The Signaling Pathway Coupling Epidermal Growth Factor Receptors to Activation of p21<sup>ras</sup>*

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Epidermal growth factor (EGF) treatment causes autophosphorylation of the epidermal growth factor receptor (EGFR) leading to increased guanine nucleotide exchange factor (GEF); Sos activity and enhanced formation of p21<sup>ras</sup>-GTP. The connection of the EGFR to p21<sup>ras</sup> activation can occur through binding of Grb2-Sos complexes to the EGFR through the adaptor protein Shc via EGFR-Shc-Grb2-Sos multimeric complexes. Therefore, we investigated the importance of Shc in coupling the EGFR to activation of ras GEF (Sos). EGF treatment led to rapid tyrosine phosphorylation of Shc. Although phosphorylated EGFR can bind to both Shc and Grb2, the predominant linkage was observed between EGFR and Shc. Similarly, more Grb2 was associated with Shc than with EGFR after EGF stimulation. Immunoprecipitation of Shc from EGF-stimulated cells removed 93% of the ras GEF activity, whereas, precipitation of EGFR had only a small effect on ras GEF activity. These data indicate that coupling to Shc provides the major pathway linking activated EGFRs to Grb2-Sos and stimulation of the p21<sup>ras</sup> pathway.

Epidermal growth factor (EGF)<sup>a</sup> stimulates the intrinsic tyrosine kinase activity of the epidermal growth factor receptor (EGFR), leading to activation of p21<sup>ras</sup>; and this process is necessary for EGF-induced cell cycle progression (1, 2). p21<sup>ras</sup> is active in its GTP-bound form, and p21<sup>ras</sup>-GTP formation can be mediated by dissociation of GDP from p21<sup>ras</sup> to facilitate GTP exchange and/or by inhibiting the hydrolysis of GTP on p21<sup>ras</sup> (3). The former process is controlled by ras guanine nucleotide exchange factor (GEF) (4-6) and the latter is controlled by ras GTPase-activating protein (7). Recent reports have provided evidence that EGF increases p21<sup>ras</sup>-GTP formation primarily by activating GEF activity, rather than by inhibition of GTPase-activating protein (8-10). Recently, the Drosophila melanogaster Son of Sevenless (Sos) protein was isolated (4), and the mammalian homologue of Sos has been proposed as the GEF that mediates signaling from growth factor tyrosine kinases to p21<sup>ras</sup> activation (5, 6). The pruned rich region of Sos binds to the SH3 domain of Grb2, which is an adaptor protein composed of one SH2 and two SH3 domains (11), and preformed Grb2-Sos complexes exist within unstimulated cells (6, 12-15). Through the Grb2 SH2 domain, Grb2-Sos complexes can bind to tyrosine phosphorylated EGFRs, providing a mechanism whereby EGF can stimulate p21<sup>ras</sup>-GTP formation (6, 12-15). Alternatively, the SH2 domain of the Grb2-Sos complex can also bind to phosphorylated Shc, which contains an SH2 domain that can recognize an EGF phosphotyrosine motif (12-16). Thus, Shc provides another pathway to couple EGFRs to p21<sup>ras</sup> activation.

In this report, we evaluated the relative contributions of the EGFR-Grb2-Sos or EGF- Shc-Grb2-Sos pathways in mediating EGF-induced GEF activation of p21<sup>ras</sup>. Our results indicate the importance of Shc as an adapter protein transducing biologic signals from activated EGFR to the p21<sup>ras</sup> pathway.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Materials**—Rat1 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 50 mg/ml gentamycin (17). The p21<sup>ras</sup> probe (c-Ha-Ras) was a gift from Dr. Alan Wolfman (Cleveland Clinic Foundation). EGF was purchased from Life Technologies, Inc. (HGHF 32 Cimnmol) was from DuPont-NEN. Electrophoresis reagents were from Bio-Rad. Enhanced chemiluminescence reagents were from Bio-Rad. Enhanced chemiluminescence reagents were from Amersham Corp. A monoclonal anti-phosphotyrosine antibody (pT20), a polyclonal and a monoclonal anti-Shc antibody, and a monoclonal anti-Grb2 antibody were from Transduction Laboratories (Lexington, KY). Polyclonal Grb2 antibodies were from Upstate Biotechnology (Lake Placid, NY) and Santa Cruz (Santa Cruz, CA). A polyclonal anti-EGF receptor antibody was kindly provided by Dr. Stuart J. Decker (Parke-Davis Pharmaceuticals, MI). All other routine reagents were purchased from Sigma.

