Tris-HCl (pH 8.0)–6 M guanidine-HCl (ultrapure)–20 mM dithiothreitol (DTT)–2 mM EDTA for 1 h at room temperature. The proteins were then renatured by incubating the gel in 1 liter of 50 mM Tris-HCl (pH 8.0)–1 mM DTT–2 mM EDTA–0.04% Tween 20 overnight at 4°C without agitation. Prior to the kinase reaction, the gel was equilibrated in kinase buffer [40 mM HEPES (pH 8.0), 1 mM DTT, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 20 mM $MgCl_2$, 100 μM sodium vanadate] for 1 h at room temperature. The kinase reaction was initiated by placing the gel in fresh kinase buffer containing 30 μM [γ-^{32}P]ATP (10 μCi/ml) and incubated for 1 h at room temperature with gentle agitation. The gel was washed extensively with repeated changes of wash buffer (5% trichloroacetic acid, 1% sodium PP_i), dried, and subjected to autoradiography.

c-Jun kinase (JNK) in vitro kinase assay. Cells (10^7) were harvested in 0.5 ml of lysis buffer (buffer J) (50 mM HEPES [pH 7.8], 0.3 M NaCl, 1.5 mM $MgCl_2$, 1.2 mM EDTA, 0.1% Triton X-100, 20 mM β-glycerophosphate, 100 mM NaF, 10 mM sodium PP_i, 0.1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin, 2 μg of aprotinin per ml, 1 μg of leupeptin per ml). The cleared extract (400 μg in 200 μl) was diluted 1:1 with 200 μl of lysis buffer (minus Triton X-100 and NaCl) plus the addition of 0.5 mM DTT and 3.5 mM $MgCl_2$ (final concentrations in 400 μl). The extracts were incubated with rotation for 4 h at 4°C with 10 μg of glutathione-*S*-transferase (GST)-c$JUN_{1–79}$ conjugated to glutathione-agarose beads (prepared as specified by the manufacturer [Pharmacia]). The agarose beads were pelleted by quick microcentrifugation, and the protein complexes were washed three times with HEPES binding buffer (20 mM HEPES [pH 8.0], 2.5 mM $MgCl_2$, 0.1 mM EDTA, 50 mM NaCl, 0.05% Triton X-100). The final wash was performed in kinase buffer (20 mM HEPES [pH 8.0], 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 0.1 mM sodium vanadate, 2 mM DTT). The kinase reaction was initiated by resuspending the pelleted beads in 30 μl of kinase buffer plus 20 μM [γ-^{32}P]ATP (0.2 μCi/ml) for 30 min at room temperature with gentle agitation. The reactions were terminated by addition of 1 ml of ice-cold HEPES binding buffer, and the beads were pelleted, resuspended in Laemmli sample buffer, and boiled for 5 min. Proteins were resolved on an SDS–10% polyacrylamide gel, stained with Coomassie blue, destained, dried, and subjected to autoradiography.

SOS in vitro kinase assay. Cells which had been transfected with mitogen-activated protein (MAP) kinase-specific phosphatase (MKP-1) or the empty vector and treated as indicated in the figures were harvested in the lysis buffer used for immunoblotting (see above). The extracts (30 μl) were incubated with 1 μg of epitope (Glu-Glu)-tagged hSOS1 bound to anti-Glu-Glu monoclonal antibodies conjugated to protein G-Sepharose (33). The kinase reaction was initiated with the addition of 100 μl of kinase buffer B (20 mM HEPES [pH 8.0], 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 0.1 mM sodium vanadate, 2 mM DTT, 40 μM ATP, 5 μCi of [γ-^{32}P]ATP). The reaction mixtures were incubated for 30 min at room temperature with gentle agitation and terminated by addition of 1 ml of ice-cold HEPES-binding buffer. The beads were then pelleted, and the proteins were heated at 100°C in Laemmli sample buffer for 5 min and resolved on an SDS–7.5% polyacrylamide gel. The gels were stained with Coomassie blue, destained, dried, and subjected to autoradiography. In the case of the fractions from the MonoQ column, 30 μl from each 1-ml fraction was assayed identically to that described above for SOS kinase activity.

MonoQ column chromatography. Cells which had been transfected with MKP-1 or empty vector and treated as indicated in the figures were harvested in the lysis buffer used for immunoblotting (with HEPES at pH 7.4 rather than 7.8). The extracts were cleared, and 1 mg of protein was made to 10 ml with buffer B (50 mM HEPES [pH 7.4], 2.5 mM EDTA, 50 mM sodium fluoride, 10 mM sodium PP_i, 2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride) and applied to an HR5/5 MonoQ fast protein liquid chromatography column. Chromatography was performed at a flow rate of 0.25 ml/min with a 20-ml linear gradient of 0 to 1 M NaCl in buffer B beginning at fraction 15. Fractions (1 ml) were collected and stored at −80°C.

