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Tyrosine Phosphorylation Sites at Amino Acids 239 and 240 of Shc Are Involved in Epidermal Growth Factor-Induced Mitogenic Signaling That Is Distinct from Ras/Mitogen-Activated Protein Kinase Activation

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Epidermal growth factor (EGF) induces tyrosine phosphorylation of the Shc adapter protein, which plays an important role in EGF-stimulated mitogenesis. Shc stimulates Ras/mitogen-activated protein kinase (MAPK) through forming a complex with Grb2 at the phosphorylated tyrosine (Y) residue 317. In this study, we identified novel phosphorylation sites of Shc, at Y239 and Y240. To define the Shc pathway further, we used NIH 3T3 cells expressing the previously characterized mutant EGF receptor (EGF-R) which lacks all known autophosphorylation sites but retains EGF-stimulated mitogenesis with selective phosphorylation of Shc. We constructed wild-type (WT) or mutant Shc cDNAs in which Y317 or/and Y239 and Y240 are replaced with phenylalanine (F) and introduced them into NIH 3T3 cells expressing WT or mutant EGF-R. In the WT EGF-R-expressing cells, the Y239/240/317F Shc, but not Y317F or Y239/240F Shc, decreased EGF-stimulated cell growth. In the mutant EGF-R-expressing cells, Y317F Shc or Y239/240F Shc decreased EGF-stimulated cell growth significantly, though Y317F was a little more potent than Y239/240F. Although cells expressing the Y317F Shc hardly activated MAPK in response to EGF, cells expressing the Y239/240F Shc fully activated MAPK. In contrast, Y239/240F Shc, but not Y317F Shc, reduced the EGF-induced c-*myc* message. These results suggest that Shc activates two distinct signaling pathways, Y317 to Ras/MAPK and Y239 and Y240 to another pathway including Myc, and that both are involved in EGF-induced mitogenic signaling.

Upon stimulation with growth factors, receptor tyrosine kinases, including the epidermal growth factor (EGF) receptor (EGF-R), activate and phosphorylate themselves (59). This receptor autophosphorylation creates binding sites for SH2 or phosphotyrosine binding (PTB) domain-containing signaling molecules such as Shc or Grb2, resulting in the activation of multiple signal transduction pathways near the plasma membrane (10, 30, 43).

An adapter protein, Shc, lacks a catalytic domain but has an SH2 domain (C terminal) and a PTB/phosphotyrosine interaction (PI) domain (N terminal) (4, 28, 44). Shc becomes tyrosine phosphorylated upon stimulation with a number of growth factors, including EGF, as well as oncogenic tyrosine kinases (18, 40, 44, 56). When the EGF-R tyrosine kinase phosphorylates Shc, the SH2 domain appears to play a key role. Shc binds to the autophosphorylated EGF-R via the SH2 or the PTB/PI domain (3, 4, 17). This appears to lead to efficient tyrosine phosphorylation of Shc (17). However, a mutant EGF-R lacking all known autophosphorylation sites still phosphorylates Shc without Shc binding to the receptor, indicating that for the tyrosine phosphorylation of Shc, autophosphorylation sites on the EGF-R appear unnecessary (18, 34). Under these conditions, some auxiliary subunits of EGF-R such as ErbB-2 or some membrane proteins may serve as binding sites for Shc (18, 27, 53). We previously showed that the Shc SH2 domain suppresses the EGF-induced mitogenesis of fibroblasts in a dominant negative manner (17). Another group has reported that microinjection of antibodies against Shc suppresses the mitogenesis of fibroblasts in response to EGF, insulin, or insulin-like growth factor 1 (52). These findings suggest that Shc is important for growth factor-induced mitogenesis.

It is widely accepted that Shc is involved in activation of Ras (2, 16–18, 43, 45, 49, 56). Ras activation is an immediate-early response to growth factors that is believed essential for mitogenesis of fibroblasts (14, 41, 54, 58). Grb2 is another adapter protein that contains SH2 and stably binds to mSOS, a Ras GDP/GTP exchange protein (6, 8, 35, 39, 50). Tyrosine-phosphorylated Shc forms a complex with Grb2-mSOS at the phosphorylated tyrosine (Y) residue 317 via the SH2 domain of Grb2 (51). This stimulates Ras activation, which leads to stimulation of mitogen-activated protein kinase (MAPK), resulting in the activation of nuclear transcription factors to induce immediate-early genes, such as c-*fos* (21, 25, 36, 38, 42, 46).

EGF-R stimulates Ras activation through at least two routes. One is through the direct binding of the Grb2-mSOS complex to the autophosphorylated EGF-R at Y1068 via the SH2 domain of Grb2 (3, 6, 15, 33, 50). The other is through the phosphorylated Shc-Grb2-mSOS complex (17, 49). These two routes seem to contribute redundantly to Ras activation (2, 17).

A mutant EGF-R in which Y1068 (the major Grb2 binding site) is substituted with phenylalanine, resulting in a decrease in the direct binding of Grb2 to the receptor, almost fully stimulates mitogenesis in response to EGF (24, 26). This finding suggests that the decreased activation of the receptor-Grb2-mSOS route for Ras does not affect strongly the mitogenic signaling of EGF-R, probably because the Shc-Grb2-mSOS route remains intact. On the other hand, the dominant

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negative Shc mutant or antibodies to Shc suppress EGF-induced mitogenesis despite the retained receptor-Grb2-mSOS route (17, 52). Therefore, Shc might send another signal distinct from Ras activation for mitogenesis of fibroblasts.

