Insulin-induced Desensitization of Extracellular Signal-regulated Kinase Activation Results from an Inhibition of Raf Activity Independent of Ras Activation and Dissociation of the Grb2-SOS Complex*

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Previous studies have suggested that the interaction between the small adaptor protein Grb2 with the Ras guanyl nucleotide exchange factor SOS is functionally important in the regulation of the Ras activation/inactivation cycle. To examine the relationship between the Grb2-SOS complex and Ras activation, we observed that insulin stimulation results in a rapid but transient activation of Ras and the extracellular-signal regulated kinase (ERK) followed by dissociation of the Grb2-SOS complex. Although treatment with the phorbol myristate acetate resulted in ERK activation and complete dissociation of the Grb2-SOS complex, there was no effect on subsequent insulin-stimulated Ras activation. Similarly, insulin stimulation followed by insulin removal resulted in a time-dependent restoration of the Grb2-SOS complex but which was significantly slower than the recovery of insulin-stimulated Ras activation. In addition, although insulin was able to activate Ras under these conditions, there was a complete desensitization of Raf and ERK activation. This apparent homologous desensitization of insulin action was specific for Raf and ERK in the insulin re-stimulation of insulin receptor autophosphorylation and protein kinase B activation were unaffected. Together, these data demonstrate the presence of a pathway independent of the Grb2-SOS complex that can lead to Ras activation but that the desensitization of Raf accounts for the homologous desensitization of ERK.

Over the past several years substantial progress has been made in identifying and characterizing signal transduction pathways linking receptor tyrosine kinase activation to nuclear transcriptional activation events (1, 2). One such pathway that has received considerable attention is the Ras/Raf/MEK1/ERK transcriptional activation events (1, 2). One such pathway that has received considerable attention is the Ras/Raf/MEK1/ERK pathway Ras functions as a molecular switch converting tyrosine kinase signals into a serine/threonine kinase cascade. In the case of insulin, activation of the insulin receptor tyrosine kinase results in the tyrosine phosphorylation of the Shc and IRS families of intracellular substrates (3, 4). The phosphorylation of these proteins generates specific recognition sites for the src homology 2 (SH2) domain of the 25-kDa adaptor protein Grb2 (5–7). In addition to a central SH2 domain, Grb2 also contains two SH3 domains that direct its association with the Ras guanyl nucleotide exchange factor SOS (8–11). The formation of the Shc-Grb2-SOS and IRS-Grb2-SOS ternary complexes are thought to result in the conversion of the inactive GDP-bound Ras to the active GTP-bound state (12–16). Once in the activated GTP-bound state, Ras associates with members of the Raf family of serine/threonine kinases (17, 18). In turn, activated Raf functions as an upstream kinase for the dual specificity kinase MEK which phosphorylates and stimulates ERK activity providing an important bifurcation point for the regulation of metabolic, transcriptional, and mitogenic events (19, 20). Thus, in this pathway Ras functions as a molecular switch converting tyrosine kinase signals into a serine/threonine kinase cascade.

Although this potential mechanism for Ras activation is quite appealing, there are several lines of evidence suggesting that the Grb2-SOS complex may not necessarily be required for Ras activation. In the case of R7 cell development in Drosophila, expression of a carboxyl-terminal SOS deletion, which cannot bind the Grb2 homologue Drk, is sufficient to rescue the SOS null phenotype (21). Similarly, constitutive expression of SOS can induce downstream signaling and cellular transformation in the absence of Grb2 binding (22–25). In contrast, there is also substantial evidence supporting a positive functional role for the Grb2-SOS complex; for example, point mutations in the Grb2 SH3 domain which prevent binding to SOS impairs vulval development in Caenorhabditis elegans (26). In addition, Ras inactivation directly correlates with the dissociation of the Grb2-SOS complex, and endothelin activation/inactivation of Ras in mammalian cells was temporally dependent upon the association state of the Grb2-SOS complex (27–31).