Immunodepletion of MEK protein. The MonoQ flowthrough fractions 4 to 12 were pooled, and 100-μl aliquots were incubated in the absence or presence of 3 μl of a MEK1/2-specific rabbit antibody (kindly provided by Alan Saltiel, Parke-Davis) or a nonspecific rabbit antibody for 16 h at 4°C. The samples were precipitated with protein A-Sepharose, and the immunocomplexes were isolated by centrifugation. The cleared supernatants were then assayed for the presence of the MEK protein by immunoblotting with a MEK1/2 rabbit polyclonal antibody (Transduction Laboratories). In parallel, the cleared supernatants were also assayed for SOS phosphorylating activity by the SOS in vitro kinase assay as described above.

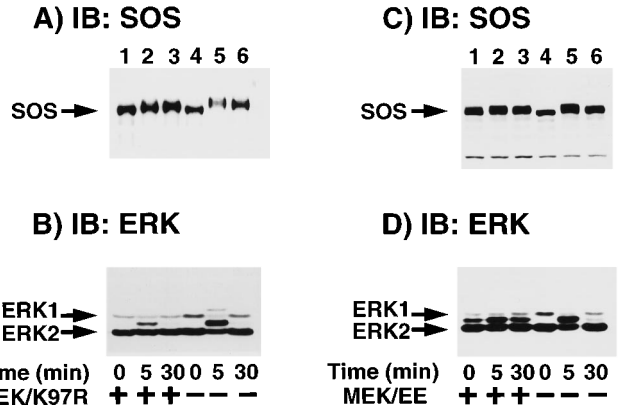

FIG. 1. Insulin stimulation of ERK and SOS phosphorylation is MEK dependent. CHO/IR cells were transfected with the empty vector (lanes 4 to 6) or the mammalian expression vector encoding the dominant-interfering MEK mutant MEK/K97R (lanes 1 to 3) (A and B) or the constitutively active MEK mutant MEK/EE (lanes 1 to 3) (C and D) as described in Materials and Methods. At 30 h following transfection, the cells were serum starved for 3 h and then untreated (lanes 1 and 4) or stimulated with 100 nM insulin for 5 (lanes 2 and 5) or 30 (lanes 3 and 6) min as indicated. Whole-cell lysates were prepared and subjected to SOS immunoblotting (A and C) or ERK immunoblotting (B and D).

RESULTS

Insulin-stimulated SOS phosphorylation is MEK dependent. It has recently been reported that activation of growth factor receptor tyrosine kinases results in the serine/threonine phosphorylation of SOS (9, 12, 49). Furthermore, SOS phosphorylation was also observed in cells transfected with v-Ras or v-Raf, suggesting the involvement of the Ras/Raf/MEK/ERK pathway. To identify the kinases directly responsible for the insulin stimulation of SOS phosphorylation in vivo, we initially expressed the MEK dominant-interfering mutant, in which lysine 97 was replaced with arginine (MEK/K97R), in CHO/IR cells (Fig. 1). In cells transfected with the empty vector, insulin stimulated a decrease in SOS electrophoretic mobility characteristic of serine/threonine phosphorylation (Fig. 1A, lanes 4 to 6). However, expression of MEK/K97R inhibited the insulin-stimulated shift in SOS (lanes 1 to 3). Consistent with MEK functioning as the upstream activator of ERK, MEK/K97R expression also inhibited the insulin stimulation of ERK phosphorylation (Fig. 1B, lanes 1 to 3) compared with that in cells transfected with the empty vector (lanes 4 to 6). The partial inhibition of ERK phosphorylation (lane 2) following expression of MEK/K97R correlated with the partial inhibition of SOS phosphorylation (Fig. 1A, lanes 2 and 3). Consistent with results of previous studies (48), the insulin stimulation of ERK