Induction of the c-*myc* message is another important immediate-early response to growth factors (21, 42, 46). EGF induces a detectable c-*myc* message within 30 min. The mechanism of c-*myc* mRNA induction remains unknown but may be distinct from the Ras pathway (1, 48). Many lines of evidence suggest that Myc is important for mitogenesis. For example, microinjection of antisense RNA specific to c-*myc* blocks entry of fibroblasts into S phase (23). The ectopic induction of Myc activity is sufficient to drive quiescent fibroblasts into the cell cycle (13). It has been reported that v-*ras* and c-*myc* transform fibroblasts cooperatively (32). This finding is consistent with the notion that Ras and Myc act cooperatively at different points for cell proliferation.

Here, we identified new tyrosine phosphorylation sites of Shc, Y239 and Y240 (collectively referred to as Y239/240). We next examined the role of the phosphorylation sites of Shc in EGF signaling, using a truncated mutant EGF-R. This mutant EGF-R lacks all of the identified autophosphorylation sites but selectively phosphorylates Shc without activating other molecules such as phospholipase C-γ1 (PLC-γ1) (17, 18). Thus, it allowed us to analyze the Shc pathway without interference by other signaling pathways. We provide evidence to support the notion that the phosphorylation of Y239/240 of Shc sends a previously unrecognized signal distinct from Ras/MAPK activation for the EGF-induced mitogenesis of fibroblasts. This pathway and the Shc-Ras pathway appear to cooperatively contribute to efficient cell proliferation stimulated by EGF. Furthermore, we show that this signal may be involved at least in part in c-*myc* mRNA induction in response to EGF.

MATERIALS AND METHODS

Construction of Shc mutants. The Y239/240F, Y239F, or Y240 Shc cDNA was engineered by PCR-mediated mutagenesis. The mutagenic oligonucleotides were 5′-CCATCAGTTCTTTAATGACTTCCCGGGGAA-3′/5′-AAGTCATTAAAGAACTGATGGTCAGGTGGCTC-3′, which changes Y239/240 to two phenylalanines, 5′-CCATCAGTTCTATAATGACTTCCCGGGGAA-3′/5′-AAGTCATTATAGAACTGATGGTCAGGTGGC-3′, which changes Y239 to F, and 5′-TACTTTAATGACTTGCCGGGGAAGGAACCCCCC-3′/5′-CCCCGGCAAGTCATTAAAGTACTGATGGTCAGGTGG-3′, which changes Y240 to F. Each PCR-synthesized *Bam*HI-*Pst*I fragment was replaced with the wild-type (WT) Shc cDNA and sequenced to ensure that the only substituted positions were modified. The *Bam*HI-*Eco*RI fragment or the *Xho*I-*Xba*I fragment of the WT, Y317F (17), Y239/240F, Y239F, or Y240F Shc cDNA was cloned into the pGEX-2T vector (Promega) or the pKU-Hyg expression vector containing a hygromycin-selectable marker.

Cell culture and expression of Shc mutants. We established the WT or mutant (DEL+F992) human EGF-R-overexpressing NIH 3T3 cells, which are devoid of functional endogenous EGF-R, as described previously (18). In the DEL+F992 EGF-R mutant, the C-terminal domain is truncated at residue 1011 and Y992 is changed to phenylalanine, resulting in a lack of most of the autophosphorylation sites. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 5% newborn calf serum (NBCS) (Gibco).

Cells were transfected with 15 μg of the expression vectors by the calcium phosphate method and selected with hygromycin (200 μg/ml for cells expressing the WT EGF-R; 50 μg/ml for cells expressing the mutant EGF-R; Wako, Tokyo, Japan).

In vitro phosphorylation of GST-Shc proteins. In vitro phosphorylation experiments were carried out as previously described (17). Briefly, cells were lysed in ice-cold lysis buffer A (50 mM HEPES [pH 7.4], 150 mM NaCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 10% glycerol, 0.5% Nonidet P-40, 10 μg of aprotinin per ml). The cell lysate was incubated with EGF (1 μg/ml) at 25°C for 30 min and subjected to immunoprecipitation with anti-EGF-R antibody (clone EGF-R1; Amersham) prebound to protein G-Sepharose at 4°C for 2 h. The washed immunoprecipitates were incubated with purified glutathione *S*-transferase (GST)–Shc and Shc mutant proteins (2 μg) in 30 μl of phosphorylation buffer (20 mM HEPES [pH 7.5], 100 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 10% glycerol, 0.1% Nonidet P-40) containing 3 μM ATP and 10 μCi of [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham) at 30°C for 30 min.

In vivo labeling of cellular phosphoproteins and stimulation of cells with EGF. Cells were starved overnight in phosphate-free DMEM containing 0.5% dialyzed NBCS, labeled with 1.5 mCi of $^{32}P_i$ (NEX-053; NEN) per ml for 4 h, and treated with EGF (50 ng/ml) at 37°C for 5 min.

Phosphopeptide mapping. To obtain in vivo-labeled Shc protein, the in vivo-labeled and EGF-stimulated or unstimulated cells were lysed, and the cell lysates were immunoprecipitated with anti-Shc antibodies (Upstate Biotechnology, Inc.). The immunoprecipitates or the in vitro-phosphorylated GST-Shc proteins were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels and visualized on dried gels by autoradiography. ^{32}P-labeled Shc was eluted from the gel, precipitated by trichloroacetic acid, and subjected to two-dimensional phosphopeptide mapping or phosphoamino acid analysis on thin-layer cellulose plates by the Hunter thin-layer electrophoresis system (CBS Scientific, Del Mar, Calif.) as described previously (5). For tryptic phosphopeptide mapping, electrophoresis was carried out for 30 min at 1,000 V in pH 1.9 buffer.

Immunoprecipitation and immunoblot analysis. Cells were starved in 0.5% NBCS overnight and subsequently treated with EGF (50 ng/ml) at 37°C for 5 min. The cells were lysed as described previously (17). Immunoprecipitation with anti-Shc antibodies (Upstate Biotechnology, Inc.) prebound to protein A-Sepharose (Pharmacia) and immunoblot analysis with antiphosphotyrosine antibody (PY-20; ICN), anti-Shc antibody (Transduction Laboratories), anti-phospho-MAPK antibody (Bio-Rad), or anti-Grb2 antibody (Transduction Laboratories) were carried out as described previously (17).

