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Tyrosine Phosphorylation of *ras* GTPase-activating Protein Does Not Require Association with the Epidermal Growth Factor Receptor*

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The importance of the carboxyl-terminal domain of the epidermal growth factor (EGF) receptor and its five autophosphorylation sites in the *in vivo* interaction and tyrosine phosphorylation of the *ras* GTPase-activating protein (*ras*GAP) has been investigated, using NIH 3T3 cells transfected with mutant EGF receptors. Phosphorylation of *ras*GAP by EGF receptor mutants, in which one to four autophosphorylation sites (Tyr-1173, -1148, -1086, and -1068) were mutated to phenylalanine, was reduced by 50–60% compared to the wild-type receptor. Elimination of these four autophosphorylation sites by truncation of 123 carboxyl-terminal residues of the EGF receptor paralleled results obtained with point mutants. Substantial inhibition (about 90%) of *ras*GAP tyrosine phosphorylation by the EGF receptor occurred only when the remaining autophosphorylation site (Tyr-992) was mutated, in the context of this truncated receptor or in the full-length receptor mutated at all four other autophosphorylation sites. However, a point mutation of only Tyr-992 in the full-length receptor suppressed tyrosine phosphorylation of *ras*GAP only by 50%. In contrast, an EGF receptor lacking the last 214 amino acid residues (Dc214), which emcompasses all five autophosphorylation sites, phosphorylated *ras*GAP to the same extent as the wild-type receptor. However, this truncated receptor was significantly impaired in its capacity to phosphorylate phospholipase C-γ1. Interestingly, while EGF receptor autophosphorylation sites are required for EGF-induced *ras*GAP association with the receptor, maximal phosphorylation of *ras*GAP by the truncated receptor Dc214 occurred without detectable formation of receptor·*ras*GAP complexes. Furthermore, the capacity of mutated EGF receptors to bring about focal transformation was correlated with their capacity to phosphorylate *ras*GAP.

The receptor for epidermal growth factor (EGF),[1] a 170-kDa transmembrane glycoprotein, is a member of the protein tyrosine kinase family (1, 2). EGF binding to the receptor triggers activation of receptor tyrosine kinase activity, which is essential for inducing cellular responses to EGF (3, 4), and leads to tyrosine phosphorylation of specific cellular substrates and autophosphorylation of the receptor (1, 2). Tyrosine autophosphorylation regulates the biological activity of the EGF receptor by influencing receptor kinase activity (5, 6) and by creating binding sites for physiologically important substrates. A number of these substrates contain sequence motifs termed *src* homology (SH2) domains, such as phospholipase C-γ1 (PLC-γ1), phosphatidylinositol 3-kinase (PI 3-kinase), src homology and collagen, GTPase-activating protein of *ras* (*ras*GAP) (for review, see Refs. 7 and 8), and phosphotyrosine phosphatases *syp* (9) and 1D (10).

*ras*GAP stimulates the intrinsic GTPase activity of *ras*, converting *ras* from the active GTP·*ras* form to the inactive GDP·*ras* form (11). Also, some evidence suggests that *ras*GAP, in addition to its regulatory properties on *ras*, may be a downstream effector of *ras* (12–14). Several studies show that *ras* is critically involved in several growth factor-induced signaling pathways (15–17). Since the formation of GTP·*ras* is stimulated by activated tyrosine kinases, such as EGF and PDGF receptors (18–21), and *ras*GAP becomes associated with (22, 23) and is phosphorylated by these tyrosine kinases (24–27), it has been proposed that *ras*GAP may connect tyrosine kinases with *ras* signaling. Also, it has been reported that the modulation of a guanine nucleotide exchange protein might be a target for the EGF-induced activation of *ras* (28, 29). The physiological consequences of growth factor-induced tyrosine phosphorylation of *ras*GAP is not known. Regulation of *ras*GAP may be highly complex, since it is associated with two other tyrosine phosphorylated proteins, pp62 and pp190, which have been recently cloned (30, 31), that may also influence *ras*GAP activity (25, 32, 33). Also, *src* (34) and some *src* family members (35) have been reported to be associated with *ras*GAP. Recently, studies *in vitro* indicate that binding of tyrosine phosphorylated *ras*GAP to activated EGF receptors leads to a small inhibition of *ras*GAP activity (36).

Association of SH2-containing substrates with activated growth factor receptors is thought to be essential for their subsequent phosphorylation and/or activation. Mutation of autophosphorylation sites on the PDGF, fibroblast growth factor, and *trk* receptors that are essential for receptor association with PLC-γ1 prevents tyrosine phosphorylation of PLC-γ1 and phosphatidylinositol hydrolysis (37–41). Tyrosine-phosphorylated peptides corresponding to PI 3-kinase binding sites on the insulin receptor substrate (IRS-1) and PDGF receptors have been reported to increase PI 3-kinase activity (42). Studies of the PDGF β-receptor indicate that PLC-γ1, *ras*GAP, and PI 3-kinase bind to specific phosphotyrosine sites differentiated by the sequence motifs adjacent to each tyrosine residue (37, 38, 43–45). Data for the EGF receptor are less definitive. The

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[1] The abbreviations used are: EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium; SH2, *src* homology region 2; GAP, GTPase-activating protein; PLC-γ1, phospholipase C-γ1; PI 3-kinase, phosphatidylinositide 3-kinase; PDGF, platelet-derived growth factor.

carboxyl-terminal domain of the EGF receptor contains all known autophosphorylation sites (Tyr-1173, -1148, -1086, -1068, and -992) (5, 46–48), and it has been demonstrated that PLC-γ1 and *ras*GAP associate with a tyrosine-phosphorylated carboxyl-terminal fragment of the EGF receptor (49). However, the exact tyrosine autophosphorylation sites involved in receptor interaction with these substrates are unknown.

In this study, we have examined the requirement of receptor autophosphorylation sites for *ras*GAP association with and/or tyrosine phosphorylation by the EGF receptor.

EXPERIMENTAL PROCEDURES

Materials—EGF was isolated from mouse submaxillary gland according to the method of Savage and Cohen (50) and iodinated as described by Carpenter and Cohen (51). ^{125}I-Labeled rabbit anti-mouse IgG and ^{125}I-protein A were obtained from ICN. Nitrocellulose membranes were from Micron Sepharose Inc. G418 and tissue culture reagents were from Life Technologies, Inc.; gentamicin, protein A-Sepharose CL-4B, and PDGF-BB were from Sigma. Protein G-Sepharose 4B was obtained from Zymed Inc.

Rabbit polyclonal antibodies to the human EGF receptor and PLC-γ1 were described previously (52, 53). Rabbit polyclonal *ras*GAP antisera (638) to bovine *ras*GAP (24) were kindly provided by Dr. J. Gibbs (Merck, Sharpe & Dome). Rabbit polyclonal phosphotyrosine antibodies and mouse monoclonal antibodies to the intracellular domain of the EGF receptor were obtained from Zymed Inc. Rabbit polyclonal antibodies to human *ras*GAP and mouse monoclonal antibodies to the extracellular domain of EGF receptor were purchased from Upstate Biotechnology, Inc. Mouse monoclonal antibodies to PLC-γ1 (54) were kindly provided by Dr. Sue Goo Rhee (National Institutes of Health). Rabbit polyclonal antibodies to the PDGF β-receptor (55) were generously provided by Dr. T. O. Daniel (Vanderbilt University).