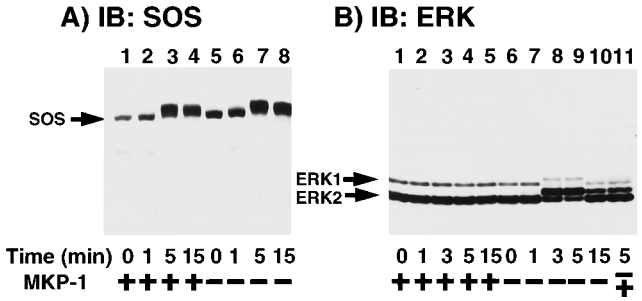

FIG. 2. Expression of MKP-1 inhibits the insulin stimulation of ERK phosphorylation but does not affect SOS phosphorylation. CHO/IR cells were transfected with the empty vector or the mammalian expression vector encoding MKP-1. At 30 h following transfection, the cells were serum starved for 3 h and then untreated or stimulated with 100 nM insulin for 1, 3, 5, or 15 min as indicated. Whole-cell lysates were prepared and subjected to SOS immunoblotting (A) or ERK immunoblotting (B). One-half of the cell extracts used in lane 4 and lane 9 (5 min) of panel B were mixed and incubated on ice for an additional 30 min prior to the addition of Laemmli sample buffer and subjected to ERK immunoblotting (lane 11).

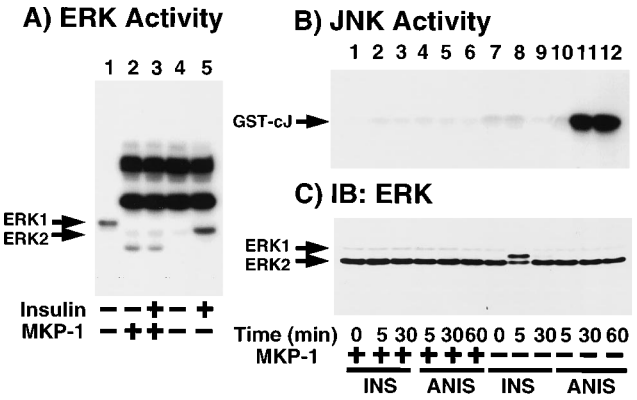

FIG. 3. Insulin is a specific activator of ERK and does not stimulate JNK activity. CHO/IR cells were transfected with the empty vector or with MKP-1 as described in the legend to Fig. 2. The cells were untreated or stimulated with 100 nM insulin (INS) or 50 μg of anisomycin (ANIS) per ml for 5 or 30 min as indicated. Whole-cell extracts were subjected to an ERK in-gel renaturation kinase assay (A), c-Jun kinase (JNK) assay (B), and ERK immunoblot (C) as described in Materials and Methods. As a positive control for the ERK in-gel kinase assay, a partially purified brain preparation of ERK1 was run as a standard (A, lane 1).

phosphorylation was transient and maximal at 5 min, whereas SOS phosphorylation was significantly more persistent.

Since expression of the dominant-interfering MEK/K97R mutant functions by complexing with and inhibiting its upstream activator Raf or its downstream substrate ERK, we also expressed a constitutively active MEK mutant, in which serines 218 and 222 were replaced with glutamic acid (MEK/EE). As controls, cells transfected with the empty vector displayed the typical insulin-stimulated phosphorylation of SOS (Fig. 1C, lanes 4 to 6) and ERK (Fig. 1D, lanes 4 to 6). In contrast, expression of MEK/EE resulted in the constitutive phosphorylation of SOS (Fig. 1C, lanes 1 to 3). Although the active MEK mutant also resulted in the constitutive phosphorylation of ERK, insulin was still capable of increasing ERK phosphorylation, albeit to a small extent (Fig. 1D, lanes 1 to 3). Taken together, these data indicate that SOS phosphorylation was dependent upon a functional MEK activity.

Expression of MKP-1 inhibits insulin-stimulated ERK phosphorylation and ERK activity. The MEK dual-function protein kinase phosphorylates ERK on threonine and tyrosine residues in a TEY motif that is essential for ERK activation (4, 6, 13, 30). Recently, several dual-function protein phosphatases which specifically dephosphorylate both of these residues were identified (1, 38, 46). As observed in Fig. 1, insulin stimulation of cells transfected with an empty vector resulted in a time-dependent phosphorylation of SOS (Fig. 2A, lanes 5 to 8) and transient phosphorylation of ERK (Fig. 2B, lanes 6 to 10). Expression of the myc epitope-tagged MKP-1 completely prevented the insulin-stimulated ERK phosphorylation (Fig. 2B, lanes 1 to 5). Surprisingly, the MKP-1 blockade of ERK phosphorylation had no significant effect on the insulin-stimulated decrease in SOS electrophoretic mobility (Fig. 2A, lanes 1 to 4). Since it was possible that the MKP-1 inhibition of ERK phosphorylation occurred after cell lysis, we also mixed the cell extract from MKP-1-expressing cells (lane 4) with the cell extract from insulin-stimulated mock-transfected cells (lane 9) prior to SDS-polyacrylamide gel electrophoresis (Fig. 2B, lane 11). The presence of both the phosphorylated and nonphosphorylated ERK isoforms indicates that MKP-1 was not active in the extraction buffer and therefore did not dephosphorylate ERK after cell lysis. In addition, immunoblotting of the expressed myc epitope tag demonstrated identical levels of MKP-1 protein expression (data not shown).

