Shc Regulates Epidermal Growth Factor-induced Activation of the JNK Signaling Pathway*

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Tyrosine autophosphorylation plays a crucial role in determining the selectivity of signaling pathways activated by growth factor receptors, such as the epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors (EGFR and PDGFR). Receptor activation leads to tyrosine autophosphorylation, resulting in the association of the receptor with cytoplasmic target proteins containing SH2 domain and phosphotyrosine binding (PTB) domain such as Grb2 and Shc (1, 2).

Grb2 is composed of one SH2 domain and two SH3 domains (3, 4). The two SH3 domains of Grb2 bind to proline-rich residues near the C terminus of Sos, a Ras guanine nucleotide exchange factor. Upon stimulation with growth factors, Grb2 binds the autophosphorylated tail of growth factor receptors through its SH2 domain. This leads to translocation of Sos to the plasma membrane where Ras is located, thus increasing the exchange of GDP for GTP on Ras (5–10). The GTP-bound active form of Ras then triggers the activation of ERK cascade, leading to phosphorylation of nuclear proteins involved in transcriptional control (11–13).

In addition to the ERK pathway, EGF has been reported to activate the JNK pathway (14–16), also leading to transcriptional changes by phosphorylating Jun family transcription factors (11–13). The following Abs were purchased: anti-human EGFR from mouse EGF, judged by activation of ERK2 and JNK. The anti-ERK2 Ab, anti-JNK1 Ab, anti-Myc Ab, and anti-Ras Ab were purchased as previously (27). Human EGFR cDNA was transfected by electroporation (550 V, 25 microfarad) and selected in the presence of 1.7 mg/ml of hygromycin (Wako Pure Chemical). Expression of the transfected EGFR was confirmed by flow cytometric analysis. Mouse EGF (TOYOBO) was used for the stimulation of cells. Mouse EGF (TOYOBO) was used for the stimulation of cells. Mouse EGF (TOYOBO) was used for the stimulation of cells.

Experimental Procedures

**Cells, Expression Constructs, and Antibodies**—Wild-type and its derivative mutant DT40 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 1% chicken serum, 50 μg/ml mercaptoethanol, 2 μg/ml insulin, and antibiotics. Various DT40 mutant cells (Grb2-negative, Shc-negative, DT40 expressing RasN17, and DT40 expressing Myc-tagged Rac1N17) have been described already (27). Human EGFR cDNA was transfected by electroporation (550 V, 25 microfarad) and selected in the presence of 1.7 mg/ml of hygromycin (Wako Pure Chemical). Expression of the transfected EGFR was confirmed by flow cytometric analysis. Mouse EGF (TOYOBO) was used for the stimulation of human EGFR (19). Parental chicken DT40 cells did not respond to mouse EGF, judged by activation of ERK2 and JNK. The anti-ERK2 Ab, anti-JNK1 Ab, anti-Myc Ab, and anti-Ras Ab were purchased as previously (27). The following Abs were purchased: anti-human EGFR from Amersham; anti-Grb2 from Santa Cruz Biotechnology; and anti-Shc and anti- phosphotyrosine (4G10) from Upstate Biotechnology.

**Flow Cytometric Analysis for Surface Expression of EGFR**—Cell surface expression of EGFR was analyzed by FACScan (Becton-Dickinson & Co., Mountain View, CA) using anti-human EGFR mAb and fluorescein isothiocyanate-labeled anti-mouse IgG (Cappel).

**Immunoprecipitation and Western Blot Analysis**—DT40 cells were solubilized in lysis buffer (50 mg/ml Tris-HCl, pH 7.5, 150 mg/ml NaCl, 5 mg/ml EDTA, 2% Triton X-100, 10 μg/ml sodium vanadate, 10 mg/ml sodium pyrophosphate, 2 mg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 2 μg/ml aprotonin). Precleared lysates were sequentially incubated with Abs and protein G-Sepharose (Amersham Pharmacia Biotech) or protein A-agarose (Pierce). Immunoprecipitates were separated by SDS-PAGE gel, transferred to nitrocellulose membrane, and detected by appropriate Abs and ECL system (Amersham Pharmacia Biotech).

