Epidermal growth factor-receptor mutant lacking the autophosphorylation sites induces phosphorylation of Shc protein and Shc–Grb2/ASH association and retains mitogenic activity

(Signal transduction)

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ABSTRACT Epidermal growth factor (EGF) receptor (EGFR) can induce cell growth and transformation in a ligand-dependent manner. To examine whether the autophosphorylation of EGFR correlates with the capacity of the activated EGFR to induce cell growth and transformation, we truncated the human EGFR just after residue 1011, removing all three major autophosphorylation sites (DEL1011). Further, a point mutation was introduced at another autophosphorylation site, Tyr-992 → Phe (DEL1011+F992). The wild-type and mutant receptors were stably expressed in a NIH 3T3 variant cell line that expresses an extremely low level of endogenous EGFR and does not grow with EGF. As expected, DEL1011 and DEL1011+F992 were found to be severely impaired in EGF-induced autophosphorylation, due to the deletion of the appropriate target tyrosines. However, mutant receptors still could induce EGFR-dependent DNA synthesis, morphological transformation, and anchorage-independent growth, although the extent of these was significantly reduced when compared with wild-type EGFR. EGF-induced tyrosine phosphorylation of Ras-GTPase activating protein-associate protein p62 and phospholipase C γ1 was dramatically reduced in the cells expressing DEL1011 and DEL1011+F992. On the other hand, tyrosine phosphorylation of Shc, complex formation of Shc–Grb2/Ash, and activation of microtubule-associated protein kinase were still fully induced upon EGF stimulation without binding of Shc or Grb2/Ash to the mutant receptor. Thus, tyrosine phosphorylation of Shc may play a crucial role for activating Ras and generating mitotic signals by the activated EGFR mutant.

Material and Methods

Constructions of EGFR Mutants. The pTJNeo-EGFR (14), which contains the complete human EGFR coding sequence (15) downstream of the cytomegalovirus immediate/early promoter, was used. Tyr-992 → Phe substitution was done by introducing a point mutation with the PCR (nucleotide sequence GAGTAC to GAATTC, a new EcoRI site). To construct human EGFR premature termination mutant, a universal termination linker (CTAGCTAGCTAG) was inserted after aa 1011 [Sac I site, blunted with T4 polymerase (DEL1011)]. The linker generated one extra amino acid after aa 1011 (serine). The point mutation for Phe-992 was introduced into DEL1011 EGFR, resulting in DEL1011+F992 EGFR (Fig. 1A). To construct the in-frame insertion mutation in the kinase domain, a 12-bp Nco I synthetic DNA linker (5′-CAGCCATGGCTG-3′) was inserted at aa 789 (Rsa I site). The linker generated five extra amino acids after aa 788 (serine, alanine, methionine, alanine, aspartate) and removed Tyr-789. The mutated nucleotide sequences were confirmed by sequencing analysis.

Establishment of Cell Lines Expressing Various EGFRs and Characterization of Their Cell Growth. We used a clonal NIH 3T3 cell line, selected upon the criterion that when grown to confluency in Dulbecco's modified Eagle's medium (DMEM)/5% heat-inactivated newborn calf serum, cells did not show any transformation in EGF (Toyobo). Further, we confirmed the negligibility of any functional endogenous EGFR in this cell line by 125I-labeled EGF binding and...

Abbreviations: EGF, epidermal growth factor; EGFR, EGF receptor; GAP, GTPase-activating protein; PLC, phospholipase C; MAP kinase, mitogen-activated protein kinase; mAb, monoclonal antibody.

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[3H]thymidine incorporation (Fig. 2B). Cells were transfected with the plasmids and subjected to G418-resistant selection, as described in the legend for Table 1. G418-resistant cell lines were picked up and were screened for 125I-labeled EGFR binding, as described (14). The following results represent the number of bound 125I-labeled EGFR molecules per cell. Data are the mean ± SD value of three independent experiments: wild type, (3.51 ± 0.14) × 10^4; F992, (2.48 ± 0.11) × 10^4; DEL1011, (3.79 ± 0.14) × 10^4; DEL1011 ± F992, (2.45 ± 0.13) × 10^4; NIH 3T3, 27 ± 24. Extent of DNA synthesis stimulated by EGFR was determined as described (14). To examine growth rate, cells were seeded at 2 × 10^4 cells in 35-mm dishes in DMEM/0.5% serum with or without EGF (20 ng/ml) and were counted every two days.