These results indicate that MKP-1 expression maintained ERK in an unphosphorylated state. To verify that the unshifted and apparently unphosphorylated ERK (in the presence of MKP-1) was indeed functionally inactive, we performed an in-gel kinase assay (Fig. 3A). As a control, partially purified bovine brain ERK1 was resolved on an SDS-polyacrylamide gel and subjected to the denaturation and renaturation protocol. Incubation of the gel with [γ-^{32}P]ATP demonstrated the presence of a phosphorylated 44-kDa protein (Fig. 3A, lane 1). In parallel, extracts from cells transfected with the empty vector possessed an insulin-stimulated ERK2 activity indicated by the phosphorylation of myelin basic protein at 42 kDa (lanes 4 and 5). In contrast, extracts from cells expressing MKP-1 completely lacked any detectable insulin-stimulated ERK activity (lanes 2 and 3).

In addition to ERK, recent studies have identified a highly related MAP kinase termed JNK (14, 19, 26). JNK has a similar substrate specificity to ERK and is also activated by both threonine and tyrosine phosphorylation on a TPY consensus motif. Although MKP-1 has been reported to dephosphorylate JNK, ERK appears to be a significantly better substrate than JNK (28, 39). Therefore, it remained possible that insulin stimulation of JNK activity was responsible for SOS phosphorylation. To address this issue, JNK activity was determined by using the amino-terminal domain of c-Jun as a GST fusion protein (Fig. 3B). Insulin treatment of mock-transfected cells (Fig. 3B, lanes 7 to 9) caused no detectable JNK activity, even though insulin did stimulate ERK phosphorylation in the identical cell extracts (Fig. 3C, lanes 7 to 9). Since insulin did not activate JNK activity, insulin treatment of MKP-1-expressing cells also did not have any effect on JNK activation (Fig. 3B, lanes 1 to 3) whereas ERK phosphorylation was completely prevented (Fig. 3C, lanes 1 to 3). As a control for the JNK assay, anisomycin was used as a potent stimulator of JNK activity (Fig. 3B, lanes 10 to 12). However, anisomycin was ineffective in stimulating ERK phosphorylation (Fig. 3C, lanes 10 to 12). Under our experimental conditions, the anisomycin stimulation of JNK activity was also completely blocked by expression of MKP-1 (Fig. 3B, lanes 4 to 6). Thus, these data demonstrate that insulin was not an activator of JNK and that JNK activation does not account for the insulin stimulation of SOS phosphorylation.

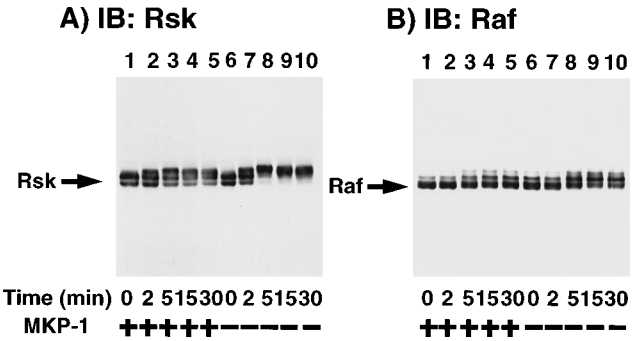

FIG. 4. Expression of MKP-1 inhibits the insulin-stimulated phosphorylation of Rsk and Raf. CHO/IR cells were transfected with the empty vector (lanes 6 to 10) or the mammalian expression vector encoding MKP-1 (lanes 1 to 5) as described in the legend to Fig. 2. The cells were then untreated (lanes 1 and 6) or stimulated with 100 nM insulin for 2 (lanes 2 and 7), 5 (lanes 3 and 8), 15 (lanes 4 and 9), or 30 (lanes 5 and 10) min as indicated. Whole-cell lysates were prepared and subjected to Rsk immunoblotting (IB) (A) or Raf immunoblotting (B).

