Insulin stimulates the Ras/Raf/MEK/ERK pathway leading to feedback phosphorylation of the Ras guanyl-nucleotide exchange protein SOS and dissociation of Grb2 from SOS. Even though epidermal growth factor (EGF) also stimulates ERK activity and phosphorylation of SOS similar to insulin, EGF induces a dissociation of the Grb2-SOS complex from Shc. To determine the molecular basis for this difference, we examined the signalling properties of a mutant EGF receptor lacking the five major autophosphorylation sites. Although EGF stimulation of the mutant EGF receptor activates ERK and phosphorylation of both Shc and SOS, it fails to directly associate with either Shc or Grb2. However, under these conditions EGF induces a dissociation of the Grb2-SOS complex suggesting a role for receptor and/or plasma membrane targeting in the stabilization of Grb2-SOS interaction. Consistent with this hypothesis, expression of an SH2 domain Grb2 mutant which is unable to mediate plasma membrane targeting of the Grb2-SOS complex results in both insulin- and EGF-stimulated uncoupling of Grb2 from SOS. Furthermore, a plasma membrane-bound Grb2 fusion protein remains constitutively associated with SOS. Together, these data demonstrate that EGF stimulation prevents the feedback uncoupling of Grb2 from SOS by inducing a persistent plasma membrane receptor targeting of the Grb2-SOS complex.

Recently, substantial progress has been made in defining the molecular mechanisms by which tyrosine kinase receptors activate Ras-dependent signalling events. For example, insulin stimulation of the insulin receptor kinase results in the tyrosine phosphorylation of several substrates including the 185-kDa insulin receptor substrates 1 and 2 (IRS1/2) and isoforms of the src homology 2 (SH2) domain-containing 2 collagen-related proteins, termed Shc (1–4). Tyrosine phosphorylation of IRS1, accounts for the increased conversion of Ras from the inactive GDP-bound state to the active GTP-bound form (16, 17). Alternatively, several studies have suggested that the carboxyl-terminal domain of SOS functions as an auto-inhibitory domain which may be derepressed by the binding of Grb2 (18–20). In either case, the interaction of SOS with Grb2 plays an important role in regulating the activation state of Ras.

In the GTP-bound state, Ras associates with and activates members of the Raf family of serine/threonine kinases (21–25). In turn, activated Raf phosphorylates and activates the dual functional protein kinase MEK which then phosphorylates the ERK family of mitogen-activated protein kinases on both threonine and tyrosine residues (12, 26). These phosphorylation events activate ERK and are required for the phosphorylation-dependent regulation of various cytosolic proteins and nuclear DNA binding transcription factors (27–29). Thus, the mitogenic actions of growth factors can be directly linked to transcriptional regulatory events utilizing Ras as a molecular switch converting upstream tyrosine kinase signals into a serine/threonine kinase cascade.

Although these data have provided a mechanism accounting for the activation and positive signaling role of Ras, Ras activation is transient and rapidly returns to its basal GDP-bound state (30–32). We and others have observed that insulin activation of the Ras/Raf/MEK/ERK pathway results in the serine/threonine phosphorylation of SOS, followed by dissociation of the Grb2-SOS complex (33–35). Furthermore, blockade of ERK activation by either expression of a dominant-interfering MEK mutant or with a specific MEK inhibitor prevents SOS phosphorylation, dissociation of the Grb2-SOS complex, and prolongs the time Ras remains GTP-bound (36, 37). These data suggest that an insulin-stimulated feedback uncoupling of Grb2 from SOS may contribute to Ras desensitization. In this study we demonstrate, however, that SOS phosphorylation following EGF stimulation does not result in dissociation of Grb2 from SOS but instead induces the dissociation of the Grb2-SOS complex from Shc. This results from an EGF-stimulated persistent plasma membrane receptor targeting of the Shc-Grb2-SOS complex which does not occur following insulin stimulation.
described previously by co-transfection of CHO/R cells with a hygromycin-resistant plasmid (pRBK, Invitrogen) and the human EGFR expression plasmid (38). These cells were maintained in α-minimal Eagle's medium supplemented with nucleosides, 200 μg/ml hygromycin, 500 μg/ml neomycin, and 10% fetal bovine serum.