Mutant Construction and Transformation Assay—EGF receptor mutants were obtained by site-directed mutagenesis by either substituting tyrosine residues with phenylalanine and/or deleting the coding sequence for different carboxyl terminus fragments. Single point mutants of Tyr-1173, -1148, -1068, or -992, double mutants (Tyr-1173 and -1148), triple mutants (Tyr-1173, -1148, and -1068), and quadruple mutants (Tyr-1173, -1148, -1086, and -1068), deletion mutants Dc63, Dc123, and Dc214 (lacking 63, 123, and 214 carboxyl-terminal residues, respectively), and receptor mutant Dc123F (Tyr-992 substituted to phenylalanine in the truncated receptor Dc123) have been previously described (5, 53, 56).

To generate the quintuple point mutant (F5), in which all five receptor autophosphorylation sites (Tyr-1173, -1148, -1086, -1068, and -992) are changed to phenylalanine, the previously described F4 construct (Tyr-1173, -1148, -1086, and -1068 substituted to phenylalanine) (56) was used and phenylalanine substitution of Tyr-992 was performed by site-directed mutagenesis in the M13mp18 vector encoding F4 EGF receptor cDNA fragment *Acc*I-*Hinc*II (3013–3625). Single-stranded template was prepared and mutagenesis performed using the primer 5′-ATGCCGACGAGTTCCTCATCCCA-3′ (992F) according to Taylor *et al.* (57) and confirmed by dideoxy sequencing (58). This quintuple mutated fragment was subcloned back into the pMMTV vector containing the EGF receptor cDNA. Subsequently the full-length EGF receptor cDNA (*Sac*II-*Xho*I) with all five point mutations was inserted in the pCO 11 vector to give rise to pMI 41 (F5).

To create the new single point mutant in which Tyr-1086 was changed to phenylalanine in the context of full-length EGF receptor (1086F), a single point mutation was introduced in the M13mp18 vector encoding the wild-type EGF receptor *Acc*I-*Hinc*II fragment (3013–3625). A single-stranded template was prepared and mutagenesis performed using the primer 5′-GAATCCTGTCTTTCACAATCAGCC-3′. Mutagenesis was performed as described above and confirmed by dideoxy sequencing. The mutated fragment was subcloned back into MMTV and into the pCO11 vectors, as described above, to give rise to pMI 39 (1086F).

Transfection and Cell Culture—For receptor transfections, NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum, penicillin, streptomycin, and glutamine. Transfections were carried out by the calcium phosphate method as previously described (59), and G418 was used for selection at the effective concentration of 0.3 mg/ml. All cell lines were maintained in DMEM containing 10% newborn calf serum and gentamicin (50 μg/ml). For experiments, cells were plated in the same medium and used when confluent.

Tyrosine Phosphorylation Studies—NIH 3T3 cells expressing wild-type or mutant EGF receptors were grown to confluence in medium containing 10% newborn calf serum and then incubated overnight in medium containing 0.5% newborn calf serum. The cells were then incubated with or without a saturating concentration of EGF (100 ng/ml) or PDGF-BB (50 ng/ml) at 4 °C for 1 h, or at 37 °C for the indicated times, in DMEM supplemented with 20 mM Hepes (pH 7.4) and 0.1% bovine serum albumin. The capacity of EGF and PDGF receptors to phosphorylate cellular substrates is not altered when cells are incubated at 4 °C (53, 55, 56), and control experiments showed that maximal EGF-induced tyrosine phosphorylation levels of *ras*GAP can be achieved under these conditions (results not shown).

After growth factor treatment, cells were washed three times with cold Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline, solubilized in lysis buffer (1% Triton X-100, 10% glycerol, 50 mM Hepes (pH 7.4), 150 mM NaCl, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 544 μM iodoacetamide) for 15 min at 4 °C, and centrifuged (10,000 × *g*, 10 min). *ras*GAP or PLC-γ1 was precipitated with specific rabbit antisera (24, 53) and protein A-Sepharose CL-4B beads. Immunocomplexes were washed three times with a buffer containing 20 mM Hepes (pH 7.2), 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, and 1 mM Na_3VO_4, resuspended in Laemmli buffer (60), and heated for 5 min at 80 °C. Samples were then electrophoresed on 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose paper. Levels of *ras*GAP and PLC-γ1 tyrosine phosphorylation were determined by Western blot analysis with a polyclonal phosphotyrosine antibody. The amount of *ras*GAP or PLC-γ1 protein in the immunoprecipitates was determined by Western blots with the corresponding polyclonal antibodies.

For quantitation, the amount of phosphotyrosine recovered from the *ras*GAP or PLC-γ1 band was normalized to the amount of *ras*GAP or PLC-γ1 protein, respectively. As determined by ^{125}I-EGF binding assay, most of the mutant receptors were expressed at low levels (7–16 × 10^4) or high levels (3–5 × 10^5) receptors per cell (see Fig. 1). For this reason, two different cell lines, expressing about 4 × 10^5 and 1 × 10^5 wild-type EGF receptors per cell, wt-1 and wt-2, respectively, were used as controls depending of the mutant receptor expression levels tested. Studies using these two cell lines revealed that the level of tyrosine phosphorylation of *ras*GAP was dependent of the number of EGF receptors per cell. Therefore, to compare results between cell lines expressing different numbers of EGF receptor, the data were normalized to the number of occupied EGF receptors determined by measuring ^{125}I-EGF binding at 4 °C in parallel cultures. Tyrosine phosphorylation of each substrate by different receptor mutants was expressed as percent tyrosine phosphorylation achieved by the wild-type receptor.

To examine tyrosine phosphorylation of total cellular proteins in response to EGF, an aliquot of cell lysates (50–100 μg) was mixed with 2 × Laemmli buffer, electrophoresed on 7.5% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and blotted with phosphotyrosine antibodies.

Receptor Association Studies—NIH 3T3 cells expressing wild-type or mutant EGF receptors were grown, treated with EGF (100 ng/ml) or PDGF-BB (50 ng/ml) for 1 h at 4 °C, and lysed as indicated above. EGF receptor precipitation was performed using mouse monoclonal antibodies against the extracellular domain of the EGF receptor and protein G-Sepharose 4B beads. Polyclonal antibodies to PDGF β-receptor and protein A-Sepharose CL-4B beads were used to precipitate the PDGF β-receptor. Immunocomplexes were washed four times, electrophoresed, and transferred to nitrocellulose paper as indicated above. Control precipitations showed that receptor antisera was not limiting in each case. Amounts of *ras*GAP or PLC-γ1 associated with growth factor receptors was detected by Western blot analysis of receptor immunoprecipitates, using polyclonal antibodies to *ras*GAP (24) or monoclonal antibodies to PLC-γ1 (54). The amount of EGF receptor immunoprecipitated was determined by immunoblot analysis, using either monoclonal antibodies to the intracellular or extracellular receptor domain, depending on the types of receptor mutants analyzed.