Grb2 Binding Assay—DT40 cells were lysed in 60 mg/ml Tris-Cl, pH 8.0 containing 150 mg/ml NaCl, 5 mg/ml EDTA, 10% glycerol, 2 mg/ml sodium pyrophosphate, 2 mg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 2 μg/ml aprotonin). Precleared lysates were sequentially incubated with Abs and protein G-Sepharose (Amersham Pharmacia Biotech) or protein A-agarose (Pierce). Immunoprecipitates were separated by SDS-PAGE gel, transferred to nitrocellulose membrane, and detected by appropriate Abs and ECL system (Amersham Pharmacia Biotech).
vanadate, 25 mM NaF, 10 μg/μl leupeptin, 10 μg/μl aprotinin, and 1% Triton X-100. Cleared lysates were precipitated with GST or GST-SH2 (Grb2 SH2 domain: amino acid 50–161 of human Grb2 fused to GST) bound to glutathione-Sepharose beads (20 μl of packed beads, 20 μg of protein). Washed precipitates were eluted with SDS-PAGE sample buffer and resolved on 8% SDS-PAGE gel and subjected to Western blotting analysis.

**In Vitro Kinase Assay**—The assay conditions were described previously (27). Briefly, cells (2–5 × 10^6) stimulated with EGF were lysed in lysis buffer. Precleared lysates were immunoprecipitated by 1 μg of anti-ERK2 Ab or 1 μg of anti-JNK1 Ab, followed by incubating with 40 μl of protein G-Sepharose. No cross-reactivity of these two Abs was already demonstrated (27). The ERK2 Ab does not recognize JNK and p38. Similarly, the JNK Ab does not recognize ERK2 and p38. The beads were washed three times with lysis buffer and two times with washing buffer. Immunoprecipitates were divided, and half of them were used for Western blotting analysis. The remaining half were washed once with kinase assay buffer and resuspended in kinase assay buffer containing [γ-^32^P]ATP (<3,000 Ci/mmole, NEN) and 5 μM cold ATP. GST-Elk or GST-c-Jun fusion protein (5 μg each) was added as a substrate for ERK2 or JNK1, respectively. After 20-min incubation at 30 °C, the reaction was terminated by the addition of SDS sample buffer followed by boiling for 5 min. The samples were separated by SDS-PAGE gel, dried, and subjected to autoradiography. Phosphorylation of the fusion protein bands was quantified using a phosphoimager (Fuji BAS 2,000).

**Ras-GTP Assay**—Bacterially expressed GST-RBD (Ras-binding domain: amino acid 1–149 of human cRaf-1 fused to GST) pre-bounded glutathione-Sepharose beads (15 μl of packed beads, 20 μg of protein) were prepared as described (28). Human H-Ras cDNA was transfected into wild-type, Grb2-deficient, and Shc-deficient chicken DT40 cells to establish genetic strategy. Human EGFR was transfected into wild-type, Grb2-deficient, and Shc-deficient chicken DT40 cells to examine the necessity or redundancy of Grb2 and Shc in ERK and JNK activation upon EGF stimulation, we took a genetic strategy. Human EGFR was transfected into wild-type, Grb2-deficient, and Shc-deficient chicken DT40 cells to obtain stable transformants. Clones expressing similar levels of EGFR in wild-type and these deficient DT40 cells, assessed by flow cytometric analysis using anti-human EGFR mAb, were selected (Fig. 1) and further characterized. EGF stimulated the EGFR autophosphorylation, and its phosphorylation extent in Grb2- or Shc-deficient DT40 cells was almost the same as that in wild-type cells (Fig. 1). These observations suggest that EGFR kinase activity is not affected by loss of Grb2 or Shc.