NIH-3T3 fibroblasts were engineered to express the human wild type EGFR receptor (EGFR-WT) and an EGFR receptor mutant (EGFR-5F) in the absence (lane 1) or presence of 100 ng insulin (lane 2) or 20 ng EGF (lane 3) for 5 min. Whole cell detergent extracts were subjected to Western blotting using an ERK antibody as described under “Materials and Methods.” CHO/R/EGFR cells were incubated in the absence (lane 1) or presence of 100 ng insulin (lane 2) or 20 ng EGF (lane 3) for 15 min. Whole cell detergent extracts were subjected to Western blotting using a SOS antibody. C, the cell extracts prepared in panel B were immunoprecipitated with a Grb2 antibody and the resultant immunoprecipitates were subjected to Western blotting using a SOS antibody. D, the Grb2 immunoprecipitates obtained in panel C were subjected to Western blotting using a Grb2 antibody.

Tyrosine Kinase-specific Dissociation of Grb2-SOS

**Fig. 1.** Insulin and EGF stimulate ERK and SOS phosphorylation but only insulin induces the dissociation of the Grb2-SOS complex. A, Chinese hamster ovary cells genetically engineered to express the human insulin and EGF receptors (CHO/R/EGFR) were incubated in the absence (lane 1) or presence of 100 ng insulin (lane 2) or 20 ng EGF (lane 3) for 5 min. Whole cell detergent extracts were subjected to Western blotting using an ERK antibody as described under “Materials and Methods.” B, CHO/R/EGFR cells were incubated in the absence (lane 1) or presence of 100 ng insulin (lane 2) or 20 ng EGF (lane 3) for 15 min. Whole cell detergent extracts were subjected to Western blotting using a SOS antibody. C, the cell extracts prepared in panel B were immunoprecipitated with a Grb2 antibody and the resultant immunoprecipitates were subjected to Western blotting using a SOS antibody. D, the Grb2 immunoprecipitates obtained in panel C were subjected to Western blotting using a Grb2 antibody.

**RESULTS**

Insulin but Not EGF Induces a Dissociation of Grb2 from SOS—To investigate the effect of insulin and EGF on SOS phosphorylation and its association with Grb2 in the same cell context, we prepared Chinese hamster ovary cells expressing both the human insulin and EGF receptors (CHO/R/EGFR). In these cells, treatment with either hormone for 5 min resulted in a characteristic mobility shift of ERK, indicative of phosphorylation and kinase activation (Fig. 1A, lanes 1–3). In addition, both insulin and EGF stimulated the phosphorylation of SOS, which also displayed a characteristic reduction in electrophoretic mobility (Fig. 1B, lanes 1–3). We and others have
reported previously that insulin-stimulated SOS phosphorylation correlated with its dissociation from Grb2 in CHO/IR and 3T3L1 adipocytes (34, 35, 37). Consistent with this result, insulin treatment of CHO/IR/EGFR cells resulted in the dissociation of the Grb2-SOS complex (Fig. 1C, lane 1). As a control, the immunoprecipitates were also subjected to Shc immunoblotting to confirm that equal amounts of Grb2 were immunoprecipitated under these conditions, (Fig. 1D, lanes 1–3). Furthermore, the inability of EGF stimulation to induce the dissociation of the Grb2-SOS complex was not unique to the CHO/IR/EGFR cells as identical results were recapitulated in NIH-3T3 cells expressing the human EGF receptor (see Fig. 3).