**Western Blotting Studies**—Cells were starved for 24 h in serum-free Dulbecco’s modified Eagle’s medium. The cells were then treated with 130 ng/ml EGF at 37 °C. After the indicated time, cells were lysed in a buffer containing 30 mm Tris, 150 mm NaCl, 10 mm EDTA, 0.5% sodium deoxycholate, 1% Triton X-100, 1 mm phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μm leupeptin, 1 mm Na<sub>2</sub>VO<sub>4</sub>, pH 7.4. The cell lysates were centrifuged to remove insoluble materials. The supernatants (50 μg of protein) were used for immunoprecipitation with the indicated antibodies for 5 h at 4 °C. The entire precipitate and all of the remaining supernatant proteins were then separated by SDS-PAGE and transferred to Immobilon-P by electrob. For immunoblotting, membranes were blocked and probed with specified antibodies. Blots were then incubated with horseradish peroxidase-linked secondary antibody followed by enhanced chemiluminescence detection, according to the manufacturer’s instructions (Amerham Corp.) (18). Based on immunoprecipitation and Western blot results, we estimate the efficiency of precipitation as 80-90% for anti Shc, 70-80% for anti Grb2, and 80-90% for anti EGFR.

**Measurement of GTP- and GDP-bound p21<sup>ras</sup>**—As described previously (19), cells were serum starved for 16 h, labeled with

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<sup>†</sup> The abbreviations used are: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; GEF, guanine nucleotide exchange factor; Sos, Son of Sevenless; PAGE, polyacrylamide gel electrophoresis.
(32P)orthophosphate, and stimulated with 130 nM EGF for varying times. After cell lysis, Ras was immunoprecipitated, and the nucleotides were eluted from the immunoprecipitate and separated by thin layer chromatography.

Measurement of GEF Activity in Membranes—Cells were starved for 16 h in serum-free Dulbecco’s modified Eagle’s medium. The cells were then treated with 130 nM EGF at 37 °C for 2 min. The cells were then collected in a buffer containing 50 mM Hepes, 150 mM NaCl, 10 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM dithiothreitol, pH 7.5. The cells were disrupted by 20 strokes of a tight fitting Dounce homogenizer. The homogenate was centrifuged at 3,000 rpm in an Eppendorf 5402 centrifuge at 4 °C for 3 min to remove the nuclear fraction. The supernatants were re-centrifuged at 220,000 × g at 4 °C for 60 min. The particulate fraction was suspended in a buffer containing 0.05% SDS, 0.1% Triton X-100, 50 mM Hepes, 150 mM NaCl, 10 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, 1 mM Na₂HPO₄, 1 mM leupeptin, 10 μg/ml aprotinin, 1 mM dithiothreitol, 100 μM GTP, 100 μM GDP, pH 7.5, and sonicated at 4 °C for 30 s. For immunodepletion studies, the extract was immunoprecipitated with specified antibodies at 4 °C for 3 h, and the supernatants were used for GEF activity assay. The GEF activity in the membranes was determined by measuring the dissociation of protein-bound 32P]GTP radioactivity using a nitrocellulose filter binding assay as described previously (19).

RESULTS

Fig. 1 shows the time course of EGF-stimulated tyrosine phosphorylation of Shc. To assess Shc phosphorylation, cell lysates were immunoprecipitated with anti-Shc antibody, and the precipitates were immunoblotted with anti-phosphotyrosine antibody. As shown in Fig. 1A, the 46-, 52-, and 66-kDa Shc isoforms were tyrosine-phosphorylated upon EGF stimulation, and the 52-kDa isomorph was the major phosphorylated species. Peak phosphorylation of Shc was observed by 30 s and declined after 2.5 min. These results are summarized in Fig. 1B. It is clear that tyrosine phosphorylation of Shc proceeds rapidly, consistent with the notion that activated EGF receptors bind to Shc directly (12–15). The time course of EGF-stimulated p21⁰⁺GTP formation is shown in Fig. 1C, and the functional form of this time course is consistent with the kinetics of Shc phosphorylation.