Cell growth assay. [^{3}H]thymidine incorporation triggered by EGF was analyzed as described previously (19). Examination of cell growth rate was carried out as described previously (18). Cells were seeded at 2×10^4 per 35-mm-diameter dish in 0.5% NBCS for WT EGF-R-expressing cells and 0.1% NBCS for mutant EGF-R-expressing cells. Subsequently, the medium was changed for DMEM containing 0.5% NBCS for WT EGF-R-expressing cells and 0.1% NBCS for mutant EGF-R-expressing cells, with or without EGF (1 ng/ml for WT EGF-R-expressing cells; 10 ng/ml for mutant EGF-R-expressing cells). The medium was changed every 2 days.

In-gel MAPK assay. Cells were starved in 0.5% NBCS overnight, treated with EGF (100 ng/ml) at 37°C for 3 min, and lysed as described previously (18). The cell lysates were separated on SDS-polyacrylamide gels containing 0.5 mg of myelin basic protein, a specific substrate for MAPK, per ml. The in-gel MAPK assay was performed as described previously (18).

Northern blot analysis. Northern blot analysis for total cellular RNA (15 μg for each sample) was carried out as described previously (55). The probe cDNA for c-*myc* was described previously (55).

RESULTS

Identification of novel tyrosine phosphorylation sites of Shc, Y239 and Y240. To examine the notion that Shc has an important function distinct from Ras activation, we analyzed the tyrosine phosphorylation sites of Shc. We performed in vitro kinase assays using immunoprecipitated EGF-R from EGF-R-overexpressing cells and the GST-fused Shc protein as a substrate. As shown in Fig. 1A, the EGF-R and Shc protein were strongly phosphorylated in response to EGF. Phosphoamino acid analysis showed that the Shc protein became phosphorylated only on tyrosine residues by EGF-R tyrosine kinase (Fig. 1C).

We also examined in vivo phosphorylation of Shc by the EGF-R. We transfected the Shc cDNA in EGF-R-expressing NIH 3T3 cells, established stable cell lines (see Fig. 3), and labeled these cells with $^{32}P_i$. We stimulated these cells with EGF, and the cell lysates were immunoprecipitated with anti-Shc antibodies and then subjected to SDS-gel electrophoresis. Upon EGF stimulation, the phosphorylation level of Shc was increased about 10-fold, based on the intensity of bands corresponding to Shc (data not shown).

We compared two-dimensional tryptic phosphopeptide maps of in vitro- and in vivo-phosphorylated Shc proteins (Fig. 1B). The in vitro map resolved the major four spots 1, 2, 2′, and 3. It appears that the minor spots below spots 2, 2′, and 3 are partially digested products, since they did not appear clearly or reproducibly. The in vivo map resolved the major six spots a, b, b′, c, d, and e, all of which appeared upon EGF stimulation (Fig. 1B and data not shown). The in vitro, in vivo, and mixed maps revealed that in vitro spots 1, 2, 2′, and 3 correspond to in vivo spots a, b, b′, and c, respectively. This finding indicates that the Shc protein becomes phosphorylated at similar sites in

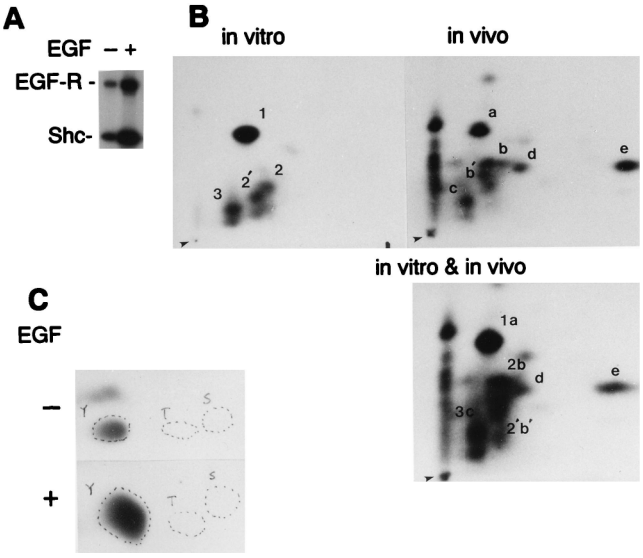

FIG. 1. Two-dimensional tryptic phosphopeptide mapping of Shc protein in vitro and in vivo. (A) In vitro kinase activity of EGF-R tyrosine kinase for Shc protein. Lysates from EGF-R-expressing cells were incubated with or without EGF (1 μg/ml) for 30 min at 30°C and immunoprecipitated with anti-EGF-R antibody. The immunoprecipitates were incubated with [γ-^{32}P]ATP and GST-fused Shc protein for 30 min at 30°C. (B) Two-dimensional tryptic phosphopeptide mapping of the labeled Shcs in vitro and in vivo. Cells expressing the WT Shc and EGF-R were labeled with $^{32}P_i$, stimulated with EGF (50 ng/ml) for 5 min, and immunoprecipitated with anti-Shc antibodies. The immunoprecipitates or in vitro-phosphorylated Shc protein as described above was subjected to tryptic phosphopeptide mapping. Arrows indicate the sample loading origin. Each map was obtained from ~800 Cerenkov cpm (400 cpm of each sample for the mix), and the plates were exposed for autoradiography for 5 days. (C) Two-dimensional phosphoamino acid analysis of in vitro-phosphorylated Shc protein. Positions of tyrosine (Y), serine (S), and threonine (T) residues are indicated.