EGF Induces a Dissociation of SOS from Shc—In addition to the SH3 domains of Grb2 binding the carboxyl-terminal proline-rich region of SOS, the Grb2 SH2 domain directs association with tyrosine-phosphorylated proteins including Shc (5–7, 44). To determine the potential effect of EGF on the interactions between Shc and the Grb2-SOS complex, we next performed Shc immunoprecipitations (Fig. 2). As expected, there was no detectable SOS protein in the Shc immunoprecipitates from cell extracts isolated from unstimulated cells (Fig. 2A, lane 1), since Shc is not tyrosine phosphorylated in the basal state (Fig. 2C, lane 1). One min of EGF stimulation resulted in the co-immunoprecipitation of SOS with Shc, due to the tyrosine phosphorylation of Shc and formation of a Shc-Grb2-SOS ternary complex (Fig. 2A, lane 2). However, as previously reported (45) longer treatments with EGF for 15 and 30 min resulted in a decreased amount of SOS that was co-immunoprecipitated with Shc (Fig. 2A, lanes 3 and 4). As a control, the Shc immunoprecipitates were also subjected to Shc immunoblotting to verify that the 66-, 52-, and 46-kDa Shc isoforms were equivalently immunoprecipitated under these experimental conditions (Fig. 2B, lanes 1–4). The dissociation of the Grb2-SOS complex from Shc was not due to Shc dephosphorylation since the tyrosine phosphorylation of the 52- and 46-kDa Shc proteins was persistent over this time period (Fig. 2C, lanes 1–4). Furthermore, insulin stimulation also resulted in a time-dependent decrease in the amount of SOS that could be co-immunoprecipitated with Shc following insulin treatment (data not shown). Together, these data demonstrate that under conditions in which insulin induces dissociation of Grb2 from SOS, EGF did not affect Grb2-SOS interactions, but instead results in an uncoupling of the Grb2-SOS complex from Shc.

EGF Receptor Targeting Prevents Dissociation of Grb2 from SOS—There are several potential mechanisms which could account for the inability of EGF to induce a dissociation of the Grb2-SOS complex compared to insulin. One obvious difference between the insulin and EGF receptors is that the autophosphorylated EGF receptor directly provides phosphorylation binding sites for both Shc and Grb2 (5, 6, 9, 11, 46). We therefore speculated that the differential localization of the Shc-Grb2-SOS and/or Grb2-SOS complexes following insulin and EGF receptor activation might contribute to the regulation of Grb2-SOS interactions. To test this possibility, we compared the signaling properties of the wild type EGF (EGFR-WT) receptor with an EGF receptor in which the five major tyrosine autophosphorylation sites were mutated to phenylalanine (EGFR-5F).

Consistent with the normal mitogenic signaling properties of the EGFR-5F receptor (39), EGF-dependent phosphorylation of ERK was identical to that observed in cells expressing the wild type EGF receptor (Fig. 3A, lanes 1–4). Similarly, EGF stimulation of both the EGFR-WT and EGFR-5F cells resulted in a comparable reduction of SOS mobility (Fig. 3B, lanes 1–4). However, in contrast to the EGFR-WT receptor, activation of the EGFR-5F receptor resulted in a marked EGF-dependent decrease in the amount of Grb2 bound to SOS (Fig. 3C, lanes 1–4). The ability of the EGFR-5F receptor to mediate dissociation of the SOS-Grb2 complex occurred without any significant changes in the amount of immunoprecipitated Grb2 protein (Fig. 3D, lanes 1–4). These data suggest that the inability of the wild type EGF receptor to signal dissociation of the Grb2-SOS complex may be an intrinsic property of the tyrosine-phosphorylated EGF receptor itself.

We next determined whether the EGFR-5F receptor could tyrosine phosphorylate Shc despite the absence of the high affinity binding sites normally provided by receptor autophosphorylation (Fig. 4). Incubation of the EGFR-WT cells with EGF resulted in the binding of the EGFR-WT receptor with Grb2 (Fig. 4A, lanes 1 and 2) as well as the association of Shc with Grb2 (Fig. 4B, lanes 1 and 2). As expected, stimulation of the EGFR-5F cells failed to induce an association of Grb2 with the mutant EGF receptor (Fig. 4A, lanes 3 and 4). Nevertheless, EGF activation of the EGFR-5F receptor resulted in association of Grb2 with Shc (Fig. 4B, lanes 3 and 4). Phosphoryrosine immunoblots of Grb2 immunoprecipitates demonstrated that the association of Grb2 with Shc correlated with tyrosine phosphorylation of Shc by the EGFR-5F receptor (Fig. 4C, lanes 1–4). These results are consistent with the mutant EGF receptor utilizing Shc as a phosphoryrosine acceptor substrate which then binds to the Grb2 SH2 domain. These data suggest further that the inability of the wild type EGF receptor to induce dissociation of the Grb2-SOS complex resulted from the targeting and/or localization of Grb2 and Shc to the EGF receptor. Disruption of this interaction, by deletion of the EGF receptor autophosphorylation sites, resulted in an EGF receptor that functions in a manner analogous to the insulin receptor with regard to Grb2/Shc binding and Grb2-SOS dissociation.