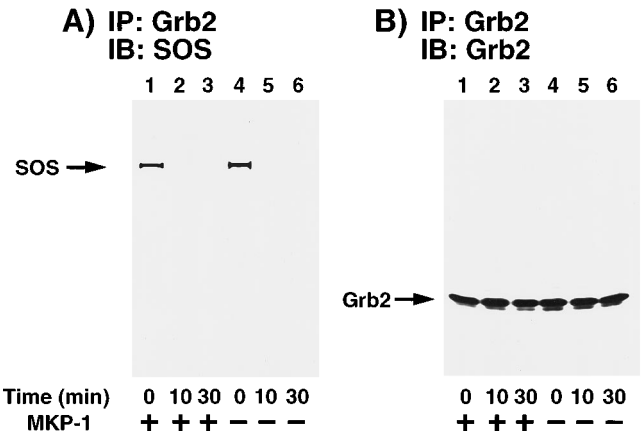

FIG. 5. The insulin-stimulated but ERK-independent phosphorylation of SOS results in a dissociation of the Grb2-SOS complex. CHO/IR cells were transfected with the empty vector (lanes 4 to 6) or the mammalian expression vector encoding MKP-1 (lanes 1 to 3) as described in the legend to Fig. 2. The cells were then untreated (lanes 1 and 4) or stimulated with 100 nM insulin for 10 (lanes 2 and 5) or 30 (lanes 3 and 6) min as indicated. Whole-cell lysates were prepared and immunoprecipitated with a Grb2 antibody. The resultant immunoprecipitates (IP) were then immunoblotted (IB) for SOS (A) or Grb2 (B).

MKP-1 inhibits ERK activity in vivo. The above analysis clearly established that ERK isolated from insulin-stimulated MKP-1-expressing cells was completely inactive. However, these data did not demonstrate that ERK was functionally inactive in the intact cells. To address this issue, we examined the effect of MKP-1 expression on the phosphorylation of the proximal ERK substrate $p90^{rsk}$ (Fig. 4). Insulin stimulation of mock-transfected cells resulted in a time-dependent reduction in SDS-polyacrylamide gel electrophoretic mobility of Rsk characteristic of serine/threonine phosphorylation (Fig. 4A, lanes 6 to 10). Expression of MKP-1 had no effect on the basal-state phosphorylation of Rsk but substantially inhibited the insulin-stimulated Rsk phosphorylation (lanes 1 to 5). In addition to Rsk, Raf lies in a feedback pathway in which the downstream kinase ERK phosphorylates Raf and inactivates Raf protein kinase activity (2, 41). As observed for Rsk, insulin stimulated the phosphorylation of Raf (Fig. 4B, lanes 6 to 10), which was inhibited by expression of MKP-1 (lanes 1 to 5). These data firmly establish that expression of MKP-1 prevented the insulin activation of ERK and phosphorylation of ERK-dependent substrates in intact cells.

Functional consequences of the ERK-independent SOS phosphorylation. It has been previously reported that the insulin stimulation of SOS phosphorylation correlated with a dissociation of the Grb2-SOS complex (11, 49). We therefore determined whether the ERK-independent phosphorylation of SOS was a functional phosphorylation event (Fig. 5A). In the absence of insulin treatment, Grb2 immunoprecipitations of cell extracts from mock-transfected cells resulted in the coimmunoprecipitation of SOS (Fig. 5A, lane 4). Following 10 or 30 min of insulin stimulation, there was a marked reduction in the amount of SOS associated with Grb2 (lanes 5 and 6). The time dependence and extent of insulin-stimulated dissociation of the Grb2-SOS complex were essentially identical in cells expressing MKP-1 (lanes 1 to 3). As controls for the efficiency of Grb2 immunoprecipitation, a Grb2 immunoblot of the identical Grb2 immunoprecipitates demonstrated identical levels of Grb2 protein under all these conditions (Fig. 5B). These data demonstrate that the insulin-stimulated ERK-independent phosphorylation of SOS had the same functional consequence as SOS phosphorylation in the absence of MKP-1 expression.

Partial purification of the insulin-stimulated but ERK-independent SOS-phosphorylating activity. Having established that SOS phosphorylation was MEK dependent but ERK independent, we designed experiments to further characterize the SOS phosphorylation activity (Fig. 6). Whole-cell extracts from insulin-stimulated cells demonstrated a marked stimulation of phosphorylation of purified baculovirus-expressed Glu-Glu-tagged hSOS protein (EE-SOS) in vitro (Fig. 6A, lanes 1 and 2). Similarly, whole-cell extracts from MKP-1-expressing cells also displayed a marked insulin stimulation of SOS phosphorylation (lanes 3 and 4). To ensure that ERK was not contributing to this activity, an ERK immunoblot of the same extracts demonstrated a complete inhibition of ERK phosphorylation in cells expressing MKP-1 (Fig. 6B, lanes 3 and 4) compared with the extracts from mock-transfected cells (lanes 1 and 2).