Based upon these data, we have examined the relationship between the Grb2-SOS association state directly with that of Ras activation and downstream signaling events. In this study we have observed that PMA pretreatment induces a complete dissociation of the Grb2-SOS complex without any significant activation of Ras. Despite the loss of the Grb2-SOS complex, insulin remains fully competent in the conversion of Ras from the inactive GDP to the GTP-bound state. Similarly, uncoupling of the Grb2-SOS complex by acute insulin stimulation only partially reduces the activation of Ras by a second round of acute insulin stimulation. In contrast, the homologous desensitization of ERK activation parallels the inability of insulin to re-activate Raf. Together, these data demonstrate that the
Grb2-SOS complex is not absolutely essential for the activation of Ras, which likely occurs through alternative mechanisms.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Chinese hamster ovary cells expressing the human insulin receptor (CHO/IR) were maintained and cultured in minimum Eagle’s medium-α supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 units/ml streptomycin plus 20 μg/ml glutamine as described previously (32). The cells were incubated in serum-free medium at 37 °C for 6 h prior to hormone addition as indicated in the figure legends. In some cases following initial insulin treatment, the cells were rapidly washed in a low pH buffer (5 mM sodium acetate, 150 mM NaCl, pH 5.0) to remove the extracellular and cell surface-bound insulin. The cells were then allowed to recover in serum-free media for various periods followed by a second round of hormone stimulation as described in the individual figure legends. The reactions were stopped by rapid aspiration of the medium and snap freezing the cells with liquid nitrogen. The frozen cells were stored at −80 °C until use.

**Preparation of Whole Cell Extracts and Immunoprecipitation—**Whole cell detergent lysates were prepared by thawing cells in a lysis buffer (50 mM Hepes, pH 7.5, 1% Triton X-100, 2.5 mM EDTA, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 2 mM pepstatin, 0.5 μg/ml inhibitory units of aprotinin, and 10 μg/ml leupeptin) for 30 min at 4 °C. Insoluble material was removed by centrifugation at 12,000 × g for 15 min at 4 °C, and the total protein in the supernatant was determined by the method of Bradford (33). Cell lysates were incubated with a polyclonal Grb2 antibody (Santa Cruz Biotechnology), and immune complexes were precipitated by incubation with protein A-agarose (Santa Cruz Biotechnology) as described previously (32). Samples for immunoblotting 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% (v/v) SDS, 100 mM dithiothreitol, 0.1% (w/v) bromphenol blue), and heated at 100 °C for 5 min.

**Immunoblotting—**Whole cell detergent extracts, GST-RBD affinity precipitates, and antibody immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis and proteins transferred to polyvinylidene difluoride membranes. The samples were immunoblotted with either a monoclonal Grb2 antibody (Transduction Laboratories), monoclonal ERK (Zymed Laboratories Inc.), polyclonal phospho-Akt (New England Biolabs), monoclonal phosphotyrosine (PY20, Santa Cruz Biotechnology), polyclonal Ras (Transduction Laboratories), or monoclonal SOS (Transduction Laboratories) antibodies. The primary monoclonal and polyclonal antibodies (except PY20-HRP) were detected with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Bio-Rad) and visualized by the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

**Ras Activation Assay—**This assay is based on the fact that only activated Ras (Ras-GTP) is capable of binding to Raf-1 Ras binding domain. Therefore, associated Ras proteins reflect the fraction of activated Ras molecules. A CDNA encoding for a glutathione transferase (GST) Raf-1 binding domain (RBD) fusion protein (GST-RBD) was kindly provided by Dr. David Shalloway (Cornell University, Ithaca, NY). This fusion protein was used to precipitate specifically according to the method of Bradford (34). Briefly, the GST-RBD bound to glutathione-Sepharose beads (20 μg, Amersham Pharmacia Biotech) was incubated with 2 μg of whole cell detergent lysates for 1 h at 4 °C. The Sepharose beads were pelleted by centrifugation and washed three times with lysis buffer followed by solubilization and resuspension in Laemmli sample buffer and separated on a 12% SDS-polyacrylamide electrophoresis gel.

**Raf Kinase Assay—**Raf protein kinase activity was determined by an immunocomplex protein kinase cascade assay using exogenous substrates according to the manufacturer’s specifications (Upstate Biotechnology Inc.). Briefly, clarified supernatants were incubated with an anti-Raf antibody (Upstate Biotechnology Inc.) and protein G-Sepharose (Santa Cruz Biotechnology), or protein G-Sepharose alone, for 1 h at 4 °C. Samples were washed three times in lysis buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.5 mM sodium vanadate, 0.1% 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 2 mM pepstatin, 0.5 μg/ml inhibitory units of aprotinin, and 10 μg/ml leupeptin) and then with kinase dilution buffer (20 mM MOPS, pH 7.2, 2-glycerol phosphate, 5 mM EGTA, 1 mM sodium vanadate, 1 mM dithiothreitol). The washed precipitates were incubated with inactive fusion proteins GST-MEK1 (0.4 μg) and GST-p42 mitogen-activated protein kinase (0.1 μg), 150 μM ATP, and 20 μM magnesium chloride, at room temperature with gentle agitation, after which a portion of each sample was incubated with 20 μg of myelin basic protein and 10 μCi of [γ-32P]ATP (3000 Ci/mmol, Amersham Pharmacia Biotech) for 10 min at room temperature with gentle agitation. Samples were transferred onto p81 phosphocellulose squares (Upstate Biotechnology Inc.), washed 3 times in 0.75% phosphoric acid, twice with acetone, and quantitated by liquid scintillation counting.