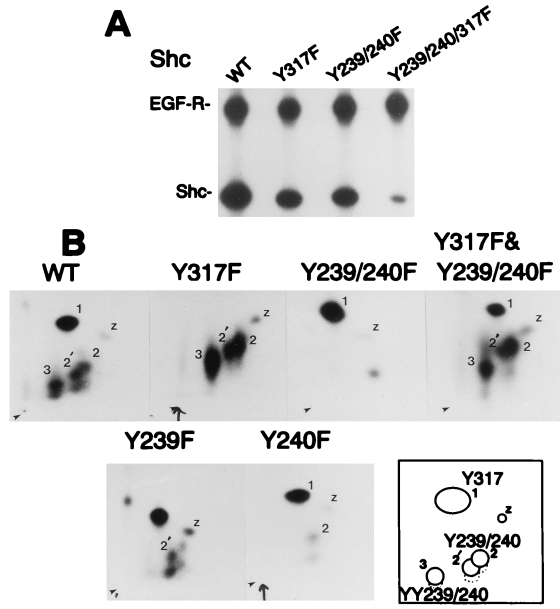

FIG. 2. Novel phosphorylation sites of Shc, Y239 and Y240, in vitro. (A) In vitro phosphorylation of the WT or mutant Shc proteins by the EGF-R tyrosine kinase. The immunoprecipitated EGF-R and the GST-fused WT or mutant Shcs were analyzed by the in vitro kinase assay as described in the legend to Fig. 1. (B) Two-dimensional tryptic phosphopeptide mapping of the in vitro-phosphorylated WT or mutant Shcs. Each map was obtained from ~2,000 cpm, and the plates were exposed for 2 days. The phosphopeptides containing Y239/240 sometimes showed their partial digestive products, indicated by dotted circles below the spots.

vitro and in vivo and that Shc has some tyrosine phosphorylation sites other than the known phosphorylation site, Y317. We searched for potential phosphoacceptor sites of Shc from the sequence. Y239 and Y240 were candidates, since they are located between the PTB and SH2 domains and this region contains Y317. We mutated Y239 and Y240 or Y317 of Shc cDNA to F, expressed them as GST-fused proteins, and performed an in vitro kinase assay. Interestingly, although the Y317F or Y239/240F Shc was clearly phosphorylated to a lesser extent than WT Shc, the phosphorylation level of Y239/240/317F Shc was markedly decreased (Fig. 2A), strongly suggesting that both Y239/240 and Y317 are major phosphorylation sites of Shc.

The in vitro-phosphorylated WT or mutant Shcs were examined by tryptic phosphopeptide mapping. As shown in Fig. 2B, spot 1 was absent from the map of the Y317F Shc and spots 2, 2′, and 3 were absent from the map of the Y239/240F Shc. The mixed maps of the Y317F and Y239/240F Shc, the Y317F and WT Shc, and the Y239/240F and WT Shc are essentially identical to that of WT Shc (Fig. 2B and data not shown). Thus, the peptide containing Y317 corresponds to spot 1 and those with Y239/240F correspond to spots 2, 2′, and 3.

To examine which spot, 2, 2′, or 3, corresponds to phosphorylation of Y239 and/or Y240, we mutated Y239 and Y240 of Shc to F, expressed them as GST-fused proteins, and examined them by in vitro kinase assay and tryptic phosphopeptide mapping. In the map of either Y239F or Y240F Shc, spot 3 was absent and the spots 2 and 2′ became single, accompanied by their partial digestion products. The doubly phosphorylated phosphoisomer should represent an increased negative charge/mass ratio and reduced hydrophobicity, which would cause it to migrate closer to the origin than the singly phosphorylated form. Thus, it appears that spot 3 corresponds to a peptide containing doubly phosphorylated Y239/240 and spots 2 and 2′ correspond to the singly phosphorylated form. We thus concluded that Shc has three major tyrosine phosphorylation sites, Y239, Y240, and Y317, in vitro.

To examine whether these sites are phosphorylated in vivo upon EGF stimulation, we transfected Y317F or Y239/240F Shc cDNA into EGF-R-expressing NIH 3T3 cells and established stably overexpressing cells (Fig. 3A). We obtained in vivo-phosphorylated Shc proteins by EGF-R and subjected it to two-dimensional tryptic phosphopeptide mapping. As shown in Fig. 4, in the map of the Y317F Shc, spot a was absent and in the map of the Y239/240F Shc, spots b, b′, and c were absent. Two minor spots, x and y, around spot a also appeared upon EGF stimulation.

To examine the tyrosine phosphorylation level of Shc in vivo when these three sites are missing, we transfected the Y239/240/317F Shc cDNA into NIH 3T3 cells expressing EGF-R and established stably expressing cells (Fig. 3A). We stimulated cells expressing WT or mutant Shcs with EGF. These cells express the same level of the EGF-R, and its autophosphorylation occurred at a similar level after EGF stimulation (data not shown). Cells were lysed, immunoprecipitated with anti-Shc antibodies, and immunoblotted with antiphosphotyrosine antibody. As shown in Fig. 3B, tyrosine phosphorylation of the Y239/240/317F Shc was decreased compared with that of the WT, Y239/240F, or Y317F Shc. The panel of vector-transfected cells in Fig. 3B showed a lower phosphorylation of the EGF-R than that of the WT Shc-expressing cells. This was due to smaller amounts of the EGF-R that were coimmunoprecipitated with anti-Shc antibodies, since the amounts of Shc in