On the basis of recapitulation of the insulin-stimulated MKP-1-independent SOS-phosphorylating activity in vitro, the cell extracts were applied to a fast phase liquid chromatography MonoQ column and the resultant fractions were assayed for SOS phosphorylating activity (Fig. 7). Extracts from insulin-stimulated cells displayed two major peaks of SOS-phos-

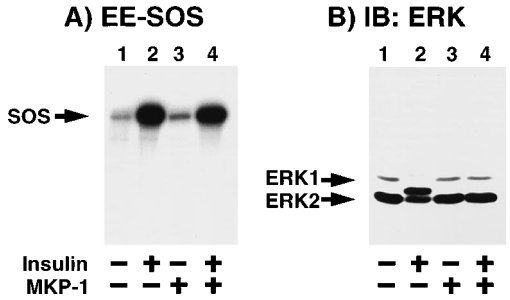

FIG. 6. The insulin-activated ERK-independent phosphorylation of SOS is recapitulated in whole-cell extracts. (A) CHO/IR cells were transfected with the empty vector (lanes 1 and 2) or the mammalian expression vector encoding MKP-1 (lanes 3 and 4) as described in the legend to Fig. 2. The cells were then incubated without (lanes 1 and 3) or with (lanes 2 and 4) 100 nM insulin for 5 min as indicated. Whole-cell lysates were prepared and incubated with 1 μg of baculovirus-purified EE-SOS followed by the addition of 5 μCi of [γ-^{32}P]ATP for 30 min as described in Materials and Methods. The samples were then subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. (B) The identical extracts used in panel A were subjected to ERK immunoblotting (IB).

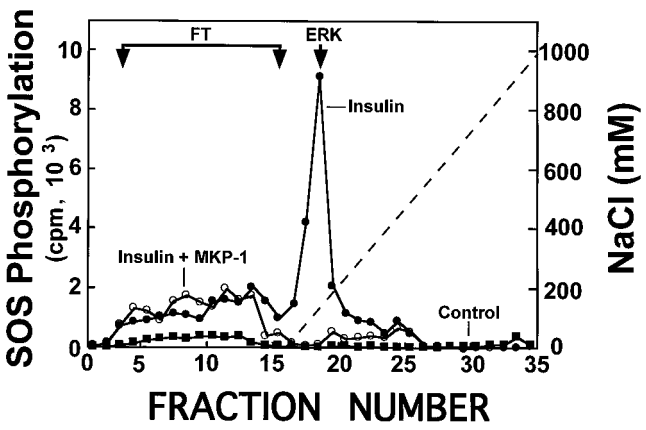

FIG. 7. Physical separation of the insulin-stimulated ERK activity from the ERK-independent SOS-phosphorylating activity. CHO/IR cells were transfected with the empty vector (solid squares and solid circles) or the mammalian expression vector encoding for the MAP kinase phosphatase, MKP-1 (open circles). At 30 h following transfection, the vector-transfected cells were left untreated (solid square) or stimulated with 100 nM insulin (solid circles) for 5 min. The MKP-1-expressing cells were also stimulated with 100 nM insulin (open circles) for 5 min. Whole-cell lysates were prepared and applied to a MonoQ column as described in Materials and Methods. The resultant fractions were assayed for SOS-phosphorylating activity. The dotted line indicates the 0 to 1,000 mM NaCl gradient applied to the MonoQ column starting at fraction 15. FT, flowthrough fractions.

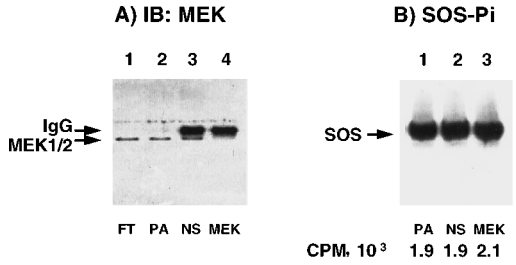

FIG. 8. MEK activity does not account for the SOS-phosphorylating activity in the MonoQ flowthrough fractions. The MonoQ flowthrough fractions from insulin-stimulated cell extracts (fractions 4 to 12), containing equivalent amounts of MEK protein as determined by MEK immunoblotting (IB), were pooled. These fractions were incubated overnight at 4°C in the absence (FT) or the presence of protein A-Sepharose (PA), nonspecific rabbit antibody (NS), or specific anti-MEK antibody (MEK) plus protein A-Sepharose as described in Materials and Methods. The samples were then cleared by centrifugation and the supernatants were immunoblotted for MEK (A) and assayed, in parallel, for SOS-phosphorylating activity (B). The numbers below the phosphorylated SOS protein in panel B are the counts per minute incorporated determined by scintillation counting of the excised SOS protein bands.