**RESULTS**

**Insulin and PMA Induce Dissociation of the Grb2-SOS Complex—**Previous studies have observed that agents that activate the Ras/Raf/MEK/ERK pathway can also induce the serine/threonine phosphorylation of SOS and dissociation of the Grb2-SOS complex (29, 35, 36). The dissociation of Grb2 from SOS has been postulated to be one mechanism that may be responsible for the transient nature of Ras activation and for the homologous desensitization of Ras activation by insulin (27, 28, 31). To compare the effects of heterologous stimulation on SOS phosphorylation and its association with Grb2, we incubated CHO/IR cells with 100 nM insulin or 100 nM PMA for various times (Fig. 1). Consistent with previous findings (27, 28, 31, 37), 5 min of insulin stimulation resulted in a characteristic mobility shift of SOS that is indicative of its serine/threonine phosphorylation (Fig. 1A, lanes 1 and 2). Furthermore, following 5 min of insulin stimulation there was a marked reduction in the amount of SOS that was co-immunoprecipitated with Grb2 (Fig. 1B, lanes 1 and 2). Similar to insulin, PMA treatment resulted in a time-dependent decrease in SOS mobility (Fig. 1A, lanes 3–7) that correlated with the time-dependent decrease in the amount of SOS that was co-immunoprecipitated with Grb2 (Fig. 1B, lanes 3–7). The addition of insulin for 5 min following PMA pretreatment had no further effect on SOS mobility or on the co-immunoprecipitation of SOS with Grb2 (Fig. 1, A and B, lanes 8–10). As a control, the immunoprecipitates were also subjected to Grb2 immunoblotting to confirm equal amounts of Grb2 were immunoprecipitated under these conditions (Fig. 1B, bottom panel). In addition, insulin and PMA stimulation also resulted in the activation of ERK as detected by its decrease in electrophoretic mobility charac-

**FIG. 1.** Insulin and PMA induces the dissociation of the Grb2-SOS complex and increases the phosphorylation of SOS and ERK. CHO/IR cells were either left untreated (lane 1) or incubated with 100 nM insulin for 5 (lane 2) or 100 nM PMA for 5 (lane 3), 15 (lane 4), 30 (lane 5), 60 (lane 6), and 90 (lane 7) min at 37 °C. Following PMA pretreatment for 30 (lane 8), 60 (lane 9), and 90 (lane 10) min at 37 °C, the cells were subsequently stimulated with 100 nM insulin for 5 min. A, whole cell detergent lysates were prepared and subjected to immunoblotting using an SOS (top) and an ERK (bottom) antibody as described under “Experimental Procedures.” B, the same whole cell detergent lysates were immunoprecipitated with a Grb2 antibody and subjected to immunoblotting with a SOS (top) or a Grb2 antibody (bottom). IB, immunoblot; IP, immunoprecipitate.
tion of Ras activation by insulin. CHO/IR cells were either left untreated (lane 1) or incubated with 100 nM insulin for 3 (lane 2), 5 (lane 3), 15 (lane 4), and 30 (lane 5) min at 37 °C. In parallel, the CHO/IR cells incubated with 100 nM PMA for 3 (lane 6), 5 (lane 7), 15 (lane 8), and 30 (lane 9) min at 37 °C. Following PMA pretreatment for 30 min the cells were subsequently stimulated with 100 nM insulin (lane 10) for 3 min at 37 °C. A, whole cell detergent lysates were prepared and precipitated with the GST-RBD fusion protein. The precipitates were then immunoblotted for Ras as described under “Experimental Procedures.” B, the same whole cell detergent lysates were immunoblotted with an ERK antibody. IB, immunoblot.