**Metabolic Labeling and Immunoprecipitation.** Cells were metabolically labeled with [35S]methionine/cysteine (1176 Ci/mmol; ICN; 1 Ci = 37 GBq), lysed in RIPA buffer (16), and immunoprecipitated with monoclonal antibody (mAb) 528IgG, a mAb specific for the extracellular domain of EGFR (17).

**Tyrosine Phosphorylation of EGFRs and Other Cellular Proteins.** Cells were starved for 18 hr in DMEM/0.5% serum and then treated with or without EGF for 5 min at 37°C. Cells were solubilized directly in Laemmli SDS sample buffer or lysis buffer (18), and an equal amount of protein was incubated with mAb 528IgG prebound with protein G-Sepharose. The samples of 20 μg of total cellular protein or the washed immunoprecipitates were resolved on SDS/10% PAGE, transferred to nitrocellulose membrane. The membrane was blotted with antiphosphotyrosine mAb PY-20 (ICN) labeled with 125I-labeled anti-mouse IgG(ab'-); fragment (500–2000 Ci/mmol; Amersham; 1 Ci = 37 GBq) or with anti-mouse horseradish peroxidase conjugate (Amersham) and visualized by autoradiography or enhanced chemiluminescence (ECL) detection system.

**Tyrosine Phosphorylation of GAP-Associated Proteins, PLC-γ1, and Grb2/Ash–Shc Complex: Coimmunoprecipitation of These Molecules with EGFR.** The lysates, prepared as above, were incubated with anti-GAP antibodies (19), a mixture of mAbs to PLC-γ1 (Upstate Biotechnology), anti-Grb2/Ash antibodies (10), anti-Shc antibodies (Upstate Biotechnology), or mAb 528IgG prebound to protein A-Sepharose. The immune complexes were immunoblotted with antiphosphotyrosine, anti-GAP, anti-PLC-γ1, or anti-Shc antibodies.

**Detection of Kinase Activity with Polyclaylamide Gels Containing Myelin Basic Proteins.** Total cell lysates (15 μg of protein) prepared as above were resolved on SDS/10% PAGE in which myelin basic protein (0.5 mg/ml) was included. The following procedures were done, essentially as described (20).

**RESULTS**

The EGFR Mutants with Carboxyl-Terminal Deletion Retain the EGF-Induced Mitogenic and Transforming Activities. To examine the ligand-dependent cellular transformation of the EGFR mutants, a NIH 3T3 cell clone that expresses the endogenous EGFR at very low levels using several criteria (21) was used for transfection with plasmid DNAs carrying the various EGFR constructs (Fig. 1A). Transfected cells were subject to G418 selection with (10 ng/ml) or without EGF, and the number of transformed foci was counted. The F992 and DEL1011 + F992 mutant EGFRs gave essentially the same level of transforming activity as that of the wild-type EGFR (Table 1). The DEL1011 EGFR showed weak, but detectable, transforming activity.

We established several stable clones that overexpressed the wild-type and mutant EGFRs and used them for further analysis. Morphological transformation of the representative clones induced with EGF are shown in Fig. 1B. These cells exhibited increased EGF surface-binding sites (1.8–6.6 × 10^4 molecules of 125I-labeled EGF binding per cell), whereas parental cells showed an extremely low amount of EGF binding (<10^2 sites per cell) (see Materials and Methods). The expected sizes of various EGFR molecules expressed in NIH 3T3 cell clones were confirmed by metabolic labeling experiments (Fig. 2A).

To assess the EGF-induced mitogenic potential, [3H]thymidine incorporation into the DNAs was measured with EGF (Fig. 2B). In contrast to the parental cells, all the mutant receptor-expressing cells showed a significant increase in DNA synthesis, reaching to approximately the same level as the wild type-expressing cells. As a control, a kinase-negative mutant EGFR having a linker-insertion in the kinase domain (EGFp, see Materials and Methods) did not induce any autophosphorylation or DNA synthesis in response to EGF (data not shown).