For quantitation, the amount of *ras*GAP that was co-precipitated with EGF receptor mutants was corrected for the amount of EGF receptor present in the immunoprecipitates and expressed as percent of *ras*GAP associated with the wild-type receptor. Control experiments using two cell lines (wt-1 and wt-2) that express quite different amount of wild-type EGF receptors, indicated that the amount of receptor associates *ras*GAP was proportional to the number of immunoprecipitated receptors.

Western Blot Analysis—Membranes were blocked for 1 h at room temperature in 5% non-fat dry milk-TBST (0.05% Tween 20, 150 mM NaCl, 50 mM Tris, pH 7.4) and probed with the indicated primary

antibody for 2 h at room temperature. Subsequently, blots were probed for 2 h at room temperature with ^{125}I-protein A, when the primary antibody was rabbit polyclonal, or with ^{125}I-goat anti-mouse, when the primary antibody was mouse monoclonal. Quantitation was performed with a PhosphorImager (Molecular Dynamics).

Binding of ^{125}I-EGF—In experiments where phosphorylation studies were performed, the total number of EGF receptors occupied by EGF was determined in parallel cultures incubated with ^{125}I-EGF (100 ng/ml). Nonspecific binding was determined with a 200-fold molar excess of unlabeled EGF. Specific cell-bound radioactivity was determined as described previously (53).

RESULTS

NIH 3T3 cells expressing transfected human wild-type or mutant EGF receptors were used to determine whether structural features in the receptor carboxyl terminus are required for *ras*GAP association with and/or tyrosine phosphorylation by the EGF receptor. Fig. 1 depicts the EGF receptor constructs used in this study and their EGF receptor expression level. Parental NIH 3T3 cells possess less than 3×10^3 mouse EGF receptors per cell.

Comparison of rasGAP Tyrosine Phosphorylation and Association with EGF and PDGF β-Receptors—Since PDGF-induced phosphorylation and interaction of *ras*GAP with the PDGF receptor has been extensively studied, and the autophosphorylation site essential for association and tyrosine phosphorylation identified (41–43, 61), we compared EGF- and PDGF-induced *ras*GAP phosphorylation and receptor association in the same cell background. NIH 3T3 cells, expressing endogenous PDGF receptors (approximately 8×10^4 receptors/cell), and high levels of human wild-type transfected EGF receptors (wt-1, approximately 4×10^5 receptors/cell) were used. After growth factor treatment, *ras*GAP was immunoprecipitated and tyrosine phosphorylation analyzed by immunoblot with a phosphotyrosine antibody.

EGF induced tyrosine phosphorylation of *ras*GAP and two other proteins, pp190 and pp62 (Fig. 2*A*, *lane 2*), that are known to be recovered in *ras*GAP immunoprecipitates from EGF-treated cells (25, 33). Interestingly, the stimulation of *ras*GAP tyrosine phosphorylation by PDGF (*lane 4*) was 2-fold greater than that elicited by EGF (*lane 2*) despite the presence of 5-fold more EGF receptors than PDGF receptors in these cells. By contrast, when tyrosine phosphorylation of another SH2-containing tyrosine kinase substrate, PLC-γ1 (Fig. 2*A*, *lanes 6* and *8*), was tested, EGF produced a 2-fold higher extent of tyrosine phosphorylation of this substrate than PDGF. Since this experiment was performed under conditions of receptor saturation by EGF or PDGF at 4 °C for 1 hr, the observed differences in substrate phosphorylation do not measure comparative rates of phosphorylation, but represent equilibrium levels of *ras*GAP and PLC-γ1 phosphorylation by each receptor tyrosine kinase.

The results shown in Fig. 2*A* (*lanes 2* and *4*) also indicate that EGF- and PDGF- induced tyrosine phosphorylation of *ras*GAP-associated proteins is quantitatively different. In *ras*GAP immunoprecipitates from PDGF-treated cells (*lane 4*), no

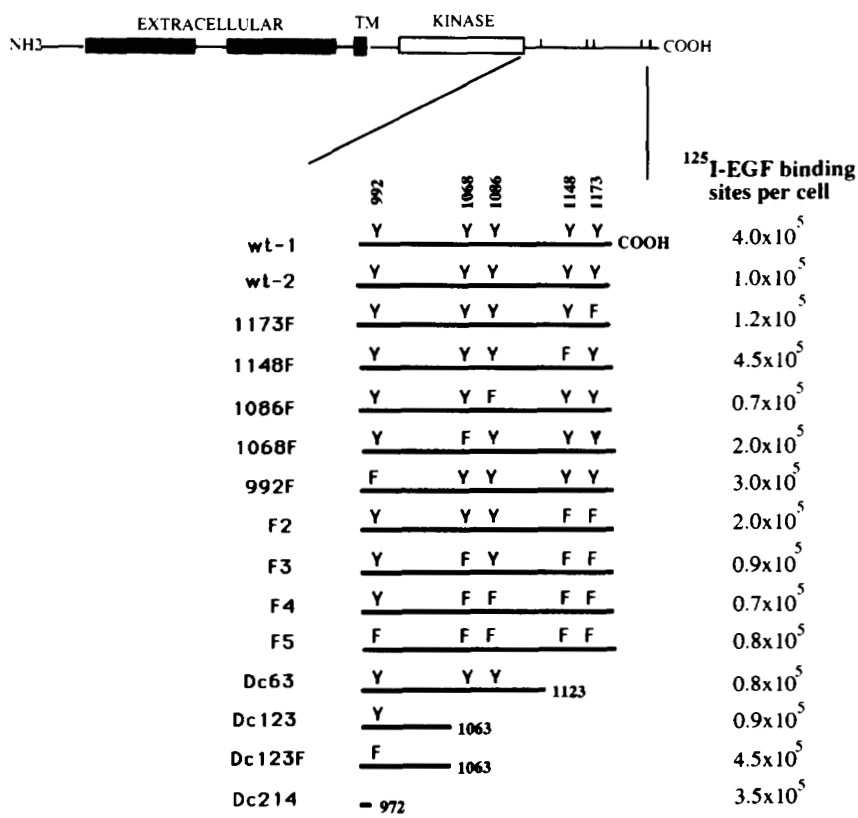

FIG. 1. **Schematic representation of EGF receptor and carboxyl-terminal receptor mutants.** The carboxyl-terminal domain of the wild-type (*wt*) human EGF receptor is shown with five autophosphorylation sites: Y992, Y1068, Y1086, Y1148, and Y1173. The point mutants with a single tyrosine (*Y*) changed to phenylalanine (*F*) are *1173F*, *1148F*, *1086F*, *1068F*, and *992F*; *F2*, receptor mutant with phenylalanine substitution of tyrosine 1173 and 1148; *F3*, receptor mutant with phenylalanine substitution of tyrosine 1173, 1148, and 1068; *F4*, receptor mutant with phenylalanine substitution of tyrosine 1173, 1148, 1068, and 1086; *F5*, receptor mutant with phenylalanine substitution of tyrosine 1173, 1148, 1068, 1086, and 992; *Dc63*, deletion of the carboxyl-terminal 63 amino acids; *Dc123*, deletion of the carboxyl-terminal 123 amino acids; *Dc123F*, deletion of the carboxyl-terminal 123 amino acids and phenylalanine substitution of tyrosine 992; *Dc214*, deletion of the carboxyl-terminal 214 amino acids. Also shown are the number of ^{125}I-EGF binding sites per cell. The transfectants have been previously published, and the number and affinities of EGF binding sites were calculated from Scatchard plot analysis of ^{125}I-EGF binding data (5, 48, 51). For these series of experiments, EGF binding was monitored in each experiment by ^{125}I-EGF saturation binding at 4 °C, as described under "Experimental Procedures." The obtained mean values, which are in agreement with previously reported data, are presented.