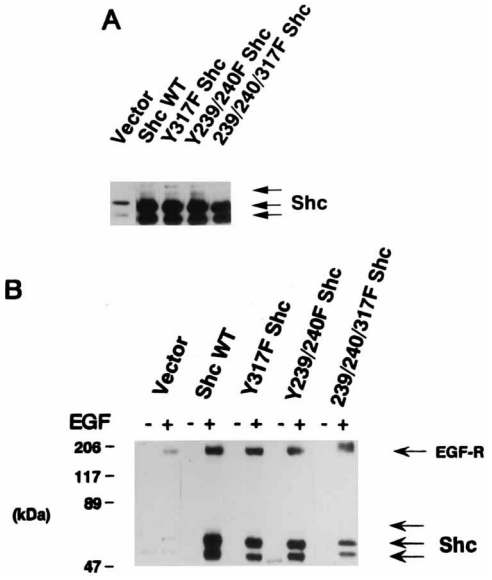

FIG. 3. Decreased tyrosine phosphorylation of the Y239/240/317F Shc stimulated with EGF. (A) Expression of the WT or mutant Shcs in EGF-R-expressing NIH 3T3 cells. Cell lysates were immunoblotted with anti-Shc antibody. (B) Cells expressing the WT or mutant Shcs were stimulated with EGF, immunoprecipitated with anti-αShc antibody, and immunoblotted with antiphosphotyrosine antibody.

vector-transfected cells were much smaller than that of WT Shc-overexpressing cells (Fig. 3A). We therefore conclude that Shc becomes phosphorylated at Y239, Y240, and Y317 in vivo and in vitro upon EGF stimulation.

Inhibition of EGF-stimulated mitogenic signaling by Y239/240F or Y317F Shc in DEL+F992-expressing cells. To examine the role of each phosphorylation site of Shc in EGF signaling, we used cells expressing the autophosphorylation site-defective mutant EGF-R (DEL+F992), since the signal transduction from this particular mutant EGF-R appears to be highly dependent on the activation of Shc (18). We transfected the WT or mutant Shc cDNAs into NIH 3T3 cells expressing the mutant EGF-R

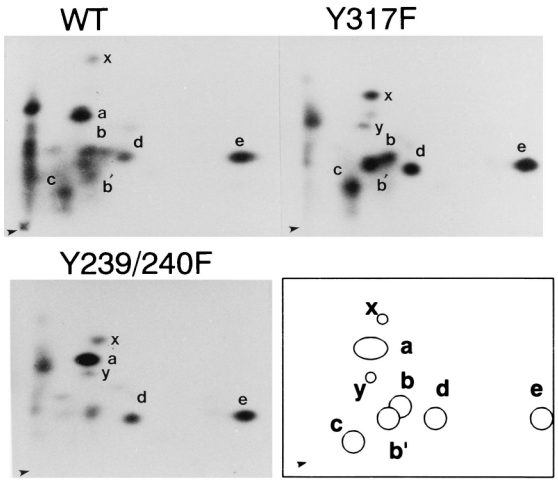

FIG. 4. Novel phosphorylation sites of Shc, Y239 and Y240, in vivo. Each two-dimensional tryptic phosphopeptide map of the in vivo-phosphorylated WT and mutant Shcs was obtained from ~300 cpm (200 cpm of each sample for the mix), and the plates were exposed for 14 days.

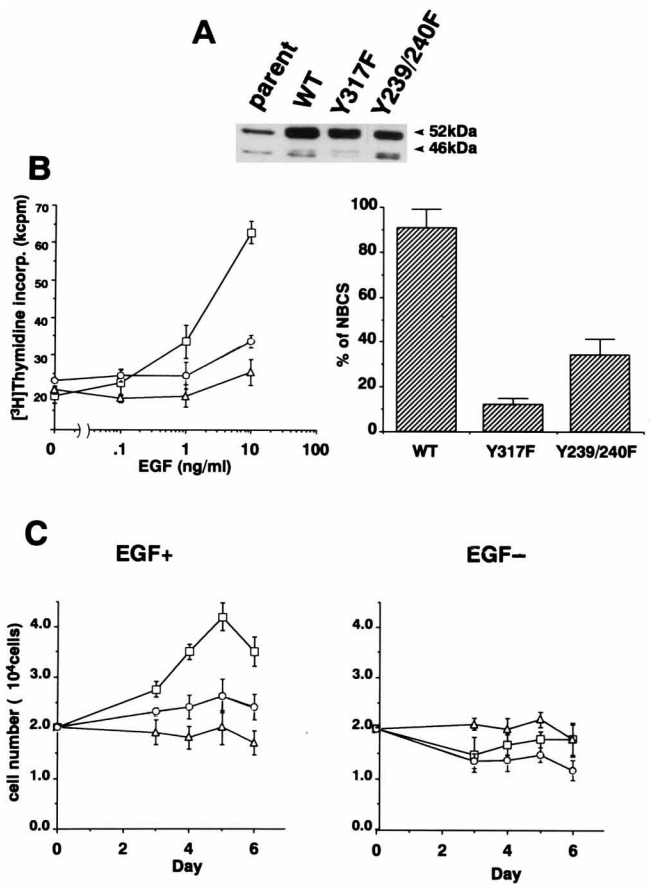

FIG. 5. Inhibition of the EGF-stimulated growth activity by the Y317F or Y239/240F Shc in the autophosphorylation site-defective mutant EGF-R-expressing cells. (A) Expression of the WT and mutant Shcs in the mutant EGF-R-expressing NIH 3T3 cells. The cell lysates were immunoblotted with anti-Shc. (B) Left, [^{3}H]thymidine incorporation (incorp.) of the WT or mutant Shc-expressing cells by EGF. Quiescent cells were incubated with various concentrations of EGF for 22 h and labeled with [^{3}H]thymidine for the last 4 h. Results in both panels are indicated by the means ± standard deviations of triplicates of three independent experiments. Similar results were obtained with two to three independent clones. □, WT Shc; ○, Y239/240F Shc; △, Y317F Shc. Right, [^{3}H]thymidine incorporation at 10 ng of EGF per ml, expressed as percentage responses upon treatment with 5% NBCS. (C) Growth curves of WT or mutant Shc-expressing cells with or without EGF. □, WT Shc; ○, Y239/240F Shc; △, Y317F Shc.

and established several lines expressing them at similar levels (Fig. 5A).