FIG. 2. PMA pretreatment does not result in the desensitization of Ras activation by insulin. CHO/IR cells were either left untreated (lane 1) or incubated with 100 nM insulin for 15 min (lane 2), 100 nM PMA for 30 (lanes 3 and 4) or 600 mM sorbitol (Sorb.) for 30 (lanes 5 and 6) min at 37 °C. Following the PMA and sorbitol pretreatments, the cells were subsequently stimulated with 100 nM insulin for 3 min. A, whole cell detergent lysates were prepared and subjected to immunoblotting using an SOS (top) and an ERK (bottom) antibody as described under “Experimental Procedures.” B, the same whole cell detergent lysates were immunoprecipitated with a Grb2 antibody and subjected to immunoblotting with an SOS (top) or a Grb2 antibody (bottom). C, in parallel, CHO/IR cells were either left untreated (lane 1), incubated with 100 nM insulin for 3 min (lane 2), 100 nM PMA for 30 (lanes 3 and 4) or 600 mM sorbitol for 30 (lanes 5 and 6) min at 37 °C. Following the PMA and sorbitol pretreatments, the cells were subsequently stimulated with 100 nM insulin for 3 min at 37 °C (lanes 4 and 6, respectively). Whole cell detergent lysates were prepared and precipitated with GST-RBD fusion protein. The precipitates were then immunoblotted for Ras as described under “Experimental Procedures.” IB, immunoblot; IP, immunoprecipitate.

teristic of threonine and tyrosine phosphorylation (Fig. 1A, bottom panel, lanes 1–7). PMA pretreatment followed by 5 min of insulin stimulation slightly enhanced the activation of ERK relative to PMA alone (Fig. 1A, bottom panel, lanes 8–10). These data indicated that under conditions in which the Grb2-SOS complex was completely dissociated (PMA pretreatment), insulin was still capable of activating ERK.

PMA-induced Dissociation of the Grb2-SOS Complex Does Not Block the Insulin Activation of Ras—Several lines of evidence have implicated the Grb2-SOS complex as a major pathway for the activation of Ras and its downstream effectors (5, 8, 9, 38–41). We therefore directly examined the relationship between the Grb2-SOS complex and Ras activation (Fig. 2). To assess Ras activation, we took advantage of the ability of GTP-bound Ras to associate with a GST fusion protein containing the Ras binding domain of Raf (GST-RBD). This assay has recently been demonstrated to reflect specifically the amount of Ras in the active GTP-bound state (30, 34). As previously observed using [32P]GTP loading assays, insulin stimulation resulted in the characteristic time-dependent Ras activation/inactivation cycle (Fig. 2A, lanes 1–5). Maximal Ras activation occurred 3–5 min in the continuous presence of insulin stimulation and gradually returned toward the GDP-bound inactive state. Consistent with Ras functioning upstream of ERK, the insulin activation of ERK paralleled the transient change in Ras activation (Fig. 2B, lanes 1–5). In contrast, PMA treatment had a marginal effect on Ras activation which did not appear to be time-dependent (Fig. 2A, lanes 6–9). Nevertheless, PMA stimulation resulted in a more persistent activation of Ras consistent with PMA activating the ERK pathway downstream or in parallel to Ras (Fig. 2B, lanes 6–9). Surprisingly, however, 30 min of PMA pretreatment had no effect on the ability of insulin to stimulate Ras activation (Fig. 2A, lane 10). This occurred despite the complete PMA-induced dissociation of the Grb2-SOS complex under these conditions (Fig. 1). Furthermore, insulin stimulation following pretreatment with PMA increased the extent of ERK activation (Fig. 2B, lane 10). Together, these data suggest that insulin can activate Ras independent of the Grb2-SOS association state.

Osmotic Shock but Not PMA Inhibits the Insulin Stimulation of Ras Activation—Similar to PMA, osmotic shock does not activate Ras but is a potent stimulator of ERK and induces the phosphorylation of SOS and dissociation of the Grb2-SOS complex (36). Therefore, we used osmotic shock treatment as an additional mechanism to examine the relationship between the Grb2-SOS complex and Ras activation (Fig. 3). As expected, insulin stimulation resulted in the typical decrease in SOS electrophoretic mobility and activation of ERK (Fig. 3A, lanes 1 and 2). Similarly, stimulation of the cells by both PMA and osmotic shock also resulted in the phosphorylation of SOS and activation of ERK (Fig. 3A, lanes 3–6). Although osmotic shock did induce a broadening and a decrease in SOS mobility, its effect was not as large as that observed by insulin or PMA treatment. Nevertheless, stimulation of the cells with all three stimuli (insulin, PMA, and osmotic shock) induced a dissociation of Grb2 from SOS (Fig. 3B, lanes 1–6). In comparison, insulin stimulation resulted in a marked activation of Ras, whereas PMA and osmotic treatment were without effect (Fig. 3C, lanes 1–3 and 5). As previously observed (Fig. 2), despite the dissociation of the Grb2-SOS complex in the PMA-stimulated cells, subsequent insulin stimulation resulted in the activation of Ras (Fig. 3C, lanes 3 and 4). In contrast, osmotic shock, which also induced the dissociation of the Grb2-SOS complex (Fig. 3B), prevented the subsequent insulin stimulation of Ras activation (Fig. 3C, lanes 5 and 6). These data demonstrate that the heterologous desensitization of Ras activation is agonist-dependent and under certain conditions can appear to directly correlate with the Grb2-SOS association state.