We next examined the capacity of EGFR to stimulate phosphorylation of cellular proteins in wild-type, Grb2-deficient, and Shc-deficient cells. As shown in Fig. 2A, comparison of the overall tyrosine phosphorylation between wild-type and Shc-deficient DT40 cells did not exhibit significant changes, except that the band corresponding to Shc itself was absent in the mutant cells. Although the overall tyrosine phosphorylation pattern upon EGF stimulation was not significantly affected by loss of Grb2, phosphorylation of some proteins (indicated by arrows) was inhibited in the mutant cells. Moreover, the tyrosine phosphorylation extent of Shc was decreased by loss of Grb2 (Fig. 2, A and B). These observations suggest that Grb2 modulates tyrosine phosphorylation on some of the cellular substrates, one of which is Shc.

To formally demonstrate that Grb2 associates with phosphorylated EGFR via its SH2 domain in DT40 B cells, we examined the associated molecules with Grb2 SH2 domain. As observed in fibroblasts (3, 4, 19, 26), both autophosphorylated EGFR and phosphorylated Shc were associated with the Grb2 SH2 fusion protein (Fig. 3A). Supporting this observation, the immunoprecipitates with anti-Grb2 Ab contained both of these molecules (Fig. 3B).

After EGF stimulation, DT40 cells were lysed and immunoprecipitated with anti-ERK2 Ab. The immunoprecipitates were assayed for in vitro kinase activity by the ability to phosphorylate Elk-1. EGF-induced ERK2 activation in DT40 cells was maximal at 1 min, after which activity declined (Fig. 4A). This ERK2 response was profoundly reduced in Grb2-deficient DT40 cells.
cells, whereas Shc-deficient cells showed the normal ERK2 response upon EGF stimulation (Fig. 4A), indicating that Grb2 is required for EGF-induced ERK2 activation.

To further demonstrate that the ERK2 defect in Grb2-deficient cells is because of loss of Ras activation, we employed a binding assay developed by Taylor and Shalloway (28). This assay is based on the observation that Raf protein has high affinity for active Ras-GTP but does not bind the inactive GDP-bound form of Ras. Thus, we used a GST fusion protein containing the Ras-binding domain (RBD) of Raf to selectively precipitate activated Ras. The recovery of activated Ras was monitored by immunoblotting with anti-Ras Ab. As shown in Fig. 5, EGF stimulation of wild-type and Shc-deficient cells both resulted in increased Ras-GTP, whereas this increase was completely abolished by loss of Grb2.

Since the slight ERK2 activation in Grb2-deficient DT40 cells was observed despite no increased Ras-GTP, this remaining ERK2 activation in the mutant cells could be explained by a Ras-independent mechanism. To examine this possibility, we transfected a dominant-negative form of Ras, RasN17, into DT40 cells expressing EGFR. As shown in Fig. 6B, expression of RasN17 inhibited the EGF-induced ERK2 activation, but the residual ERK2 response was still observed like that in Grb2-deficient DT40 cells (Figs. 4A and 6B). Thus, these results suggest that Ras-independent pathway, at least to some extent, contributes to full ERK2 activity upon EGF stimulation in DT40 cells.

As reported previously, EGF stimulated JNK activity in wild-type DT40 cells expressing EGFR. The JNK activity was determined in an immunocomplex kinase assay with c-Jun as a substrate. The kinetics of JNK activation were distinct from those of ERK2, being marked at 30 min and declined by 60 min (Fig. 4B, and data not shown). Grb2-deficient cells showed the normal JNK response upon EGF stimulation, whereas this JNK response was abolished by loss of Shc (Fig. 4B). These results indicate that Shc, but not Grb2, is involved in EGF-mediated JNK activation. Previous results have shown that EGF-induced JNK activation can be completely inhibited by RasN17 and inhibited approximately 50% by a dominant negative form of Rac1, Rac1N17, in fibroblasts (14, 15). To examine this possibility in DT40 B cells, we transfected Rac1N17 into wild-type cells expressing EGFR (Fig. 6A). As shown in Fig. 6C, expression of Rac1N17 in DT40 cells almost completely abolished the EGF-mediated JNK activation, whereas this response was not affected by expression of RasN17.