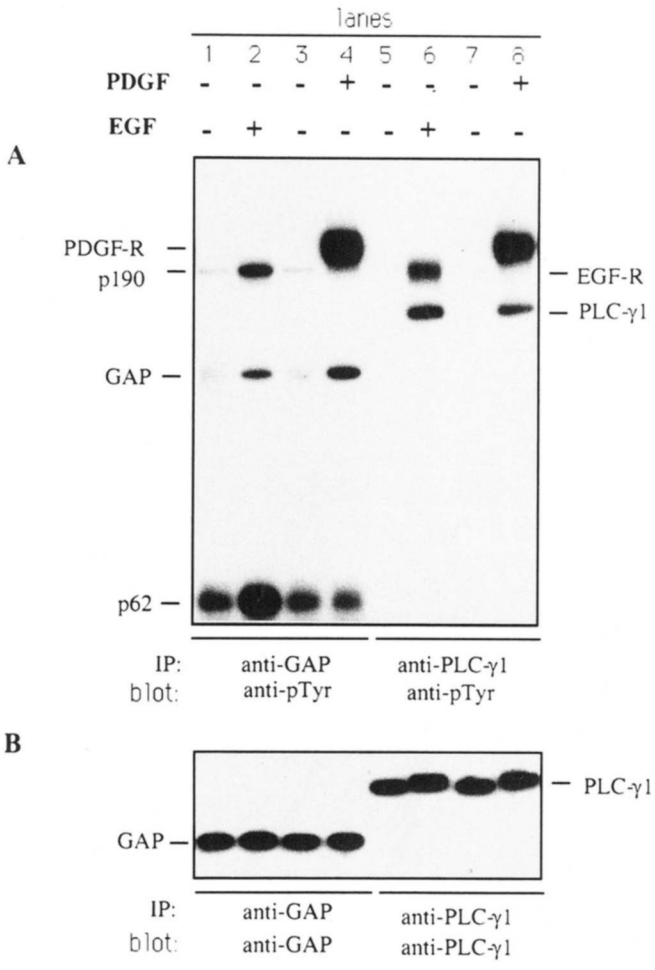

FIG. 2. **Tyrosine phosphorylation of rasGAP, rasGAP-associated proteins and PLC-γ1 by EGF and PDGF-BB.** NIH 3T3 cells transfected with wild-type EGF receptor were serum-starved overnight, treated with or without EGF (100 ng/ml) or PDGF-BB (50 ng/ml) for 1 h at 4 °C, and then solubilized as described under "Experimental Procedures." *ras*GAP and PLCγ-1 were immunoprecipitated with antiserum to each, electrophoresed and transferred to nitrocellulose membranes. *Panel A*, anti-phosphotyrosine Western blot analysis of *ras*GAP and PLCγ-1 immunoprecipitates. *Panel B*, anti-GAP and anti-PLCγ-1 Western blot analysis of the same immunoprecipitates analyzed in *panel A*. Positions of *ras*GAP and *ras*GAP-associated proteins (*p190* and *p62*), PLC-γ1, PDGF β-receptor, and EGF receptor are indicated.

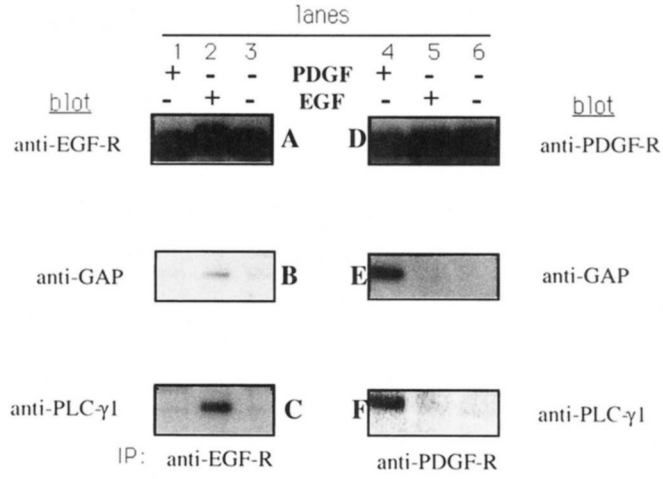

FIG. 3. **Comparison of rasGAP and PLC-γ1 association with EGF and PDGF receptors *in vivo*.** NIH 3T3 cells transfected with human wild-type EGF receptors were serum-starved overnight, treated with or without EGF (100 ng/ml) or PDGF-BB (50 ng/ml) for 1 h at 4 °C, and then solubilized in lysis buffer. EGF receptor and PDGF β-receptor were immunoprecipitated as described under "Experimental Procedures," electrophoresed, and transferred to nitrocellulose membranes. For relative quantitation, different amounts of whole cell lysates (5.0, 2.5, 1.2, and 0.5% volumes of the immunoprecipitate lysates) were simultaneously electrophoresed and transferred to nitrocellulose membranes (not shown). *Panel A*, anti-EGF receptor Western blot analysis of EGF receptor immunoprecipitates. *Panel B*, anti-*ras*GAP Western blot analysis of the same EGF receptor immunoprecipitates analyzed in *panel A*. *Panel C*, anti-PLC-γ1 Western blot analysis of the same EGF receptor immunoprecipitates analyzed in *panel A*. *Panel D*, anti-PDGF β-receptor Western blot analysis of PDGF β-receptor immunoprecipitates. *Panel E*, anti-*ras*GAP Western blot analysis of the same PDGF β-receptor immunoprecipitates analyzed in *panel D*. *Panel F*, anti-PLCγ-1 Western blot analysis of the same PDGF β-receptor immunoprecipitates analyzed in *panel D*. Positions of *ras*GAP, PLC-γ1, PDGF β-receptor, and EGF receptor are indicated.

significant increase in tyrosine phosphorylation of either pp62 or pp190 proteins was observed, whereas EGF treatment (*lane 2*) produced significant increases in the tyrosine phosphorylation of both proteins. The tyrosine-phosphorylated protein migrating slightly slower than pp190 in *ras*GAP immunoprecipitates from PDGF-treated cells (*lane 4*) was identified by Western blotting as the PDGF β-receptor. However, we were not able to detect EGF receptors in *ras*GAP immunoprecipitates from EGF-stimulated cells (data not shown). By comparison, tyrosine phosphorylated EGF or PDGF receptors were detected in PLC-γ1 immunoprecipitates from EGF- (*lane 6*) or PDGF- (*lane 8*) treated cells, respectively. These results indicate that, compared to *ras*GAP·PDGF β-receptor complexes, *ras*GAP·EGF receptor complexes are less stable and/or more transient. Also, EGF receptor·*ras*GAP complexes seem more labile than EGF receptor complexes with PLC-γ1.