Insulin Stimulation Induces Homologous Desensitization of ERK but Not Ras Activation—It has been previously reported that insulin stimulation results in the homologous desensitization of downstream signaling events (28, 31, 42). To investigate further the desensitization of insulin signaling, we directly investigated the effect of homologous hormone treatment on
SOS phosphorylation, Grb2-SOS association, Ras and ERK activation. To mimic the pulsatile nature of insulin exposure that occurs in vivo, we incubated the cells for 15 min with insulin followed by a rapid acidic wash to remove the free and cell surface-bound insulin and then allowed the cells to recover for various times in serum-free media (Fig. 4). As is apparent, insulin stimulation for up to 15 min resulted in a time-dependent phosphorylation of SOS, activation of ERK, and dissociation of the Grb2-SOS complex (Fig. 4, A and B, lanes 1–5). Removal of insulin at 15 min resulted in a slower time-dependent dephosphorylation of SOS which returned to basal state within 120–240 min (Fig. 4A, lanes 6–11). Since ERK activation is transient even in the continuous presence of insulin, there was a more rapid recovery of ERK back to the basal state. In any case, there was a return of the Grb2-SOS complex that was fully recovered by 240 min and which directly paralleled the dephosphorylation of SOS (Fig. 4B, lanes 6–11).

By having determined the time frame for the recovery of the Grb2-SOS complex following insulin-stimulated dissociation, we next examined the ability of insulin to re-stimulate the dissociation of the Grb2-SOS complex and to re-activate ERK (Fig. 5). In these studies, cells were treated with insulin for up to 10 min; the insulin was removed by a rapid low pH wash, and the cells were recovered for various times followed by a second 3-min insulin stimulation. As expected, insulin stimulation resulted in a time-dependent phosphorylation of SOS and activation of ERK (Fig. 5A, lanes 1–3). Following insulin removal, there was a slow time-dependent dephosphorylation of SOS, with a more rapid inactivation of ERK (Fig. 5A, lanes 4, 6, 8, and 10). However, 30 min subsequent to the insulin washout, a second acute insulin stimulation was unable to induce the phosphorylation of SOS or the activation of ERK (Fig. 5A, lanes 4 and 5). Similarly, 60 min after insulin washout, an acute insulin stimulation still had no effect on either SOS phosphorylation or ERK activation (Fig. 5A, lanes 6 and 7). In contrast, 120 min subsequent to insulin washout there was a partial restoration of acute insulin-stimulated SOS phosphorylation and ERK activation which fully recovered by 240 min following the initial insulin washout (Fig. 5A, lanes 8–11). In parallel, insulin stimulation resulted in the dissociation of the Grb2-SOS complex, which remained dissociated for up to 60 min following insulin washout (Fig. 5C, lanes 1–6). As observed for SOS phosphorylation, 120 min following the insulin washout there was a partial recovery of the Grb2-SOS complex which was fully reassociated by 240 min (Fig. 5C, lanes 7 and 9). At these time points, the recovery of insulin-stimulated SOS phosphorylation directly correlated with the ability of acute insulin stimulation to induce a second round of Grb2-SOS dissociation (Fig. 5C, lanes 8 and 10). These data demonstrate that subsequently re-stimulated by the re-addition of 100 nM insulin for 3 min at 37 °C. Whole cell detergent lysates were then prepared and precipitated with the GST-RBD fusion protein. The precipitates were then immunoblotted for Ras as described under “Experimental Procedures.” C, CHO/IR cells were either left untreated (lane 1) or incubated with 100 nM insulin for 3 (lane 2) or 10 (lane 3) min at 37 °C. Following the 10 min of insulin pretreatment, the cells were washed in a pH 5.0 containing buffer and placed in serum-free media for an additional 30 (lanes 4 and 5), 60 (lanes 6 and 7), 120 (lanes 8 and 9), and 240 (lanes 10 and 11) min at 37 °C. Following the various second incubation times the cells were subsequently re-stimulated by the re-addition of 100 nM insulin for 3 min at 37 °C. Whole cell detergent lysates were then immunoprecipitated with a Grb2 antibody and subjected to immunoblotting with an SOS (top) or a Grb2 antibody (bottom). IB, immunoblot; IP, immunoprecipitate.