**DISCUSSION**

Our results support the previous contention that Grb2 plays a critical role in a highly conserved process for the control of Ras signaling by receptor tyrosine kinases (3, 5–10). The slight
phospholipase C- may be involved in Ras-independent ERK activation through stimulation of its catalytic activity (29, 30), PKC activation in vitro kinase assay were described as in Fig. 4.

Expression of dominant-negative Ras and dominant-negative Rac1 (A). EGF-stimulated cells were lysed and immunoprecipitated with anti-ERK2 Ab (B) or anti-JNK Ab (C). Stimulation conditions and in vitro kinase assay were described as in Fig. 4.

EGF-mediated ERK2 activation by loss of Grb2 or expression of RasN17 (Figs. 4A and 6B) suggests that EGF utilizes an additional signaling pathway, leading to the ERK response. Given the evidence that PKCα phosphorylates Raf-1, thereby leading to stimulation of its catalytic activity (29, 30), PKC activation may be involved in Ras-independent ERK activation through phosphorylation of Raf-1. In fact, EGF was able to induce phospholipase C-γ activation, leading to PKC activation in DT40 cells expressing EGFR (data not shown).

Stimulation of PC12 cells with nerve growth factor (NGF) activates the NGF receptor (NGFR) Trk, and thereby induces neurite extension, in a response that is dependent on the Ras pathway (31). Overexpression of Shc in PC12 cells induces neurite extension; furthermore, this extension is blocked by expression of a dominant negative Ras (RasN17) (26). Based on these data, it has been proposed that Shc-Grb2 complex is involved in Ras pathway. However, the data presented here demonstrate that Shc does not participate in EGF-independent Ras activation in DT40 B cells (Fig. 5). Since a recent report has shown that EGFR and NGF can utilize Shc in different ways to promote their activation (25), one explanation for this disparity is that EGFR utilizes more dominantly through the direct binding of Grb2-Sos complex to the autoprophosphorylated EGFR, whereas NGFR utilizes Shc-Grb2-Sos complex for Ras activation. It is also possible that this difference may reflect the difference in these cell types (lymphoid cells versus neuronal cells).

In contrast to no effect of disruption of Shc on EGF-mediated ERK response, Shc-deficient cells failed to induce EGF-mediated JNK response (Fig. 4B). The effect of Shc on the JNK pathway is presumably mediated by Rho family GTPases because Rac1N17, but not RasN17, inhibited the EGF-mediated JNK response completely (Fig. 6C). Although Rho family GTases mainly contribute to the JNK signaling pathway, previous results using fibroblasts have shown that the EGF-mediated JNK pathway is inhibited by expression of not only Rac1N17 but also RasN17 (14, 15), suggesting a cross-talk between these GTases (32). These somewhat inconsistent findings might reflect the fact that the existence of this cross-talk between Rho family and Ras varies depending on different cell types. Indeed, RasN17 inhibited only the ERK pathway without affecting the JNK pathway in DT40 B cells (Fig. 6, B and C).

Analogous to the mechanism by which Grb2 activates Ras, Shc might bring members of the family of guanine nucleotide exchange factors (GEFs) for the Rho family GTases (of which Vav is a member) to the plasma membrane where these GTases are located. Assuming that Vav is a sole target, one would predict that the EGF-mediated JNK response is transient like the ERK response. However, as shown in this study, the EGF-mediated JNK response in DT40 B cells was marked at 30 min although the activation was observed at 10 min. Thus, our data suggest that two distinct GTases might be involved in the initial phase and the sustained JNK activation, respectively. Reminiscent of this type of regulation is NGF-mediated ERK activation; the early phase of the ERK activation is mediated by Ras, but sustained activation of this pathway is because of activation of Rap1 (33). Because Rac1N17 is able to inhibit not only Rac1, but also other Rho family GTases, an almost complete block of the EGF-mediated JNK response by introduction of Rac1N17 could be accounted for by simultaneous blockade of two distinct GTases by Rac1N17.

Acknowledgments—We thank Dr. D. Cantrell for providing the GST-RBD expression plasmid and thank K. Gotoh for technical assistance.

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