Expression of Grb2 Mutants Affects Both Insulin and EGF-stimulated Dissociation of Grb2 from SOS—To test the hypothesis that the binding of the Grb2 SH2 domain to the EGF receptor prevented the dissociation of the Grb2-SOS complex, we expressed a myc epitope-tagged wild type Grb2 protein (Grb2-myc) and a mutant Grb2 protein defective in binding to phosphotyrosine residues (Grb2/R86K-myc). To insure that expression of the mutant Grb2/R86K-myc protein would not function in a dominant-interfering manner for Ras activation, we adjusted the amount of transfected plasmid to produce protein levels approximately equal to that of endogenous Grb2 (data not shown). Under these conditions, immunoprecipitation with
The differences in Grb2-SOS targeting induced by insulin and EGF stimulation—Redistribution of Grb2 from the Cytosolic to Particulate Fraction. Occurs to Different Extents following Insulin and EGF Stimulation—To compare directly the effect of insulin and EGF on the subcellular localization of Grb2, we examined the cytosolic/particulate distribution of the expressed wild type and mutant Grb2 proteins (Fig. 6). In the basal state, the wild type Grb2-myc fusion protein was exclusively localized to the cytosolic fraction (Fig. 6A, lanes 1 and 2). However, following 5 min of insulin stimulation there was a small amount of the Grb2-myc protein which translocated to the particulate fraction (Fig. 6A, lanes 3 and 4). Since only a small amount of the Grb2-myc was redistributed, there was no significant decrease in the cytosolic fraction. In contrast, EGF stimulation resulted in a marked appearance of Grb2-myc in the particulate fraction with a concomitant reduction in the cytosolic fraction (Fig. 6A, lanes 5 and 6). Consistent with the Grb2 SH2 domain mutant (Grb2/R86K-myc) having a reduced affinity for phosphoryl-rose binding sites, this mutant was exclusively localized to the cytosolic fractions in the basal, insulin, and EGF-stimulated cells (Fig. 6B, lanes 1–6). As expected, expression of the membrane-bound form of Grb2 (A2/Grb2-myc) also associated with SOS in unstimulated cells (Fig. 5C, lane 1). However, neither insulin nor EGF induced dissociation of this Grb2 fusion protein from SOS (Fig. 5C, lanes 2 and 3). Thus, under these conditions the insulin regulation of Grb2-SOS interaction was essentially identical to that observed for EGF.

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lanes 1, 4, and 7). In contrast, incubation of low concentrations of GST-Grb2 with extracts isolated from insulin-stimulated cells demonstrated a marked reduction in the amount of bound SOS compared to extracts from unstimulated cells (Fig. 7, lanes 2, 5, and 8). However, at high concentrations of GST-Grb2 (30 and 100 μg), SOS binding was essentially saturated (Fig. 7, lanes 10, 13, and 16).

Furthermore, at these high concentrations of GST-Grb2, there was no significant difference in the amount of bound SOS between the unstimulated and insulin-stimulated cell extracts (Fig. 7, compare lanes 13 with 14 and lanes 16 with 17). These data demonstrate that decreased binding between Grb2 and SOS following insulin stimulation results from a reduction in the apparent specific binding affinity ($K_d$) without any significant affect on the total amount of bound SOS protein ($B_{\text{max}}$).