It has been shown that both Shc and Grb2 can associate with the EGFR following EGF stimulation (12–15), and therefore the time course and the amount of Shc and Grb2 association with the EGFR were assessed. After EGFR treatment, cell lysates were immunoprecipitated with anti-Shc antibody, and the precipitates and supernatants were immunoblotted with anti-phosphotyrosine antibody. As seen in Fig. 2A, a 175-kDa phosphoprotein band was co-precipitated with the anti-Shc antibody, and the identity of this band as the EGFR was confirmed by using specific EGFR antibodies (data not shown). The EGFR:Shc association was rapid, with peak complex formation detected at 30 s, declining thereafter (Fig. 2A). Thus, the time course of the EGFR:Shc complex formation was comparable to the kinetics of Shc phosphorylation as seen in Fig. 1. Similar experiments were performed using anti-Grb2 antibody. Following EGF stimulation, cell lysates were immunoprecipitated with anti-Grb2 antibody, and the precipitates and supernatants were immunoblotted with anti-phosphotyrosine antibody. Tyrosine-phosphorylated EGF-Rs were co-precipitated by the anti-Grb2 antibody, but the magnitude of EGFR-complex formation was much less than for EGFR-Shc complexes (Fig. 2B). For example, at 1–2.5 min, ~55% of EGFR precipitates with anti-Shc, compared to ~20% with anti-Grb2. EGF treatment can also lead to tyrosine phosphorylation of erbB2 (8), which is present in rat fibroblasts (20). ErbB2 has a reported mobility of ~185 kDa on SDS-PAGE (20), which should allow distinction from the 175-kDa EGFR. Interestingly, as seen in Fig. 2, there is a variable EGF stimulated phosphoprotein band, mostly in the supernatants, which runs above the EGFR, consistent with erbB2. By Western blotting with anti-EGFR antibody we have confirmed that the 175-kDa phosphoprotein band is the EGFR and no higher molecular mass bands were identified. Nevertheless, we cannot exclude the possibility that a small amount of erbB2 is co-mingled in the EGFR band.

We next assessed the association of Grb2 with Shc and/or the EGFR. After EGF treatment, cell lysates were immunoprecipitated with anti-Shc or anti-EGFR antibody, and the precipitates and supernatants were immunoblotted with anti-Grb2 antibody. Formation of complexes containing Shc:Grb2 (Fig. 3A) and/or EGF-Grb2 (Fig. 3B) was rapid. Although the kinetics of Grb2 association with Shc or EGFR were similar, a substantially larger amount of Grb2 associated with Shc than with EGFR.

Another way to quantitate the association of Grb2 with Shc and EGFR is to conduct sequential immunoprecipitation studies using anti-Shc and anti-EGFR antibodies. Following EGF stimulation, cell lysates were first immunoprecipitated with the anti-Shc antibody, and the remaining supernatants were re-immunoprecipitated with anti-EGFR antibody. The anti-Shc
body precipitates and supernatants were analyzed by immunoblotting treated with EGF for the indicated times. Cell lysates were immunoprecipitated with Shc antibody. A, separate experiments. B, Grb2 association with EGFR (175 kDa) is shown by an arrow. The anti-Shc precipitates and supernatants were analyzed by immunoblotting with anti-phosphotyrosine antibody. Molecular mass of Grb2 (25 kDa) is shown by an arrow.

FIG. 2. EGF-induced Shc and Grb2 association with EGFR receptor. A, Shc association with EGFR. Serum-starved cells were treated with EGF for the indicated times. Cell lysates were immunoprecipitated with anti-Shc antibody. The anti-Shc precipitates and supernatants were analyzed by immunoblotting with anti-phosphotyrosine antibody, identical to that described for Fig. 1. Molecular mass of EGFR (175 kDa) is shown by an arrow. B, Grb2 association with EGFR. The cells were treated as described above, and anti-Grb2 antibody precipitates and supernatants were analyzed by immunoblotting with anti-phosphotyrosine antibody. Results are representative of three separate experiments.

Most likely these represent EGFR-Shc-Grb2 containing complexes. However, a much greater amount of Grb2 could be precipitated by anti-Shc antibody even when the lysates were first immunodepleted by anti-EGFR antibody (Fig. 4, lane 8).

It has been shown that Sos contains GEF activity toward p21<sup>ras</sup> in vitro and that stimulation with EGF does not alter total cellular GEF activity (14). Rather, a major proportion of Sos is translocated from the cytosol to the plasma membrane fraction after EGF stimulation (14). Consequently, we measured GEF activity in membrane fractions following EGF stimulation and found that EGF increased by 2.3-fold the ability of the membrane fraction to enhance ras guanine nucleotide dissociation (from 19.4 ± 4% to 45.6 ± 5% [3H]GDP released). It has been suggested that preformed Grb2-Sos complexes exist in cells (6, 12–15), and consistent with this, we found that immunodepletion by anti-Grb2 antibody removed 88.4 ± 4% of the EGF-stimulated membrane GEF activity (Table I). EGF stimulation leads to direct association of these complexes with either Sos or EGFR (12–15), and to evaluate the relative magnitudes of these effects, we measured GEF activity in the membranes before and after immunoprecipitation of the fractions with anti-Shc and/or anti-EGFR antibody. As can be seen in Table I, precipitation of Shc from EGF-stimulated preparations removed 92.5 ± 2% of the total membrane GEF activity, while immunoprecipitation by anti-EGFR antibody only removed 39.3 ± 17% of total membrane GEF activity.