All cell lines did not show any significant growth in the absence of EGF in 0.5% serum. However, EGF addition stimulated the growth rate of various receptor-expressing

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Total no.</th>
<th>%</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>23/497</td>
<td>4.63</td>
</tr>
<tr>
<td>F992</td>
<td>23/428</td>
<td>5.37</td>
</tr>
<tr>
<td>DEL1011</td>
<td>(16)*/456</td>
<td>(3.50)*</td>
</tr>
<tr>
<td>DEL1011 + F992</td>
<td>20/461</td>
<td>4.34</td>
</tr>
<tr>
<td>Vector</td>
<td>0/721</td>
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NIH 3T3 cells were transfected with 5 μg of plasmid DNA per dish (14). The transfected cells were grown in the presence of G418 at 0.2 mg/ml with EGF at 10 ng/ml. After 2 weeks the number of G418-resistant colonies and transformed foci were counted. Total no. indicates the sum of transformed foci and of G418-resistant colonies in three independent experiments (SD, ±20%). No transformed foci were seen without EGF. *Typical transformed foci were not developed, but weak ones could be observed (in parentheses).
cells, except in the parental cells (Fig. 2C): DEL1011+F992-expressing cells were at an intermediate rate, and the DEL1011-expressing cells grew rather slowly in comparison. In soft agar, all the various receptor-expressing cells, but not parental cells, could form colonies in an EGF-dependent manner (data not shown).

DEL1011 and DEL1011+F992 EGFR Mutants Cannot Efficiently Phosphorylate the Receptor Itself, GAP Complex, or PLC-γ1 on Tyrosine Residues. To examine EGF-induced tyrosine phosphorylation of cellular proteins EGF-stimulated total cell lysates were immunoblotted by phosphotyrosine antibody. For the wild type and F992, we could detect significant autophosphorylation of the receptors (Fig. 3A). However, as expected from deletion of the major autophosphorylation sites, the DEL1011 and DEL1011+F992 mutants did not show appreciable phosphorylation of the receptor in the total cell lysates (Fig. 3A) or in the immunoprecipitates of the receptors (Fig. 3B). Parental cells did not show any autophosphorylation of endogenous mouse receptors at detectable levels. In the DEL1011- and DEL1011+F992-expressing cells, several proteins other than EGFR were phosphorylated on tyrosine in an EGF-dependent manner (Fig. 3A), suggesting that the tyrosine kinase itself in these mutants is still active. We detected tyrosine phosphorylation on 180-kDa c-ErbB2 protein (data not shown). Thus, at least a portion of the phosphorylated bands around 180 kDa in Fig. 3A is c-ErbB2.

Because the mutant EGFRs, lacking most of the autophosphorylation sites, still retain mitogenic activities, we examined whether EGF induces tyrosine phosphorylation of several important candidates for direct targets of EGFR. GAP is associated with 62 kDa (p62) and 190 kDa (p190) proteins, which are rapidly tyrosine-phosphorylated in response to EGF.

FIG. 2. Molecular sizes and mitogenic activity of various EGFR mutants. (A) Metabolically labeled EGFRs were immunoprecipitated with anti-extracellular domain of EGFR. Wild-type (lane 1) and F992 (lane 2) EGFRs migrated at 170 kDa (large arrowhead), whereas both DEL1011 (lane 3) and DEL1011+F992 (lane 4) migrated at positions consistent with their predicted size of 130 kDa (small arrowheads). Lanes 5, parental NIH 3T3 cells. (B) [3H]Thymidine incorporation (incorp.) of various EGFR-expressing cells by EGF. Quiescent cells were incubated with various concentrations of EGF for 22 h and labeled with [3H]thymidine for the last 4 h. Results are indicated by the mean ± SD of triplicates of at least three independent experiments with two to three independent clones. (C) Growth curves of NIH 3T3 cells overexpressing various EGFRs with or without EGF. □, Wild type; A, F992; B, DEL1011; ○, DEL1011+F992; lower lines, parental NIH 3T3. Values within parentheses in C indicate cell numbers cultured without EGF.

FIG. 3. EGF-induced tyrosine phosphorylation of EGFRs and cellular proteins in cells bearing various EGFR mutants. Quiescent cells were treated with or without EGF (100 ng/ml) for 5 min at 37°C and solubilized; the cell lysates containing an equal amount of protein were then immunoprecipitated with anti-EGF extracellular domain. Twenty-microgram samples of protein (A) or immunoprecipitates (IP) (B) were electrophoresed on SDS/PAGE and immunoblotted with antiphosphotyrosine (anti-PY) antibody PY-20. Cont., control; WT, wild type. Arrowhead, EGFR.

EGF (8). Upon EGF stimulation, GAP-associated p62 was markedly phosphorylated in the cells expressing wild-type EGFR (Fig. 4A). On the other hand, in the DEL1011 and DEL1011+F992 EGFR-expressing cells, phosphorylation of GAP complex was hardly detectable, although lysates contained equal amounts of GAP p120, as indicated in Fig. 4A. GAP itself was not clearly phosphorylated, as reported by others (22).