phorylating activity (solid circles). The sharp peak of activity eluting at approximately 200 mM NaCl (fractions 16 to 20) contains activated ERK1/2 proteins as detected by immunoblotting (data not shown). Consistent with previous studies (15), we did not detect the ERK proteins in any of the other fractions. The other region of SOS-phosphorylating activity was a broad peak which did not bind to the MonoQ column but which was present in the flowthrough fractions (fractions 3 to 13). As expected, extracts from unstimulated cells had relatively low levels of SOS-phosphorylating activity in all the column fractions. Importantly, extracts from insulin-stimulated cells expressing MKP-1 also displayed essentially no SOS-phosphorylating activity in fractions 16 to 20, which contained the inactive ERK1/2 proteins as detected by immunoblotting (data not shown). However, the SOS-phosphorylating activity in the flowthrough was completely unaffected by the expression of MKP-1. These data further document that the insulin-stimulated SOS-phosphorylating activity was physically distinct from the ERK proteins.

MEK is not the insulin-stimulated SOS-phosphorylating activity. As previously reported (15), ERK is not present in the MonoQ flowthrough fractions; however, immunoblotting with a MEK antibody demonstrated the presence of the MEK proteins in these fractions. Therefore, to assess the potential contribution of MEK as an SOS-phosphorylating activity, we next determined the amount of SOS phosphorylating activity in the MonoQ flowthrough fractions that were immunodepleted of MEK protein (Fig. 8). Immunoblotting of the MonoQ flowthrough from insulin-stimulated cell extracts demonstrated the presence of the MEK proteins, which were not reduced by incubation with protein A-Sepharose (Fig. 8A, lanes 1 and 2). As a control for nonspecific immunoprecipitation, incubation with a nonimmune rabbit antibody followed by incubation with protein A-Sepharose also did not reduce the amount of MEK protein remaining in the supernatant (lane 3). The band immediately above the MEK protein represents the immunoglobulin heavy chain from the initial precipitating antibody. In contrast, incubation with the MEK specific antibody followed by protein A-Sepharose resulted in a substantial reduction in the amount of MEK protein in the supernatant (lane 4). In comparison, the amount of SOS-phosphorylating activity was essentially unaffected in the protein A-Sepharose, nonspecific or MEK-specific immunodepleted supernatants (Fig. 8B). The near complete immunodepletion of MEK protein from the MonoQ flowthrough fractions without any effect on SOS phosphorylation directly demonstrated that MEK was not the kinase responsible for SOS phosphorylation.

DISCUSSION

Ras functions as an important molecular switch converting upstream tyrosine kinase signaling events into downstream activation of serine/threonine protein kinase activities. Since Ras itself has low intrinsic GTP exchange and GTPase activities, the GTP-GDP cycle is determined by the dynamic equilibrium between SOS and RasGAP function. Recent studies have indicated that tyrosine kinase receptor activation results from a targeting of SOS to the plasma membrane location of Ras, thereby catalyzing the exchange of GTP for GDP (3, 21, 34). In addition, several studies have suggested that the carboxyl-terminal region of SOS may function as an autoinhibitory domain which may be derepressed by the binding of Grb2 (24, 31, 45). Once in the GTP-bound active state, the Ras effector domain associates with and via an undefined mechanism and activates the Raf family members of serine/threonine kinases, resulting in the activation of a kinase cascade (Fig. 9). In this model, ERK activation serves to modulate the activity of various downstream effectors but also functions in a feedback pathway mediating Raf phosphorylation and inactivation (2, 41). In a similar manner, activation of the Ras/Raf/MEK/ERK cascade by various growth factors, serum, v-Ras, v-Raf, and phorbol esters has also been observed to phosphorylate SOS on serine/threonine residues (11, 12, 48, 49).