Surprisingly, cell extracts from EGF-stimulated cells also displayed a reduced association of SOS with Grb2 when incubated with low concentrations of the GST-Grb2 fusion protein in an identical manner to extracts from insulin-stimulated cells (Fig. 7, lanes 3, 6, and 9). Incubation with higher GST-Grb2 concentrations (30–100 μg) also resulted in an apparent saturation of SOS binding with no significant difference in association between unstimulated and EGF-stimulated cell extracts (Fig. 7, compare lanes 13 with 14 and lanes 16 with 17). These data demonstrate that the functional differences between insulin and EGF-stimulated dissociation of the Grb2-SOS complex is not intrinsic to the SOS protein itself and thus cannot be accounted for by differences in receptor-mediated post-translational modifications of SOS.
Recent studies have begun to define a pathway directly linking tyrosine kinase growth factor signaling to Ras activation. In the basal state, the guanylnucleotide exchange protein SOS is bound to the SH3 domains of the small adapter protein Grb2. Following receptor kinase activation, either through receptor autophosphorylation and/or tyrosine phosphorylation of Shc, the SH2 domain of Grb2 targets the cytosolic Grb2-SOS complex to the plasma membrane location of Ras (14, 15). In addition, several studies have suggested that the SOS carboxy-terminal Grb2 binding domain functions as an autoinhibitory region (14, 18–20). In either case, the targeting and/or allosteric regulation of SOS provides a mechanism for the interaction of SOS with its substrate Ras, thus allowing for the productive exchange of GTP for GDP. Although these mechanisms account for the rapid growth factor-mediated activation of Ras, even in the continuous presence of growth factors Ras-GTP binding is transient and returns to the inactive GDP-bound state within 30 to 60 min (30–32). This desensitization of Ras activation is an important event, as disruption of normal Ras regulation can lead to oncogenesis with nearly 15% of all human tumors containing mutant forms of Ras that maintain the protein in its active GTP-bound state (47).

In addition to the GTP for GDP exchange activity of SOS, Ras is also regulated by the GTPase activating protein (Ras-GAP) which hydrolyzes GTP-bound Ras to GDP (48). Since Ras itself has relatively low intrinsic guanylnucleotide exchange or GTPase activities, the dynamic equilibrium between SOS and Ras-GAP activities within a cell defines the relative activation state of Ras. Thus, modulation of either of these two effector proteins could be responsible for the inactivation of Ras. However, insulin does not have any significant effect on Ras-GAP phosphorylation, activity, and/or localization, whereas EGF has been reported to inhibit Ras-GAP activity (49). Based upon these previous findings, it is unlikely that Ras-GAP plays a role in growth factor-stimulated desensitization of Ras activation.

To address the mechanism of Ras desensitization, we and others have observed recently that following insulin-mediated Ras activation there is a Raf/MEK/ERK pathway feedback phosphorylation of SOS (33, 35–37). SOS phosphorylation temporally correlates with dissociation of the Grb2-SOS complex which also parallels the return of Ras back to the inactive GDP-bound state. Furthermore, prevention of the Grb2-SOS dissociation by inhibition of MEK activity results in a prolongation of GTP-bound Ras (36, 37). Thus, these data support a model in which a feedback uncoupling of the Grb2-SOS complex removes the Ras activation signal and thereby limits the duration of Ras activation by insulin.

Similar to insulin, various agents which activate the ERK pathway (serum, platelet-derived growth factor, v-Ras, v-Raf) induce phosphorylation of SOS and result in dissociation of the Grb2-SOS complex (35). However, although several studies have also observed an EGF-induced phosphorylation of SOS, there are marked discrepancies with regard to Shc-Grb2-SOS interactions. In one study, EGF was observed to induce the dissociation of Grb2 from SOS in an identical fashion to that of insulin (36). In contrast, other studies have not observed any effect of EGF on the association state of the Grb2-SOS complex (45, 50, 51). In two of these studies, SOS phosphorylation resulted in an uncoupling of the Grb2-SOS complex from Shc due to a reduction in affinity of the Grb2 SH2 domain without any effect on the Shc tyrosine phosphorylation state (45, 51). Alternatively, another report indicated that the uncoupling of the Grb2-SOS complex from Shc correlated with the tyrosine dephosphorylation of Shc (50). Although the basis for these differences in EGF action are not readily apparent, the data presented in this article are consistent with a rapid insulin-stimulated formation of a Shc-Grb2-SOS ternary complex followed by an insulin-induced dissociation of Grb2 from SOS, thereby decreasing the amount of SOS indirectly bound to Shc. However, in the case of EGF stimulation the net result of uncoupling Shc from SOS also occurred but was a consequence of an EGF-induced dissociation of Shc from the Grb2-SOS binary complex without any significant effect on Grb2-SOS interactions. The inability of EGF to induce a dissociation of Grb2-SOS was surprising since EGF also stimulates ERK activation and SOS phosphorylation in an apparently identical manner to that of numerous agents which result in Grb2-SOS dissociation (33–37).