To further study the association of *ras*GAP and PLC-γ1 with EGF and PDGF receptors, the presence of these substrates in receptor immunoprecipitates was tested (Fig. 3). Cells were treated with EGF or PDGF, and receptors were immunoprecipitated. After electrophoresis and transfer to nitrocellulose, each sample was blotted with antisera to the respective receptors (*panels A* and *D*), to *ras*GAP (*panels B* and *E*) or to PLC-γ1 (*panels C* and *F*). *Ras*GAP and PLC-γ1 were detected in receptor immunoprecipitates from EGF-treated cells (*lane 2*), and PDGF-treated cells (*lane 4*). The small amount of *ras*GAP or PLC-γ1 detected in immunoprecipitates of basal receptors (*lanes 1*, *3*, *5*, and *6*) is nonspecific, since a similar signal was obtained when control antibodies were used.

To estimate the percentage of total cellular *ras*GAP and PLC-γ1 found associated with activated EGF or PDGF receptors, aliquots, ranging from 0.5 to 5% of the total cellular lysates used for the immunoprecipitations presented in Fig. 3, were electrophoresed, transferred to nitrocellulose, and blotted with antisera to *ras*GAP and PLC-γ1 to determine the total cellular amount of each of these proteins (results not shown). Quantitation of these results together with the data in Fig. 3 revealed that approximately, 0.1 and 0.8% of the total cellular *ras*GAP and PLC-γ1, respectively, were co-precipitated with activated EGF receptors. In contrast, approximately 1.2% and 0.9% of total cellular *ras*GAP and PLC-γ1, respectively, were co-precipitated with activated PDGF β-receptors. Therefore, while equivalent levels of PLC-γ1 are associated with these activated receptors, there is approximately 10-fold more *ras*GAP associated with activated PDGF receptors than EGF receptors. These results help to explain why EGF receptors were not detected in *ras*GAP immunoprecipitates from EGF-treated cells, unlike PDGF receptors from PDGF-treated cells (Fig. 2, *lanes 2* and *4*). The data in Figs. 2 and 3 indicate that *ras*GAP has a relatively weak, but consistently detectable, capacity to interact with activated EGF receptors, which allowed us to examine the role of EGF receptor autophosphorylation sites in receptor interactions with *ras*GAP.

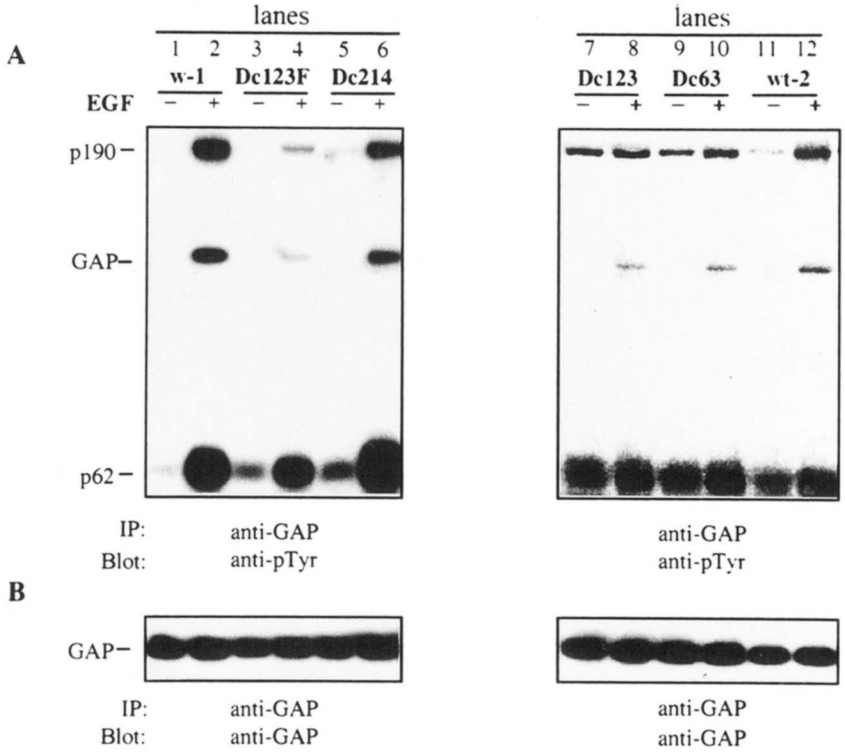

FIG. 4. **EGF-induced tyrosine phosphorylation of *ras*GAP and *ras*GAP-associated proteins by truncated EGF receptors.** NIH 3T3 cells expressing the wild-type or mutant EGF receptors were incubated with or without EGF (100 ng/ml) for 1 h at 4 °C and solubilized in lysis buffer, and then *ras*GAP was immunoprecipitated as indicated under "Experimental Procedures." *Panel A*, anti-phosphotyrosine Western blot analysis of GAP immunoprecipitates. *Panel B*, anti-*ras*GAP Western blot analysis of the same *ras*GAP immunoprecipitates analyzed in *panel A*. Positions of *ras*GAP and *ras*GAP-associated proteins (*p62* and *p190*) are indicated.

TABLE I
Quantitation of EGF-induced tyrosine phosphorylation of rasGAP by truncated EGF receptor

Cells expressing the indicated EGF receptor constructs were tested for *ras*GAP phosphorylation as described in Fig. 4. The amount of phosphotyrosine in *ras*GAP was corrected for the amount of *ras*GAP present and then for the number of EGF binding sites in each cell line, as described under "Experimental Procedures." Data are expressed as percent of phosphotyrosine per *ras*GAP in cells expressing wild-type (wt) EGF receptors and correspond to the average ± S.E. of at least three independent experiments.

EGF receptor	Phosphotyrosine per *ras*GAP
	% of wt
wt	100
Dc63	75 ± 1
Dc123	38 ± 4
Dc123F	11 ± 6
Dc214	83 ± 12

Tyrosine Phosphorylation of rasGAP by EGF Receptor Mutants—Initially, EGF receptor mutants with increasing deletions of the carboxyl terminus, removing two (Dc63), four (Dc123), or all five (Dc214) autophosphorylation sites, were investigated. The data in Fig. 4, which are quantitated in Table I, show that in cells expressing truncated receptors Dc63 (*lanes 9* and *10*) and Dc123 (*lane 7* and *8*), *ras*GAP tyrosine phosphorylation induced by EGF was decreased by approximately 25 and 60%, respectively, compared to cells expressing wild-type receptors (*lanes 11* and *12*). EGF-induced tyrosine phosphorylation of *ras*GAP was decreased by 90% in cells expressing the receptor mutant Dc123F (*lane 3* and *4*), in which the only remaining autophosphorylation site (Tyr-992) was mutated to phenylalanine. Surprisingly, when all five autophosphorylation sites were removed by truncation, the kinase activity of this truncated receptor (Dc214) toward *ras*GAP (*lanes 5* and *6*) was not significantly different from the wild-type receptor.

Equivalent results to those shown in Fig. 4 were obtained with a different *ras*GAP polyclonal antibody (Upstate Biotechnology, Inc.). Also, similar amounts of *ras*GAP were recovered in phosphotyrosine immunoprecipitates from EGF-treated cells expressing comparable numbers of wild-type or Dc214 EGF receptors. It is significant to note that receptor Dc214 is not phosphorylated at other tyrosine residues in the presence of EGF, as judged by Western blot with a phosphotyrosine antibody.[2] These results indicate that tyrosine phosphorylation of the EGF receptor is not essential for maximal *ras*GAP phosphorylation, at least in the context of this truncated receptor.