We starved the cells in a low-serum medium for 48 h, stimulated them with increasing concentrations of EGF, and measured the ability to trigger DNA synthesis (Fig. 5B, left). In cells expressing the WT Shc, DNA synthesis was efficiently triggered upon EGF stimulation, consistent with our previous results (18). In the cells expressing Y239/240F Shc, DNA synthesis did not occur significantly at 1 ng of EGF per ml. Upon stimulation of these cells with a saturating concentration of EGF (10 ng/ml), DNA synthesis was triggered but to lesser extent: about 40% of that of the cells expressing WT Shc (Fig. 5B, right). In the cells expressing Y317F Shc, DNA synthesis was more impaired: about 15% of that of the WT Shc-expressing cells at a saturating concentration of EGF (Fig. 5A and B).

We next measured the EGF-stimulated growth rate of the cells expressing WT or mutant Shcs. None of the cells proliferated in 0.1% serum without EGF (Fig. 5C, right). As shown

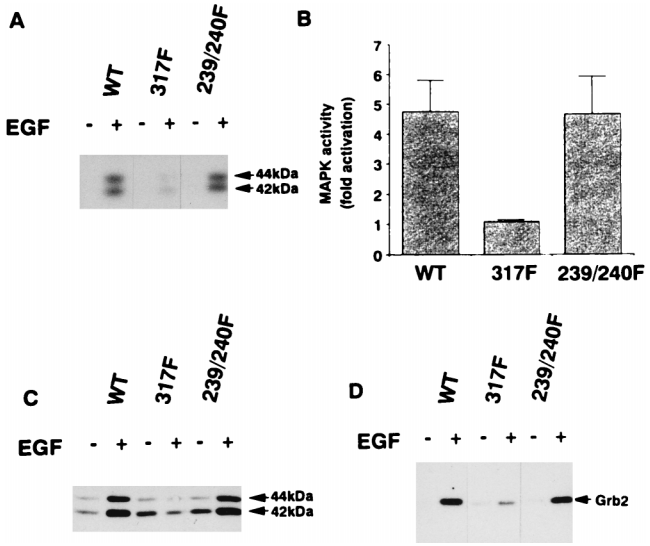

FIG. 6. Inhibition of EGF-stimulated MAPK activation by the Y317F Shc but not by the Y239/240F Shc. The WT or mutant Shcs and the autophosphorylation site-defective mutant EGF-R-expressing cells were stimulated with or without EGF. (A) The cell lysates were resolved on an SDS-polyacrylamide gel containing myelin basic protein, and MAPK activity was analyzed by the in-gel kinase assay. (B) The increase in MAPK activity analyzed by the in-gel kinase assay is expressed relative to that of unstimulated activity. Results are indicated by the means ± standard deviations of triplicates of three independent experiments with two to three independent clones. (C) The cell lysates were resolved on an SDS-polyacrylamide gel and immunoblotted with anti-phospho-MAPK antibody. (D) The cell lysates were immunoprecipitated with anti-Shc antibody and immunoblotted with anti-Grb2 antibody.

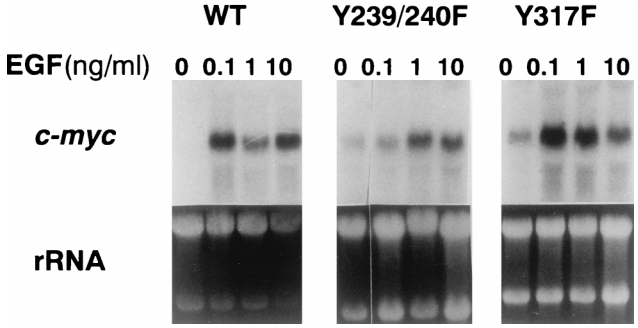

FIG. 7. Inhibition of c-*myc* message induction by the Y239/240F Shc but not by the Y317F Shc. Cells were stimulated with various concentrations with EGF for 30 min. Northern blot analyses were performed with probes representing portions of c-*myc*.

in the left panel of Fig. 5C, the cells expressing WT Shc grew efficiently in response to EGF. However, the cells expressing Y239/240F Shc grew to a lesser extent, and those expressing Y317F Shc did not grow in response to EGF. Thus, Y239/240F Shc and Y317F Shc inhibited EGF-stimulated growth activity in cells expressing the mutant EGF-R, and this effect was a little more potent in the Y317F Shc than in the Y239/240F Shc.

Inhibition of EGF-stimulated MAPK activation by Y317F Shc but not by Y239/240F Shc. Shc stimulates Ras/MAPK activation through forming a complex with Grb2 at phosphorylated Y317 (51). To examine whether the mutant forms of Shc coimmunoprecipitated with Grb2 after EGF stimulation, we stimulated cells expressing both the mutant EGF-R and the mutant forms of Shc with EGF. Cells were lysed, immunoprecipitated with anti-Shc antibodies, and immunoblotted with anti-Grb2 antibody. Upon stimulation with EGF, WT and Y239/240F Shc strongly bound to Grb2 at similar levels (Fig. 6D). In contrast, Y317F Shc bound to Grb2 only slightly (Fig. 6D).

We next examined the effects of the Shc mutants on MAPK activation. We stimulated cells with EGF, and the cell lysates were subjected to an in-gel kinase assay using myelin basic protein as a specific substrate of MAPK. As shown in Fig. 6A and B, cells expressing the Y317F Shc did not activate MAPK to detectable levels in response to EGF. However, cells expressing the WT or Y239/240F Shc activated MAPK efficiently to similar levels in response to EGF.