To explore these apparent differences between insulin and EGF signaling, we considered that one unique feature of the EGF receptor pathway compared to the insulin receptor is the direct association of the EGF receptor itself with Grb2-SOS (5, 6, 9, 11). In contrast, insulin stimulation does not result in a stable association of the Shc-Grb2-SOS and/or Grb2-SOS complexes with the insulin receptor (16, 43, 44, 52, 53). We therefore speculated that EGF would induce dissociation of the Grb2-SOS complex if membrane EGF receptor targeting was disrupted. This was initially accomplished by expression of an autophosphorylation defective but kinase active EGF receptor mutant in which the five major tyrosine acceptor sites were mutated to phenylalanine (39). Interestingly, despite the lack of detectable autophosphorylation and/or association with Grb2, this EGF receptor mutant functions to phosphorylate Shc and induces both ERK and SOS phosphorylation. It is unlikely that these signaling properties are due to dimerization of the mutant EGF receptor with either ErbB3 or ErbB4 receptors since the only tyrosine-phosphorylated proteins detected in Grb2 immunoprecipitates was Shc.

The effect of EGF receptor binding on Grb2-SOS interaction was further substantiated by expression of a targeting defective Grb2 mutant which does not associate with either the tyrosine-phosphorylated EGF receptor or Shc. Under these conditions, EGF stimulated dissociation of Grb2 from SOS to the same degree as insulin. Furthermore, a membrane localized chimeric Grb2 protein remained bound to SOS following insulin or EGF stimulation. Thus, in terms of Grb2-SOS interaction, expression of a targeting defective Grb2 mutant confers an insulin-like response to EGF stimulation, whereas a membrane bound form of Grb2 prevents the insulin stimulated dissociation of Grb2 from SOS. In any case, persistent membrane association of the Shc-Grb2-SOS and/or Grb2-SOS complexes via the EGF receptor apparently overrides the effect of SOS phosphorylation to induce its uncoupling from Grb2. Instead, association of the EGF receptor with the Shc-Grb2-SOS ternary complex seems to influence the interaction of Shc with the Grb2-SOS complex, without any significant effect on Grb2-SOS binding. Thus, these data suggest that the binding interaction between Shc, Grb2, and SOS are dependent upon the intracellular location of this complex following growth factor stimulation.

One additional possibility to account for the ability of insulin but not EGF to induce dissociation of the Grb2-SOS complex is a difference in site-specific phosphorylation of SOS. However, this is unlikely since in vitro reconstitution of Grb2-SOS binding in extracts from both insulin and EGF-stimulated cells demonstrated an equivalent reduction in the apparent binding affinity of SOS for Grb2. Despite this reduction in apparent affinity, it is possible that the effective concentration of Grb2 and SOS might be sufficient to overcome this lower binding interaction following EGF but not insulin stimulation. This would be consistent with the targeting of the Shc-Grb2-SOS complex.
and/or Grb2-SOS complexes to the EGF receptor but not to the insulin receptor. Alternatively, it is also possible that EGF receptor targeting of the Grb2-SOS complex induces the association of an additional accessory factor(s) that might influence the binding interaction between Shc, Grb2, and SOS. For example, the tyrosine-phosphorylated EGF receptor associates with the GTPase activating protein and phospholipase Cγ which are not bound by the insulin receptor.

In either case, it is important to recognize that even though EGF does not uncouple Grb2 from SOS, Ras activation is transient and recovers to the basal GDP-bound state within 30 min following both insulin and EGF stimulation in numerous cell types including the CHO/IR/EGFR cells.2 We and others have recently reported that the insulin-stimulated dissociation of the Grb2-SOS complex is an important mechanism involved in the Ras inactivation process (36, 37). Thus, the essential feature of uncoupling SOS from Shc appears to be the necessary event in both the insulin- and EGF-dependent recovery of activated GTP-bound Ras to the inactive GDP-bound state. Currently, we are attempting to determine the molecular basis for the EGF receptor-targeted dissociation of Shc from the Grb2-SOS complex versus the insulin induced dissociation of Grb2-SOS interactions.

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