To determine whether the data obtained with truncation mutants could be extrapolated to the full-length EGF receptor, we examined receptor mutants with single or multiple substitution(s) of tyrosine autophosphorylation sites to phenylalanine (Table II). Compared to the wild-type receptor, tyrosine phosphorylation of *ras*GAP was substantially reduced (more than 90%), only when all five known autophosphorylation sites of the receptor were mutated (F5). Mutation of four (F4), three (F3), two (F2) or any single tyrosine residue, except Tyr-1086, decreased, but did not abolish EGF-induced *ras*GAP phosphorylation in cells expressing these mutant receptors, compared to cells expressing wild-type receptors. In contrast to the PDGF receptor (43–45), these data do not reveal one or two particular autophosphorylation sites that are specifically required for *ras*GAP phosphorylation.

Differential Role of the Receptor Carboxyl-terminal Domain in Tyrosine Phosphorylation of PLC-γ1 and rasGAP—The capacity of the EGF receptor mutant Dc214, which lacks all known autophosphorylation sites, to effectively phosphorylate

[2] C. Soler, A. Sorkin, and G. Carpenter, unpublished results.

TABLE II

Quantitation of EGF-induced tyrosine phosphorylation of rasGAP by EGF receptor autophosphorylation site mutants

Cells expressing the indicated EGF receptor constructs were tested for *ras*GAP phosphorylation as described in Fig. 4. The amount of phosphotyrosine in *ras*GAP has been corrected for the amount of *ras*GAP present and then for the number of EGF binding sites in each cell line, as described under "Experimental Procedures." Data are expressed as percent of phosphotyrosine per *ras*GAP in cells expressing wild-type (wt) EGF receptor and correspond to the average ± S.E. of at least three independent experiments.

EGF receptor	Phosphotyrosine per *ras*GAP
	% of wt
wt	100
1173F	46 ± 6
1148F	42 ± 5
1086F	95 ± 4
1068F	33 ± 5
992F	52 ± 7
F2	50 ± 14
F3	50 ± 10
F4	36 ± 15
F5	6 ± 5

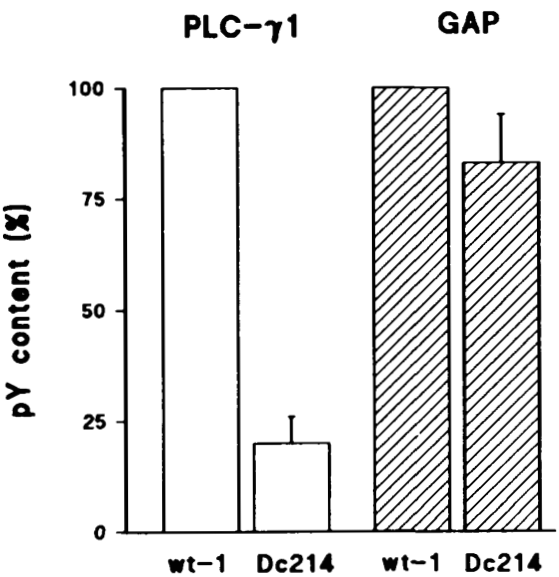

FIG. 5. **EGF-induced tyrosine phosphorylation of *ras*GAP and PLC-γ1 by the Dc214 mutant and wild-type EGF receptors.** NIH 3T3 cells expressing the wild-type (*wt*) or the truncated EGF receptor Dc214 were incubated without or with EGF (100 ng/ml) for 1 h at 4 °C and solubilized in lysis buffer, and *ras*GAP and PLC-γ1 were immunoprecipitated with antiserum to each, electrophoresed, and transferred to nitrocellulose membranes. Tyrosine phosphorylation levels were analyzed by immunobloting using a polyclonal phosphotyrosine antibody. The amount of phosphotyrosine (*pY*) in *ras*GAP and PLC-γ1 has been corrected for the amount of *ras*GAP and PLC-γ1 present and normalized to the number of EGF binding sites present in each cell line as described under "Experimental Procedures." Data are expressed as percent of phosphotyrosine per *ras*GAP detected in cells expressing wt receptor. Values for *ras*GAP correspond to the average of eight independent experiments and PLC-γ1 data correspond to the average of three independent experiments.

*ras*GAP was unexpected. Therefore, we determined the capacity of this mutant to phosphorylate PLC-γ1, another SH2-containing substrate of the EGF receptor (7, 8). The data in Fig. 5 clearly demonstrate that, unlike *ras*GAP, PLC-γ1 was only weakly phosphorylated (20%) by the Dc214 receptor compared to the wild-type receptor. To determine whether the observed differences in EGF-induced phosphorylation of *ras*GAP and PLC-γ1 by the Dc214 receptor mutant represented differences in phosphorylation kinetics, we analyzed the time course of EGF-induced *ras*GAP and PLC-γ1 phosphorylation at 37 °C (Fig. 6). The results shown in *panel A* demonstrate that the time courses of *ras*GAP tyrosine phosphorylation by wild-type and the Dc214 receptors were very similar. However, the capacity of the truncated (Dc214) receptor to phosphorylate PLC-γ1 was significantly impaired at all time points examined (*panel B*). The data in Fig. 6 (*panel C*), show that the *ras*GAP associated proteins pp190 and pp62 are also phosphorylated in response to EGF in cells expressing the Dc214 receptor mutant. Therefore, whereas the EGF receptor carboxyl terminus is essential for efficient phosphorylation of PLC-γ1, it is not obligatory for efficient phosphorylation of *ras*GAP and *ras*GAP-associated proteins.

The general tyrosine kinase activity of the EGF receptor mutant Dc214, which is defective in its capacity to phosphorylate PLC-γ1, but not *ras*GAP, and the EGF receptor mutant Dc123F, which is reduced in its capacity to phosphorylate both *ras*GAP (Fig. 4, Table I) and PLC-γ1 (56), was determined. As seen in Fig. 7*A*, the EGF-induced tyrosine phosphorylation of total cellular protein in cells expressing the Dc214 receptor (*lanes 5* and *6*) was equivalent to that of cells expressing wild-type receptors (*lanes 1* and *2*), while in cells expressing the Dc123F receptor (*lanes 3* and *4*), EGF-induced tyrosine phosphorylation of total cellular proteins was clearly decreased. EGF receptor Western blot of total cellular lysates showed similar levels of receptor expression in these three cell lines (Fig. 7*B*).

Since *ras*GAP was maximally phosphorylated by the Dc214 receptor mutant, we determined whether *ras*GAP was associated with this truncated receptor, which lacks detectable phosphotyrosine residues.[2] As shown in Fig. 8, both PLC-γ1 and *ras*GAP were found to co-precipitate with the wild-type receptor in response to EGF (*lane 2*). However, neither of these substrates was detected in the Dc214 mutant receptor immunoprecipitates (*lane 4*). Therefore, *ras*GAP phosphorylation does not require a relatively stable receptor-substrate association mechanism, whereas receptor association does seem to be necessary for efficient PLC-γ1 phosphorylation.