**Fig. 4.** The Grb2-SOS complex re-associates in a time-dependent manner following insulin removal. CHO/IR cells were either left untreated (lane 1) or incubated with 100 nM insulin for 1 (lane 2), 3 (lane 3), 5 (lane 4), and 15 (lane 5) min at 37 °C. Following the 15 min of insulin pretreatment, the cells were washed in a pH 5.0 containing buffer and placed in serum-free media for an additional 5 (lane 6), 15 (lane 7), 30 (lane 8), 60 (lane 9), 120 (lane 10), and 240 (lane 11) min at 37 °C. A, whole cell detergent lysates were prepared and subjected to immunoblotting using an SOS (top) and an ERK (bottom) antibody as described under “Experimental Procedures.” B, the same whole cell detergent lysates were immunoprecipitated with a Grb2 antibody and subjected to immunoblotting with an SOS (top) or a Grb2 antibody (bottom). IB, immunoblot; IP, immunoprecipitate.

**Fig. 5.** The re-associated Grb2-SOS complex can undergo a second round of acute insulin-stimulated dissociation. A, CHO/IR cells were either left untreated (lane 1) or incubated with 100 nM insulin for 3 (lane 2) or 10 (lane 3) min at 37 °C. Following the 10 min of insulin pretreatment, the cells were washed in a pH 5.0 containing buffer and placed in serum-free media for an additional 30 (lanes 4 and 5), 60 (lanes 6 and 7), 120 (lanes 8 and 9), and 240 (lanes 10 and 11) min at 37 °C. Following the various second incubation times the cells were subsequently re-stimulated by the re-addition of 100 nM insulin for 3 min at 37 °C. Whole cell detergent lysates were then immunoprecipitated with a Grb2 antibody and subjected to immunoblotting with a SOS (top) or a Grb2 antibody (bottom). IB, immunoblot; IP, immunoprecipitate.
our previous observations, insulin stimulation resulted in a transient activation of Ras with a marked activation at 3 min followed by a decline to near basal levels by 10 min (Fig. 5B, lanes 1–3). There was a small but further slow return of Ras to the GDP-bound state following insulin removal over a 30 to 240-min time period (Fig. 5B, lanes 4, 6, 8, and 10). However, following the 30-min recovery period acute insulin treatment resulted in the re-activation of Ras (Fig. 5B, lanes 4 and 5). The ability of a second insulin stimulation to re-activate Ras was observed at all the time points examined and occurred even in the complete absence of any detectable Grb2-SOS complexes (Fig. 5B, lanes 4–11). Thus, in an analogous fashion to PMA pretreatment, insulin stimulation results in a complete dissociation of the Grb2-SOS complex yet is still capable of activating Ras, albeit to a slightly lesser extent than in naive cells.

**Effect of Continuous Versus Pulsatile Insulin Stimulation on Ras Activation**—Our data demonstrating that insulin pretreatment does not induce homologous desensitization of Ras activation is in disagreement with a recent report suggesting that Ras does display homologous desensitization to insulin (42). However, in this latter study, cells were continuously exposed to insulin during the addition of a second insulin treatment. Thus, to determine if this could account for these apparent differences, we examined the activation of Ras in the continuous presence of insulin (Fig. 6). As previously observed, 3 min of insulin stimulation resulted in a marked activation of Ras and ERK (Fig. 6, A and B, lanes 1 and 2). Even in the continuous presence of insulin, there was a partial recovery of Ras back toward the basal GDP-bound state with a complete dephosphorylation of ERK (Fig. 6, A and B, lanes 3 and 5). Under these conditions, the concentration of insulin in the medium did not significantly change, and the re-addition of 100 nM insulin was unable to induce the reactivation of Ras (Fig. 6 A, lanes 4 and 6). Thus, the apparent homogeneous desensitization of Ras activation only occurs in the continuous presence of insulin but not when insulin is administered in a pulsatile fashion.