Next we confirmed the inhibition of MAPK activation in Y317F Shc cells but not in Y239/240F Shc cells by examining MAPK phosphorylation. Activated MEK phosphorylates serine, threonine, and tyrosine residues of MAPK, which leads to MAPK activation. We stimulated cells expressing both the mutant EGF-R and the mutant forms of Shc with EGF. Cells were lysed, and the total cell lysates were immunoblotted with anti-phospho-MAPK antibody specific to the phosphorylated and activated MAPK. As shown in Fig. 6C, cells expressing Y317F Shc did not activate MAPK to detectable levels. This finding is consistent with the results obtained by the in-gel kinase assay (Fig. 6A and B). Thus, we conclude that in vivo, phosphorylated Y239/240 Shc activates MAPK only slightly whereas phosphorylated Y317 does so significantly. It therefore appears that in cells expressing the mutant EGF-R, the main route for activation of Ras/MAPK pathway starts from Shc and the responsible tyrosine residue of Shc is Y317.

Suppression of EGF-stimulated c-*myc* message induction by the Y239/240F Shc but not by the Y317F Shc. Since we obtained evidence that Y239/240 of Shc contributes little, if at all, to the Ras/MAPK pathway, we examined whether it contributes to other signaling pathways, such as c-*myc* message induction. Numerous mechanisms are thought to operate in growth factor-stimulated c-*myc* message induction (57). If Shc affects c-*myc* message induction directly, the initial response of c-*myc* might be strongly affected, since Shc phosphorylation occurs rapidly within 5 min after EGF stimulation.

We stimulated cells with EGF for 30 min, and then total RNAs were extracted and subjected to Northern blotting with a c-*myc* probe. Figure 7 shows that in cells expressing WT or Y317F Shc, c-*myc* message was efficiently induced and saturated at 0.1 ng of EGF per ml. In cells expressing Y317F Shc, the c-*myc* message tended to be decreased at high concentrations of EGF. Interestingly, in cells expressing Y239/240F Shc, c-*myc* message was hardly detectable at 0.1 ng of EGF per ml, and it was induced with increased concentrations of EGF, reaching saturation between 1 and 10 ng of EGF per ml. Therefore, it appears that Shc affects signaling pathways to induce c-*myc* and that the responsible tyrosine residue of Shc is Y239/240, not Y317.

Inhibition of EGF-stimulated mitogenic signaling by Y239/240/317F Shc in WT EGF-R-expressing cells. We examined the role of the phosphorylation sites of Shc in the WT EGF-R signaling. We transfected the WT, Y317F, Y239/240F, or Y239/240/317F Shc cDNA cloned in the expression vector which contained the hygromycin-selectable marker into NIH 3T3 cells expressing WT EGF-R. When many hygromycin-resistant colonies appeared, we picked up several independent colonies to establish stable cell lines (Fig. 3A). We mixed the remaining colonies and cultured them. We called this mass culture.

To examine the growth activity of the mutant forms of Shc-expressing cells, we analyzed the mass cultures. Under serum-

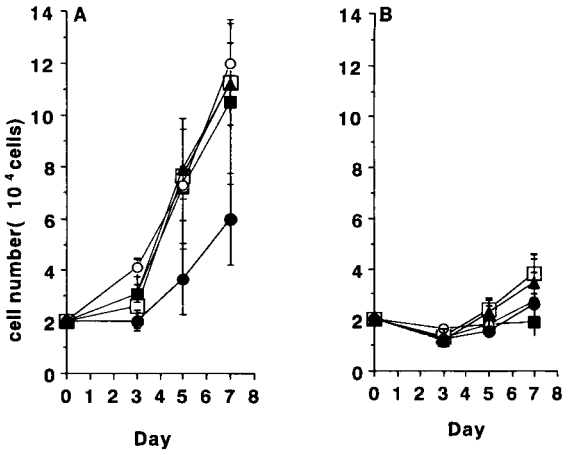

FIG. 8. Inhibition of the EGF-stimulated growth activity by Y239/240/317F Shc in the WT EGF-R-expressing cells. Shown are growth curves of cells expressing WT or mutant Shcs with (A) or without (B) EGF. □, WT Shc; ▲, Y317F Shc; ○, Y239/240F Shc; ●, Y239/240/317F Shc; ■, vector control.

starved conditions without EGF, none of the cells proliferated (Fig. 8B). As shown in Fig. 8A, cells expressing WT Shc efficiently stimulate cell growth in response to EGF. On the other hand, proliferation of cells expressing Y239/240/317F Shc lacking all three sites was about 60% suppressed compared to that of WT Shc-expressing cells. Cells expressing Y239/240F or Y317F Shc lacking either site proliferated rather efficiently, reaching a level similar to that of WT Shc. Analysis of several stable cell lines showed similar results (data not shown).

These results suggest that Y239/240 and Y317 on Shc play a role in EGF-induced mitogenesis through WT EGF-R, although a defect in either site did not affect it remarkably.

DISCUSSION

In this report, we describe novel phosphorylation sites of Shc, Y239 and Y240. We show that phosphorylation of Y239/240 plays an important role in EGF-stimulated mitogenesis by sending a signal distinct from that for Ras/MAPK activation, the established role of Shc. Furthermore, we show that this signal may be involved in c-*myc* induction and that the Shc-Ras pathway and this pathway appear to cooperatively stimulate mitogenesis of fibroblasts.

Phosphorylation sites on Shc other than Y239, Y240, and Y317. In the in vivo map, there appeared two major spots, d and e, that were absent in the in vivo map, in addition to those of Y239, Y240, and Y317 (Fig. 4). The intensity of spots d and e also increased remarkably upon EGF stimulation. Spots d and e may represent peptides which are phosphorylated on tyrosine residues in vivo but not efficiently in the context of GST-fused Shc in vitro, or they may represent the peptides phosphorylated on serine or/and threonine residues by some kinases other than the EGF-R.

In the in vivo map, there appeared two minor spots, x and y, that were clearer in the map of Y317F or Y239/240F Shc than that of WT Shc (Fig. 4). In the map of WT Shc, spot y was hardly detectable. The peptides corresponding to spots x and y might be phosphorylated when appropriate sites Y317 or Y239/240 are lost.