Autophosphorylation Site Requirements for rasGAP·EGF Receptor Association—The experiments presented in Fig. 8 demonstrate that the EGF receptor carboxyl terminus is required for *ras*GAP association with the receptor *in vivo*, in agreement with *in vitro* association studies (49). To examine which autophosphorylation site(s) might be involved in EGF receptor·*ras*GAP association, cells expressing EGF receptor mutants with autophosphorylation sites mutated or removed by truncation were used. As measured by co-precipitation studies analogous to that shown in Fig. 8, the extent of association of *ras*GAP with several EGF receptor autophosphorylation single site mutants (1173F, 1148F, 1086F, 1068F, or 992F) was not statistically different from the association with the wild-type receptor (Table III). This indicates that there is not a unique single autophosphorylation site essential for *ras*GAP·EGF receptor association. Results depicted in Table III also show that deletion of a carboxyl-terminal receptor fragment that contains Tyr-1173 and -1148 (Dc63) or the simultaneous mutation of these two tyrosines in the full-length receptor (F2) decreased *ras*GAP association with the EGF receptor by 60% ($p < 0.01$, Student's t test). Consistent with these results, mutation of the three major autophosphorylation sites of the EGF receptor (Tyr-1173, -1148, and -1068) (45, 46) reduced *ras*GAP association with the receptor by 82% ($p < 0.001$, Student's t test). The truncated receptor Dc123, which possesses only one known potential autophosphorylation site (Tyr-992) (5, 47), failed to associate with *ras*GAP. Similarly, the EGF

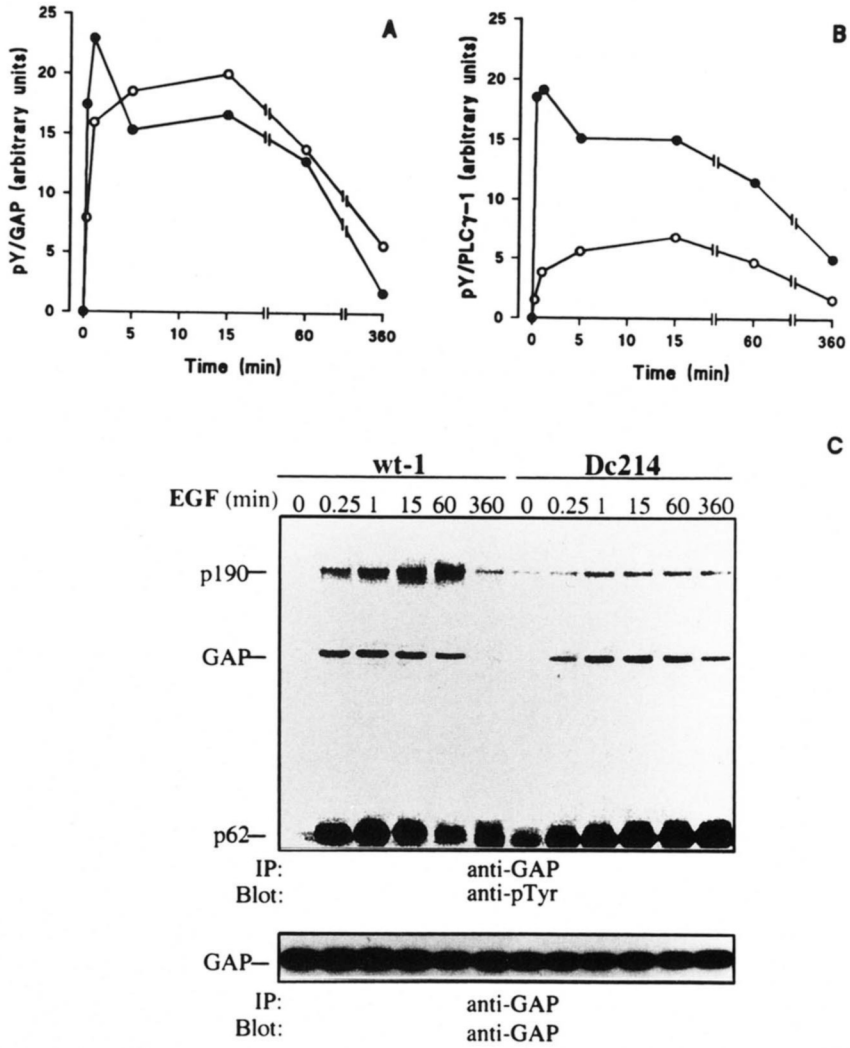

FIG. 6. **Time course of EGF-induced tyrosine phosphorylation of rasGAP and PLC-γ1 by Dc214 and wild-type EGF receptors.** NIH 3T3 cells expressing similar levels of the wild-type (●) or truncated EGF receptor Dc214 (○) were incubated without or with EGF (100 ng/ml) for the indicated times at 37 °C. Cells were solubilized in lysis buffer, and *ras*GAP and PLC-γ1 were immunoprecipitated with antiserum to each, electrophoresed, and transferred to nitrocellulose membranes. *Panel A*, Amount of phosphotyrosine (pY) in *ras*GAP corrected for the amount of *ras*GAP. *Panel B*, Amount of phosphotyrosine (*pY*) in PLC-γ1 corrected for the amount PLC-γ1 present. *Panel C*, anti-phosphotyrosine Western blot analysis of *ras*GAP immunoprecipitates. *Panel D*, anti-*ras*GAP Western blot analysis of the same *ras*GAP immunoprecipitates analyzed in *panel A*. Data of *panels E* and *F* are expressed in arbitrary units. The average value is from three independent experiments. Positions of *ras*GAP and *ras*GAP-associated proteins (*p62* and *p190*) and PLC-γ1 are indicated.

receptor did not associate with *ras*GAP when all five autophosphorylation sites were mutated (F5) or, as previously shown in Fig. 8, removed by truncation (Dc214). Taken together, these results indicate that multiple and perhaps compensatory autophosphorylation sites are essential for stable *ras*GAP association with the EGF receptor.

Phosphorylation of rasGAP and Transforming Activity of EGF Receptor Mutants—To determine whether the capacity of EGF receptor truncation mutants to induce focal transformation could be correlated with changes in *ras*GAP tyrosine phosphorylation, these parameters were measured and compared (Fig. 9). Deletion of 63 or 123 residues from the carboxyl terminus of the EGF receptor decreased both focal transformation capacity as well as *ras*GAP tyrosine phosphorylation. Also, the Dc123F receptor mutant, which is generally deficient in its capacity to phosphorylate *ras*GAP as well as other exogenous substrates, has very weak transforming activity. However, truncation of 214 residues (Dc214) results in recovery of both parameters, cellular transformation and *ras*GAP phosphorylation, to a level equivalent to the wild-type receptor. The capacity of EGF receptor mutants to transform cells, therefore, correlates with their capacity to phosphorylate *ras*GAP.

DISCUSSION

Current evidence indicates that tyrosine kinase autophosphorylation creates selective binding sites for SH2-containing substrates and serves as a regulatory mechanism for substrate phosphorylation and/or activation (7, 8, 37, 45). The results of this report show that, while EGF receptor autophosphorylation sites are required for EGF-induced *ras*GAP association with the receptor, this type of interaction is not essential for maximal tyrosine phosphorylation of this SH2-containing substrate.