## DISCUSSION

EGF and other growth factors stimulate the formation of p21<sup>ras</sup>-GTP, and this plays an important role in mediating the overall mitogenic response (2, 3). For EGF, this occurs largely by stimulation of GDP dissociation from p21<sup>ras</sup>, with replacement by GTP (8–10), and this guanine nucleotide exchange is mediated by Sos proteins (4–6). Since Grb2 exists in preformed complexes containing EGFR could be demonstrated (Fig. 4, lane 7).
complexes with Sos in unstimulated cells (6, 12–15), Grb2 is also a critical signaling molecule connecting EGFRs to p21ras. Recent studies have clearly established the importance of Grb2 in growth factor action. For example, microinjection of Grb2, together with H-ras protein, into quiescent rat embryo fibroblasts resulted in enhanced DNA synthesis (11). In addition, microinjection of an anti-Grb2 antibody into normal rat kidney-derived fibroblasts inhibited EGF and platelet-derived growth factor stimulation of cell cycle progression (21). Therefore, a key to understanding how EGF causes increased p21"GTP lies in the identification of the upstream linkages which connect the EGFR to Grb2-Sos.

Although the SH2 domain of Grb2 can directly bind to a phosphotyrosine motif in the EGFR, forming EGFR-Grb2-Sos complexes, the SH2 domain of Grb2 can also bind to a phosphotyrosine motif in Shc (12–15). Since Shc binds to phosphorylated EGFR via its SH2 domain, the EGFR has another signaling pathway to activate p21GTP (12–15). Consequently, EGFRs can couple to the p21"GTP pathway either directly by associating with Grb2-Sos complexes, or by utilizing the adapter protein Shc and forming EGFR-Shc-Grb2-Sos complexes, or both.

After EGF stimulation, there are four possible mechanisms of molecular linkage from the EGFR to Sos and stimulation of p21"GTP, and these are summarized schematically in Fig. 5. First, the tyrosine phosphorylated EGFR could bind directly to the Grb2 SH2 domain forming an EGFR-Grb2-Sos complex (Fig. 5A). However, it is unlikely that this mechanism is a major one, at least in Rat1 fibroblasts, since anti-EGFR antibody precipitated a relatively small amount of Grb2, compared to anti-Shc antibody and, equally importantly, the anti-EGFR antibody precipitated a negligible amount of Grb2 from lysates which had been already precipitated with anti-Shc antibody. Second, the EGFR could complex with Grb2-Sos complexes via Shc, forming a multimeric unit consisting of EGFR-Shc-Grb2-Sos (Fig. 5B). Our data provide strong evidence that this is an important component of EGFR signaling, since anti-Shc antibody co-precipitates a large amount of phosphorylated EGFR, as well as much of the cellular Grb2 and GEF activity. Likewise, after anti-Shc antibody precipitation, when the remaining EGFRs were precipitated with anti-EGFR antibody, only a negligible amount of Grb2 and GEF activity was recovered. Our results also exclude the possibility that appreciable amounts of Shc alone bind to EGFRs without forming Shc-Grb2-Sos complexes, since almost all tyrosine phosphorylated Shc was co-precipitated by anti-Grb2 antibody (data not shown). Third, it is possible that a single EGFR can concomitantly associate with both Grb2-Sos and Shc-Grb2-Sos complexes (Fig. 5C). Obviously, it is experimentally difficult to separate this situation from the EGFR-Shc-Grb2-Sos complexes as in Fig. 5B. However, in both of these cases, Shc would remain a quantitatively more important linkage between the EGFR and Grb2-Sos complexes, since anti-Shc antibody precipitates the greater amount of EGFR and GEF activity. Finally, following EGF stimulation, Shc-Grb2-Sos complexes could exist, independent of the EGFR (Fig. 5D). Our data provide strong evidence for this possibility, since following immunodepletion of cell lysates from EGF-stimulated cells with anti-EGFR antibody, anti-Shc antibody was able to co-precipitate a relatively large amount of Grb2. In addition, anti-Shc antibody precipitation removed far more GEF activity than did simple anti-EGFR antibody precipitation, consistent with the presence of Shc-Grb2-Sos complexes independent of the EGFR.