**Insulin Does Not Induce Homologous Desensitization of Insulin Receptor Autophosphorylation or PKB Activation**—Since insulin pretreatment resulted in the homologous desensitization of ERK activation but not Ras, we next assessed another downstream target of insulin action (Fig. 7). To ensure that there was no effect on insulin receptor kinase activation, we initially examined insulin receptor β subunit autophosphorylation by phosphotyrosine immunoblotting. Insulin stimulation for 3 min resulted in a dramatic increase in β subunit autophosphorylation which partially declined in the continuous presence of insulin for 10 min (Fig. 7A, lanes 1–3). As was the case for Ras activation, following insulin removal there was a further slow tyrosine dephosphorylation of the β subunit over the 240-min time course (Fig. 7A, lanes 4, 6, 8, and 10). Nevertheless, at each time point a second acute insulin stimulation was fully capable of inducing the full extent of insulin receptor β subunit autophosphorylation (Fig. 7A, lanes 5, 7, 9, and 11). In parallel, we also determined the relative phosphorylation and activation of protein kinase B (PKB) using the serine 473 phospho-specific PKB antibody (Fig. 7B). Insulin stimulation resulted in a time-dependent serine phosphorylation of PKB (Fig. 7B, lanes 1–3). Removal of insulin also resulted in a relatively slow dephosphorylation (Fig. 7B, lanes 4, 6, 8, and 10). Similar to β subunit phosphorylation and Ras activation, a second acute insulin stimulation resulted in the full extent of PKB serine phosphorylation at all the time points examined (Fig. 7B, lanes 5, 7, 9, and 11). Together, these data demonstrate that insulin stimulation does not result in any significant amount of heterologous desensitization to insulin receptor autophosphorylation or PKB phosphorylation. In contrast, insulin does induce a state of homologous desensitization of ERK activation.

**Insulin Induces the Homologous Desensitization of Raf-1 Activation**—By having established that following insulin stimulation, a second bolus of insulin can activate Ras but does not result in ERK phosphorylation suggests the presence of a mechanism(s) that either prevents ERK activation and/or accelerates ERK inactivation. However, previous studies have observed that the induction of the mitogen-activated protein kinase phosphatase responsible for the dephosphorylation of ERK requires substantially longer times than we have currently observed for the insulin-induced desensitization of ERK (43). Thus, we hypothesized that insulin might induce desensitization of a factor upstream of ERK but downstream of Ras. We therefore examined the insulin-induced desensitization of Raf activity (Fig. 8). As expected, insulin stimulation for 3 min
Following the various second incubation times, the cells were subsequently re-stimulated by the re-addition of 100 nM insulin for 3 min at 37 °C. Whole cell detergent lysates were prepared and assayed for Raf-1 protein kinase activity as described under “Experimental Procedures.” Data are fold increase in activity when compared with control cells and represent the mean ± S.E. of the mean from four independent experiments.

resulted in an approximate 5-fold increase Raf-1 protein kinase activity. Thirty min following insulin removal, Raf-1 kinase activity decreased to slightly less than the control basal activity but was refractory to a second acute activation by insulin. However, 120 and 240 min after insulin removal Raf-1 kinase activity recovered back to the control values. More importantly, at these longer times there was a progressive increase in the ability of an acute second insulin stimulation to activate the Raf-1 kinase. These data directly demonstrate that insulin pretreatment induces a state of homologous desensitization of Raf-1 activation which has a refractory period similar to that of ERK.

**DISCUSSION**

It has been well established that insulin stimulation results in the activation of several intracellular signaling cascades including the Ras/Raf/MEK/ERK pathway (44, 45). Although this signaling cascade regulates multiple downstream effectors, it also results in a feedback pathway leading to multi-site serine/threonine phosphorylation of the upstream activator SOS (29, 46–48). In turn, SOS phosphorylation reduces the affinity of its carboxyl-terminal domain for the SH3 domain of Grb2, resulting in a dissociation of the Grb2-SOS complex (29, 37, 49). Since these events parallel the activation/inactivation cycle of Ras, it has been suggested that the feedback dissociation of the Grb2-SOS complex may account for the transient nature of insulin-stimulated Ras activation (27, 28, 31).

However, the specific roles of Grb2 in the regulation of SOS function and the activation/inactivation of Ras by various growth factors are quite controversial. Initial genetic studies in *Drosophila* and *C. elegans* indicated that disruption of the cognate Grb2 homolog function (Drk and Sem-5, respectively) markedly impaired the developmental regulation of Ras signaling (26, 50). Subsequently, Grb2 was found to constitutively associate with SOS and associate with tyrosine-phosphorylated receptors and effectors that were necessary for Ras activation (8–10, 51). Since the membrane localization of SOS was then demonstrated to be sufficient for Ras activation, these data provided a model in which Grb2 functioned as an adaptor protein targeting SOS to the plasma membrane location of Ras (22, 52).