Although a precise functional role for SOS phosphorylation has not been established, in the case of insulin, this phosphorylation event correlated with a dissociation of the Grb2-SOS complex and the inactivation phase of Ras (11, 48, 49). In this regard, the SOS carboxyl-terminal domain contains several MAP kinase consensus phosphorylation sites in the same proline-rich region responsible for binding to the Grb2 SH3 domains. In addition, overexpression of ERK resulted in an in-

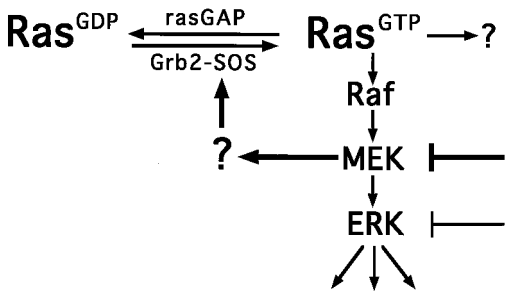

FIG. 9. Schematic model representing a proposed mechanism for the MEK-dependent phosphorylation of SOS. The relative activation state of Ras (GTP versus GDP bound) is determined by the activities of RasGAP and SOS. SOS-mediated exchange of Ras GDP for GTP results in the activation of Raf. Activated Raf phosphorylates and stimulates MEK. In turn, MEK phosphorylates ERK, resulting in the activation of ERK and subsequent downstream phosphorylation events. Blockade of ERK activity has no effect on SOS phosphorylation, whereas inhibition of MEK activity prevents SOS phosphorylation through an as yet unidentified kinase.

sulin-stimulated hyperphosphorylation of SOS and purified activated ERK was capable of phosphorylating SOS in vitro (12, 41, 49). Taken together, these data suggest that ERK may account for cellular SOS phosphorylation activity and perhaps for the dissociation of the Grb2-SOS complex.

However, in these studies, either high levels of in vivo ERK expression or in vitro assays were used to identify ERK as a potential SOS kinase. We therefore undertook an approach to inhibit the activities of endogenous kinases and determine the subsequent effect on SOS phosphorylation and dissociation of the Grb2-SOS complex. On the basis of our previous studies demonstrating stimulation of SOS phosphorylation and dissociation of the Grb2-SOS complex by v-Raf expression (49), we anticipated that the functional kinases involved were at the level of a MAP kinase kinase kinase. As expected, expression of a dominant-interfering MEK mutant, which is thought to function by competing for Raf activity, was consistent with a Raf dependence of insulin-stimulated SOS phosphorylation. Furthermore, expression of a constitutively active form of MEK resulted in the persistent phosphorylation of both ERK and SOS. These results are consistent with recent studies with a specific allosteric inhibitor of MEK activation and further support an insulin-stimulated MEK-dependent SOS phosphorylation mechanism (48).

Surprisingly, however, blockade of ERK activity with MKP-1 had no significant effect on either insulin stimulation of SOS phosphorylation or dissociation of the Grb2-SOS complex. This was not due to incomplete inhibition of ERK function, as MKP-1 abolished insulin-stimulated ERK phosphorylation, protein kinase activity, and in vivo phosphorylation of two ERK-dependent substrates. Thus, these data strongly indicated the presence of an insulin-stimulated SOS-phosphorylating activity that was MEK dependent but ERK independent. A distinct but related ERK family member of MAP kinases is the c-Jun kinase, JNK (14, 19, 26). Thus, it was possible that insulin activated JNK through a MEK-dependent mechanism which in turn was responsible for SOS phosphorylation. However, JNK contains an activation motif (TPY) which is distinct from that in ERK (TEY), and we were unable to detect any significant insulin stimulation of JNK activity. Furthermore, MKP-1 was an effective inhibitor of anisomycin-stimulated JNK activity. Thus, neither ERK nor JNK can fully account for the insulin stimulation of SOS phosphorylation.

Interestingly, the insulin-stimulated ERK-independent SOS-phosphorylating activity was not retained by a MonoQ anion-exchange column whereas ERK was specifically bound, further documenting a distinct physical separation of ERK from this activity. Although MEK does not bind to MonoQ and appears in the flowthrough fractions (15), it is unlikely that MEK directly phosphorylates SOS. MEK is a highly specific protein kinase requiring the presence of a TEY motif which is not present in SOS. Furthermore, MEK is a dual-function kinase which phosphorylates ERK in an ordered reaction utilizing the tyrosine phosphoacceptor site prior to threonine (18). In this regard, phosphoamino acid analysis and phosphotyrosine immunoblotting have not detected the presence of any phosphotyrosine in SOS (reference 12 and data not shown). More importantly, immunodepletion of MEK from the MonoQ flowthrough fractions had no effect on the SOS-phosphorylating activity. On the basis of these findings, we hypothesize that a specific SOS kinase functions downstream of MEK and is probably responsible for the observed insulin-stimulated dissociation of the Grb2-SOS complex. Thus, the identification and characterization of this potentially novel insulin-stimulated kinase is an important next step to determine the physiological role of SOS phosphorylation in regulating Ras desensitization.

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