In the map of Y239F Shc (Fig. 2), the intensity of spot 2, which represents phosphorylation of Y240, was stronger than that of spot 2, which represents phosphorylation of Y239 in the map of Y240F. There might be a tendency for Y240 to become phosphorylated prior to the phosphorylation of Y239.

Usefulness of mutant EGF-R-expressing cells for analyzing Shc. To investigate the role of each phosphorylation site of Shc in EGF signaling, we took advantage of the autophosphorylation-minus mutant EGF-R (DEL+F992) system (11, 12, 17, 18, 22, 37, 60). This mutant EGF-R is truncated at residue 1011 and has a Y-to-F substitution at the minor phosphorylation site Y992, resulting in autophosphorylation-minus EGF-R, but is still able to transduce mitogenic signals. In the mutant EGF-R system, signaling pathways requiring autophosphorylation appear to be eliminated.

For EGF-R-to-Ras activation, there are two routes that do and do not require autophosphorylation (6, 17, 18). The EGF-R activates Ras through direct binding to Grb2-mSOS complex (2, 3, 6). This route requires phosphorylated Y1068 of the EGF-R as a binding site of the SH2 domain of Grb2 and is eliminated in the mutant EGF-R system. The EGF-R also activates Ras through tyrosine-phosphorylated Shc, which forms a complex with Grb2-mSOS via the SH2 domain of Grb2 (2, 17, 18, 45, 49). Since the autophosphorylation-minus mutant EGF-R can phosphorylate Shc, this route does not require autophosphorylation of the EGF-R and is retained in the mutant EGF-R system (18, 34).

Another signaling pathway from WT EGF-R is activation of PLC-γ. The pathway is also eliminated for the most part in the mutant EGF-R system, since binding of PLC-γ to the autophosphorylated EGF-R via the SH2 domain of PLC-γ is essential for increase in catalytic activity of PLC-γ (29, 47). Therefore, the mutant EGF-R signaling, in which the Shc pathway is selectively retained, allowed for simplified and clear analysis.

Is Y239/240 involved in Grb2/mSOS/Ras activation? The known consensus sequence for potential Grb2 binding is phosphotyrosine-X-asparagine. The surrounding sequence of Y239/240 is YYND. When Y239 but not Y240 is phosphorylated, the surrounding sequence containing Y239/240 forms the phosphotyrosine-X-asparagine. However, as shown in Fig. 6D, Y317F Shc only slightly bound to Grb2. This very weak binding might be due to the Grb2 binding of Y317F Shc that was phosphorylated on Y239 or the Grb2 binding to the endogenous Shc. Based on the low level of Grb2 binding, Y239/240 of Shc seems to contribute very little to Ras/MAPK activation. Thus, in cells expressing the mutant EGF-R, activation of the Ras/MAPK pathway appears mainly dependent on phosphorylation of Y317 of Shc.

Modulation of c-*myc* induction through Y239/240. In the mutant EGF-R-expressing cells, c-*myc* message induction was not totally dependent on Shc, since at saturating concentrations of EGF, the c-*myc* message emerged to some extent in the Y239/240F Shc-expressing cells. Although c-*myc* is one of the immediate-early genes, the half-life of Myc is very short and its expression appears to be regulated at various points by multiple and complex mechanisms, such as transcription, translation, and degradation (57). We do not know exactly at which point Shc is involved.

Ras and Myc pathways appear to act cooperatively for efficient cell proliferation in response to EGF. This is reminiscent of the previous observation that the v-*ras* and c-*myc* oncogenes cooperatively transform rat embryo fibroblasts (32). We tried to obtain stable cell lines expressing Y239/240/317F Shc in the autophosphorylation-minus mutant EGF-R-expressing cells but did not succeed. This also suggests that both pathways are important for mitogenesis.

Barone and Courtneidge (1) recently been reported that platelet-derived growth factor- or EGF-induced mitogenesis

requires Src kinase activity that and Src kinase may activate the Ras-independent pathway: they call it the Src pathway, which is important for growth factor-induced mitogenesis and is involved in expression of c-Myc (1). Shc is a good substrate of Src kinase (40); thus, Shc might be one of the mediators contributing to the Src pathway.

Possible involvement of Y239/240 in cytokine signaling. It is possible that the phosphorylation of Y239/240 also contributes to other biological activities. Recently, we have found that upon stimulation with interleukin 3, Shc becomes phosphorylated at these three residues in a hematopoietic cell line, Ba/F3, as is observed with EGF (20). In this cell line, interleukin 3-stimulated phosphorylation of Shc Y239/240 appears to play a role in antiapoptotic activity. Signaling pathways activated through Shc may affect various biological phenomena dependent on cell types.

Effect of the mutant Shcs on WT EGF-R-expressing cells. We also examined these pathways in the background of WT EGF-R signaling. This is important because we cannot exclude the possibility that the effect observed only in the mutant EGF-R-signaling is aberrant due to the receptor mutation. We found that Y239/240/317F Shc inhibited the EGF-induced mitogenesis, though the Y239/240F or Y317F Shc that carried the mutation on either site did not strongly affect it (Fig. 8). These observations suggest that both Y239/240 and Y317 of Shc play an important role in WT EGF-R mitogenic signaling.

Shc: a multiple signal acceptor. It has recently been reported that phosphorylation of Y317 of Shc is important for activation of Ras not only in growth factor signaling but also in G protein-coupled receptor or integrin receptor signaling (9, 61). It is of interest to examine what kind of role Y239/240 of Shc plays in the various signaling systems.

What is the nature of the signaling pathway from Y239/240? Probably, some effector molecule(s) binds to phosphorylated Y239/240 and sends signals downstream. The identification of the signaling molecules involved in this new pathway will provide further insight into growth factor-induced signal transduction.

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