Taken together, our data are consistent with the formulation that following autophosphorylation of EGFR, Shc binds to the EGFR through its SH2 domain (12–15). This facilitates tyrosine phosphorylation of Shc and association of Shc with Grb2-Sos complexes which then, in turn, mediate the formation of p21"GTP within a multimeric EGFR-Shc-Grb2-Sos complex (12–15). However, we also find that a substantial amount of Shc-Grb2-Sos complexes exist in the absence of associated EGFR. This could mean that during the process of EGF action, dissociation of Shc-Grb2-Sos from the EGFR occurs, creating the scenario depicted in Fig. 5D. Alternatively, it is possible that EGF stimulation could lead to Shc phosphorylation, and subsequent formation of Shc-Grb2-Sos complexes, without any direct interactions between the EGFR and Shc. This would involve an intermediate tyrosine kinase, which is stimulated by the activated EGFR, and phosphorylates Shc directly. This formulation is consistent with recent observations showing that truncated EGFRs, which lack all of the major autophosphorylation sites, still induce Shc tyrosine phosphorylation and complex formation of Shc with Grb2 (22). Even if such a pathway exists, involving an intermediary tyrosine kinase situated between the EGFR and Shc, our results clearly demonstrate the presence of direct linkage between Shc and the EGFR, indicating that both pathways leading from the EGFR to Shc phosphorylation could be operative.

Increasing evidence has accrued in several systems indicating that Shc is the important adaptor molecule linking Grb2-Sos to surface receptors. Thus, it has been reported that Shc is a linking molecule coupling activated T cell receptors (29), interleukin-2 receptors (24), and Trk receptors (25) to Grb2. She has also been reported as the predominant coupling molecule linking insulin receptors to Grb2SOS and activation of the ras pathway (26, 27). Furthermore, Pronk et al. (26) have...
found Grb2 and Sos in Shc immunoprecipitates from EGF-treated cells, consistent with the current results implicating Shc as a key adaptor molecule in EGF action. In addition, Grb2 forms complexes with the platelet-derived growth factor receptor by binding to Syp, also called SHPTP2, PTPC2, or PTP1D, which in turn binds to the platelet-derived growth factor receptor through its SH2 domain (28). Taken together with the current results, the association of Grb2-Sos complexes with membrane receptors via Shc, or possibly other adaptor molecules such as Syp, appears to be the physiologically relevant mechanism for activation of p21W. This line of reasoning is also consistent with our recent studies which demonstrate the functional role of Shc in mediating EGF’s biologic action. Thus, we have conducted single-cell microinjection studies showing that microinjection of anti-Shc antibody or Shc SH2 GST fusion proteins into living Rat1 fibroblasts inhibited EGF-induced cell cycle progression by 80% (18).

It is important to consider how activation of this pathway leads to increased formation of p21W-GTP. Clearly, two possibilities exist. First, formation of the multiprotein complexes could serve to translocate Grb2.Sos complexes to the cytoplasmic side of the plasma membrane, where Sos would then gain access to membrane anchored p21W. Alternatively, formation of Grb2-Sos complexes with Shc could lead to activation of the catalytic activity of Sos to mediate GDP dissociation. The current results (Fig. 4), as well as those of others, demonstrating translocation of Sos or GEF to the plasma membrane, plus our recent finding that EGF stimulation does not change total cellular GEF activity, are more consistent with the former translocation hypothesis (14).

It is of interest to note that in our studies, the EGF-induced Shc phosphorylation peaked at 1–2 min and then declined by ~80% by 20 min. The time course of Shc-Grb2 complex formation (Fig. 3A) showed a similar pattern (Fig. 1), but only declined by ~35% by 20 min. From these differences, it is possible to speculate that within Shc-Grb2 complexes, since the Shc phosphotyrosine is buried in the Grb2 SH2 domain, the phosphatases and relatively protected from dephosphorylation. Clearly, future experiments will be necessary to evaluate this notion.

In summary, our studies indicate the importance of Shc in forming the molecular linkage between EGFR and Grb2-Sos complexes. Following EGF stimulation, Shc associates with and becomes tyrosine-phosphorylated by the EGFR. This leads to the formation of EGFR-Shc-Grb2-Sos complexes. Free Shc-Grb2-Sos complexes also exist, consistent with either dissociation of Shc-Grb2-Sos from the EGFR, or a parallel pathway containing an intermediary tyrosine kinase which phosphorylates Shc following EGFR activation. Either scenario would highlight the importance of Shc as an adaptor protein linking the EGFR to the p21W pathway. In previous studies, it has been shown that Shc is the major adaptor molecule linking the activated insulin receptor to Grb2-Sos complexes and the p21W pathway (26, 27). In combination with the current results, this suggests the general principle that adaptor proteins like Shc are the dominant mechanisms linking activated growth factor receptors to the downstream components of the p21W pathway.

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