In contrast, more recent studies have suggested that Grb2 may not be necessary for the SOS-dependent activation of Ras. For example, expression of carboxyl-terminal SOS deletions that do not interact with Grb2 are still capable of inducing cellular transformation (23–25). Although the Grb2-binding site was apparently unnecessary, deletions of the amino-terminal domains prevented cellular transformation. Consistent with this observation, expression of the SOS amino-terminal domain functions in a dominant interfering manner with respect to serum and epidermal growth factor signaling and inhibits the ability of SOS to transform cells (24, 53). Similarly, studies in *Drosophila* show that expression of a SOS carboxy-terminal truncation protein promoted R7 cell development in SOS null flies, whereas amino-terminal truncations did not (21). Taken together, these data suggest that Grb2 function is not required for Ras-dependent downstream cellular responsiveness of ERK activation. Furthermore, they support the concept that the SOS amino-terminal region, which contains functional pleckstrin homology and Dbl homology domains, plays a more significant role in the regulation of Ras activation.

On the other hand, several studies have proposed that the Ras/Raf/MEK/ERK feedback dissociation of the Grb2-SOS complex provides a mechanism to terminate Ras activation by inactivating SOS function. This model is supported by the observation that prevention of Grb2-SOS dissociation by use of the MEK inhibitor PD98059 or by expression of a dominant-interfering MEK mutant prolongs the Ras activation state concomitant with an attenuation of SOS phosphorylation and dissociation of the Grb2-SOS complex (27, 31). Similarly, following SOS phosphorylation and dissociation of Grb2 from SOS, endothelin-1 was unable to activate Ras (30). Furthermore, the ability of endothelin-1 to activate Ras directly correlated with a time-dependent dephosphorylation of SOS and reassembly of the Grb2-SOS complex. Despite these data suggesting a potential role for Grb2 in the regulation of Ras inactivation, additional signaling events must also be involved. For example, although epidermal growth factor stimulation also results in a transient activation of Ras, this agonist induces a persistent and in some cases an increase in the amount of Grb2 complexed to SOS (32). Similarly, activation of the T cell antigen receptor fails to induce the dissociation of the Grb2-SOS complex yet also results in a transient activation of Ras (54). Together, these data suggest that multiple mechanisms exist to regulate the Ras activation state and subsequent downstream signaling events that are probably both cell context and agonist-dependent.

Thus, in order to examine further the requirement for the assembled Grb2-SOS complex in regulating Ras function, we compared the effect of Grb2-SOS dissociation on the ability of insulin to stimulate Ras-GTP binding. This was accomplished by initially inducing the dissociation of the Grb2-SOS complex by pretreatment with either PMA or by osmotic shock, two potent activators of MEK and ERK (36, 55). Both these agonists were found to induce the phosphorylation of SOS with essentially a complete dissociation of the Grb2-SOS complex. Although PMA pretreatment did not impair the insulin-stimulated activation of Ras, osmotic shock pretreatment totally abrogated this response. These data directly demonstrate that the uncoupling of the Grb2-SOS complex is strictly correlative rather than causal with respect to heterologous desensitization, at least for insulin-stimulated Ras activation.

Since these data suggest that the heterologous desensitization of Ras activation is dependent upon the initial agonist, we next examined whether Ras undergoes homologous desensitization to insulin action. Previous studies have observed that in the continuous presence of insulin Ras was maximally activated within 1–3 min but subsequently returned to the inactive
GDP-bounded state within 30–60 min (28, 31, 42). Under these conditions, there was persistent SOS phosphorylation and dissociation from Grb2, and re-addition of insulin had no effect on the Ras activation state (42). Thus, these data would suggest that insulin stimulation results in the homologous desensitization of Ras. However, all these studies were performed in the continuous presence of insulin, and under normal physiological conditions insulin levels are only elevated for relatively short periods before returning to resting levels. This pattern of insulin secretion parallels the postprandial absorption and release of glucose, amino acids, and fatty acids into the circulation. Although this approach may mimic the chronic elevation of glucose, amino acids, and fatty acids into the circulation, this does not reflect normal physiologic insulin signaling properties.

To address this issue, we examined the functional properties of a repeated acute insulin treatment on Ras signaling. Our data demonstrate that following acute insulin stimulation and removal, there was a relatively slow time-dependent dephosphorylation of SOS, and reassembly of the Grb2-SOS complex. Surprisingly, however, a second insulin treatment during the removal, there was a relatively slow time-dependent dephosphorylation of insulin found in insulin-resistant states, this does not reflect although this approach may mimic the chronic elevation of glucose, amino acids, and fatty acids into the circulation. Periods before returning to resting levels. This pattern of insulin levels are only elevated for relatively short conditions insulin levels are only elevated for relatively short

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In vivo, there is significant homologous desensitization of Ras activation. This occurs despite a complete dissociation of the Grb2-SOS complex demonstrating that Grb2 function is not necessary for Ras activation. In contrast, insulin does induce the homologous desensitization of ERK activation in a time-dependent manner consistent with the desensitization of insulin-stimulated Raf activation.

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