Furthermore, in the DEL1011 and especially the DEL1011+F992 EGFR-expressing cells, EGF-triggered tyrosine phosphorylation of PLC-γ1 decreased dramatically (Fig. 4B). These results suggest that tyrosine phosphorylation of GAP-associated p62 and PLC-γ1 are not essential for the ligand-induced mitogenesis in these mutants.

DEL1011+F992 EGFR Mutant Can Induce Tyrosine Phosphorylation of Shc–Grb2/Ash Complex and Activation of Mitogen-Activated Protein (MAP) Kinase in an EGF-Inducible Manner. Recently, in EGF-stimulated cells Grb2/Ash has been reported to complex with phosphorylated EGFR and tyrosine-phosphorylated 46- and 52-kDa Shc proteins (23). It was also suggested that these complexes are upstream of Ras signaling, regulating proliferation and transformation of mammalian cells. In the wild-type EGF-expressing cells, the ligand EGF induced association of Grb2/Ash with tyrosine-phosphorylated 170-kDa and 52-kDa proteins (Fig. 5A). The 170-kDa protein was identified as EGFR by immunoprecipitation of cell lysates with anti-Grb2/Ash antibodies and then immunoblotting with anti-EGF antibody (data not shown). The 52-kDa protein was found to be Shc (Fig. 5B and C). Grb2/Ash itself was not appreciably tyrosine-phosphorylated as previously reported (9).

Surprisingly, DEL1011+F992 EGFR could also induce tyrosine phosphorylation of Shc and complex formation of Grb2/Ash with Shc without stable association of the receptor with Grb2/Ash–Shc complex (Fig. 5 A–C); in the mutant receptor-expressing cells immunoblotting of antiphosphotyrosine antibody for immunoprecipitates with anti-Shc (Fig. 5B) or anti-Grb2/Ash (Fig. 5A) antibodies showed the phosphorylation of Shc on tyrosine and association of Grb2/Ash.
with the phosphorylated Shc protein. Shc protein stably associated with wild-type EGFR in an EGFR-dependent manner, but this association was hardly detectable in EGF-stimulated DEL1011+F992 mutant receptor (Fig. 5C) (we will discuss this point further).

MAP kinase is reported to be activated in response to EGF and tyrosine-phosphorylated by MEK (MAP kinase kinase). MAP kinase is thought to be downstream of Ras (24). In total cell lysate, 42-kDa (and 44-kDa) proteins were tyrosine-phosphorylated in both wild-type and DEL1011+F992 EGFR-expressing cells but not in the parental cells after EGF addition (Fig. 5D Left). To confirm that these proteins were the activated MAP kinase, we performed a gel kinase assay and detected kinase activity for myelin basic protein, a good substrate for MAP kinase (Fig. 5D Right). By using anti-MAP kinase antibody, tyrosine-phosphorylated p42 was confirmed as MAP kinase (data not shown).

Further, in both wild-type and DEL1011+F992 EGFR-expressing cells, EGF remarkably increased the proportion of the GTP-bound active form of Ras, reaching approximately similar levels, although the parental cells induced it only slightly (data not shown).

These results clearly show that although DEL1011+F992 EGFR cannot induce autophosphorylation efficiently, it can induce tyrosine phosphorylation of Shc, complex formation of Shc–Grb2/Ash, activation of Ras signaling pathway, mitogenesis, and transformation in an EGF-dependent manner.

**Fig. 4.** EGF-induced tyrosine phosphorylation of GAP complex and PLC-γ1 in cells expressing various EGFRs. Quiescent cells were treated with or without EGF (250 ng/ml) and solubilized; the cell lysates containing equal amounts of protein were immunoprecipitated with anti-GAP (A) or anti-PLC-γ1 antibodies (B). Washed immune complexes (A, upper bands, and B) or 25 µg of cell lysate protein (A, lower bands) were separated by SDS/PAGE and were immunoblotted with antiphosphotyrosine antibody (A and B, upper bands), anti-GAP antibodies (A, lower bands), or anti-PLC-γ1 antibodies (B, lower bands).

**Fig. 5.** EGF-induced tyrosine-phosphorylation of Shc, complex formation of Shc with Grb2/Ash, association of Shc with EGFR, and activation of MAP kinase. Quiescent cells were treated with or without EGF (50 ng/ml in A, B, C; 10 ng/ml in D) and solubilized; cell lysates containing equal amounts of protein were then immunoprecipitated with anti-Grb2/Ash antibodies (A), anti-Shc antibodies (B), or anti-EGFR extracellular domain mAb (C). Immune complexes were washed, separated by SDS/PAGE, and immunoblotted with antiphosphotyrosine antibodies (A and B) or anti-Shc antibodies (C). Total cell lysates were separated by SDS/PAGE, and “in gel” kinase assay was done (D). Arrowheads indicate Shc proteins (>52 kDa; >46 kDa) and MAP kinase (>42 kDa). Cont., control; WT, wild type; IP, immunoprecipitate.

**DISCUSSION**

In this report, we have shown that the truncated forms of the EGFR lacking the distal portion of the carboxyl-terminal domain in combination with the Tyr-992 → Phe substitution (DEL1011 and DEL1011+F992) show very low, if any, autophosphorylation in vivo but retain EGF-induced mitogenic and transforming activities. Very recently, an EGFR mutant lacking autophosphorylation sites by point mutations was also reported to have mitotic activity (25).

Although the molecular basis is not yet clear, DEL1011 induces DNA synthesis but has lower activities in cell growth and cell transformation. Because the Tyr-992 → Phe substitution recovers these activities to some extent, we think that Tyr-992 in the background of DEL1011 negatively affects cell growth and transformation. A weakly phosphorylated Tyr-992 (Fig. 3) or Tyr-992 itself might be involved in suppressing signal transduction.

These DEL1011 and DEL1011+F992 mutants could not phosphorylate GAP-associated p62 or PLC-γ1 on tyrosine efficiently (Fig. 4). On the other hand, the truncated mutant DEL1011+F992 was able to fully induce tyrosine phosphorylation of Shc, complex formation between Shc and Grb2/Ash, and activation of Ras signaling pathway in an EGF-
inductive manner. These biological activities of the autophosphorylation-negative mutant were rather unexpected because the autophosphorylation sites of EGFR and their association with Grb2/Ash or Shc have been thought important for activation of the Ras signaling pathway (23). Therefore, our results strongly suggest that the region of tyrosine kinase domain in the EGFR molecule, without most of the carboxyterminal region containing the autophosphorylation sites, alone is sufficient for activation of the downstream signaling pathway (for Shc-Grb2/Ash and Ras proteins) in this system.

There appear two possibilities for phosphorylation of Shc: (i) A "direct" mechanism, in which the EGFR tyrosine kinase domain itself can directly interact with Shc and phosphorylate it on a tyrosine residue. This phosphorylation of Shc may induce complex formation of Shc with Grb2/Ash and activate the Ras pathway through Sos, guanine nucleotide-exchange factor for Ras, as recently reported (26, 27). (ii) An "indirect (two step)" mechanism, in which some membrane protein(s) are first phosphorylated on tyrosine by EGF-activated mutant receptor; then SH2 domain-containing Shc is recruited near the receptor and phosphorylated on tyrosine by the activated mutant receptor. At this moment, the indirect mechanism cannot be completely ruled out.

Shc is reported to have transforming activity apparently mediated through the Ras pathway. Further, overexpressed Shc protein has been shown to induce Ras-dependent neurite outgrowth in PC-12 cells (23). Because we detected a clear tyrosine phosphorylation of Shc and formation of Shc–Grb2/Ash complex, which were associated with mitogenic and transforming activity of the mutant EGFR, we suggest that the efficient phosphorylation of Shc is crucial for signal transduction in this mutant receptor system. A similar case, in which a stable association of tyrosine-phosphorylated Shc with Grb2/Ash, but very low binding of this complex to tyrosine kinase receptor, has been observed in nerve growth factor-Trk receptor system in PC-12 cells (29). Very recently, Grb2/Ash has been reported to form a ternary complex with the wild-type EGFR and Sos (26, 27). It would be interesting to study whether EGF induces Shc–Grb2/Ash–Sos complex formation in our mutant-expressing cells.

On the basis of recent studies, it seems clear that the autophosphorylation sites in EGFR are used for the binding and activation of signal transducers in vivo. However, in the avian v-ErbB product and in a structural alteration of EGFR in human glioblastoma, the carboxyterminal region of EGFR is significantly deleted (5, 28). Our studies on EGFR deletion mutants, such as DEL1011+992, suggest the presence of an alternative pathway for cell transformation and growth stimulation by EGFR.

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