The association of *ras*GAP with activated EGF receptors occurs at a very low stoichiometry *in vivo*, indicating that the interaction is very transient and/or of low affinity. Previously, only *in vitro* studies of this association have been reported, showing that activated EGF receptor binds to a TrpE-GAP-SH2 fusion protein (22, 23, 49). Those studies also showed that the activated EGF receptor binds much more efficiently *in vitro* to TrpE-v-*crk* (an SH2-containing oncoprotein) fusion protein than to TrpE-GAP-SH2 fusion protein, indicating that SH2 substrates can interact with the same phosphoprotein, but with markedly different affinities (22). Our results show that significantly less *ras*GAP (about 10-fold) interacts with the EGF

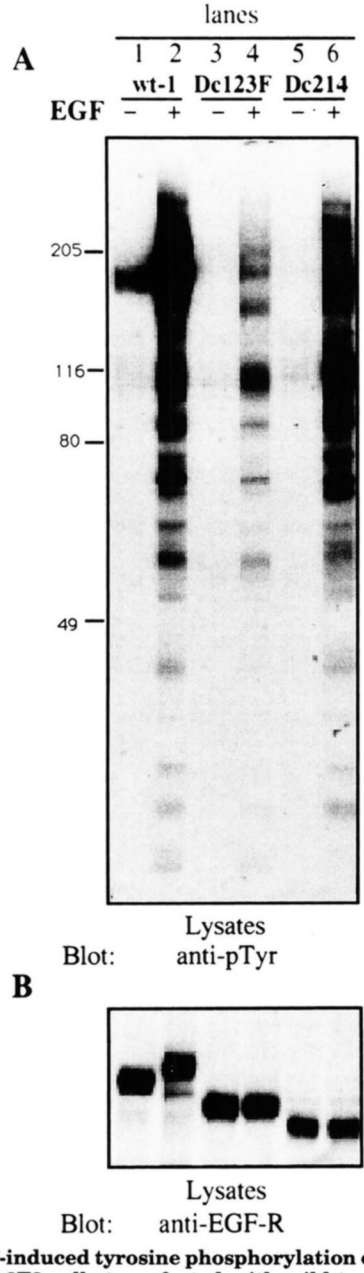

FIG. 7. **EGF-induced tyrosine phosphorylation of total cell proteins in NIH 3T3 cells transfected with wild-type, Dc123F, and Dc214 mutants EGF receptors.** Cells serum-starved overnight were treated with or without EGF (100 ng/ml) for 1 h at 4 °C and then solubilized in lysis buffer. Aliquots of cell lysates were electrophoresed and transferred to nitrocellulose membranes. *Panel A*, anti-phosphotyrosine (*pTyr*) Western blot analysis of total cell lysates. *Panel B*, anti-EGF receptor Western blot analysis of total cell lysates.

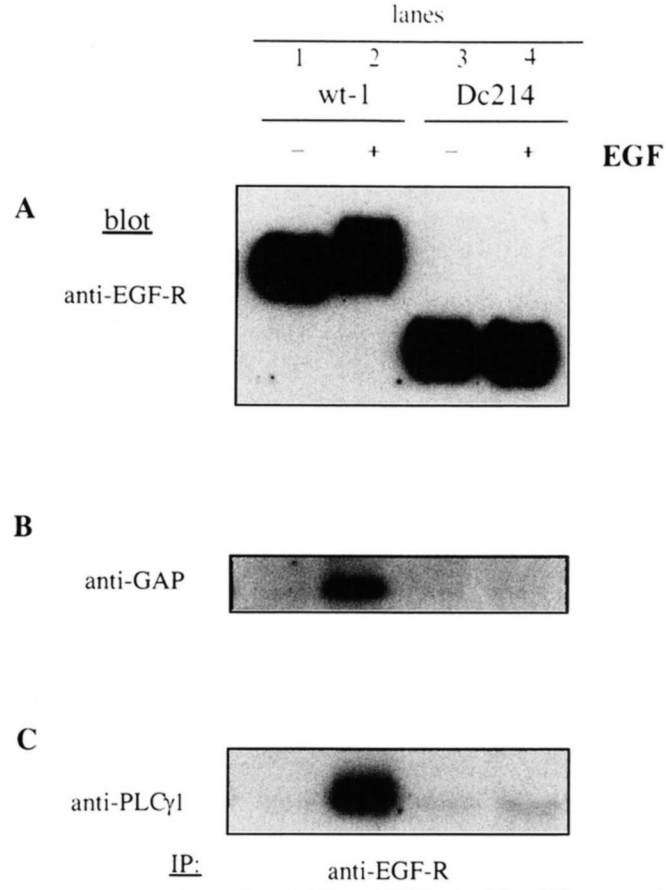

FIG. 8. **Association of rasGAP and PLC-γ1 with wild-type and Dc214 mutant EGF receptors.** NIH 3T3 cells expressing similar number of wild-type (*wt-1*) or truncated (*Dc214*) EGF receptors per cell were incubated with or without 100 ng/ml EGF for 1 h at 4 °C and then solubilized in lysis buffer. EGF receptor was immunoprecipitated as indicated under "Experimental Procedures," electrophoresed, transferred to nitrocellulose membranes, and receptor-associated *ras*GAP and PLC-γ1 were analyzed by immunoblotting. *Panel A*, anti-EGF receptor Western blot analysis of EGF receptor immunoprecipitates. *Panel B*, anti-*ras*GAP Western blot analysis of the same immunoprecipitates shown in *panel A*. *Panel C*, anti-PLC-γ1 Western blot analysis of the same immunoprecipitates shown in *panel A*.

receptor than with the PDGF β-receptor in the same cell background (Figs. 2 and 3).

By contrast, the amount of pp62 associated with *ras*GAP was much higher in EGF- than in PDGF-treated cells. Since phosphorylated pp62 and activated EGF or PDGF receptors bind to the same site in the NH_2-terminal SH2 domain of *ras*GAP (43, 62), the differing affinities of these molecules (*i.e.* activated receptor *versus* pp62) for the same *ras*GAP binding site may determine their relative association in the intact cell. The affinity of pp62 for *ras*GAP may be lower than the affinity of PDGF β-receptor, but higher than the affinity of EGF receptor, explaining the lower amount of EGF receptor·*ras*GAP complexes detected.

EGF autophosphorylation sites are necessary for *in vivo* receptor:*ras*GAP association, since complexes were not detected when all autophosphorylation sites were mutated to phenylalanine (F5) or removed by truncation (Dc214). These results are in agreement with previous studies showing that *in vitro ras*GAP associates with a phosphorylated EGF receptor carboxyl-terminal fragment (49). Furthermore, our data show that there is not a unique autophosphorylation site required for the *in vivo* association of the EGF receptor with *ras*GAP. Rather multiple or compensatory sites seem to be involved. We calculated that tyrosine residues 1173, 1148, and 1068, which are the major autophosphorylation sites of EGF receptor *in vivo* (5, 46–48), account for almost 70–80% of the total amount of *ras*GAP associated with the wild-type receptor. Since the EGF receptor mutant F3 (phenylalanine at residues 1173, 1148, and 1068) still binds a small amount of *ras*GAP (20% of wild type), an additional contribution of minor autophosphorylation sites, such as Tyr-992 or -1086 (5, 46–48) cannot be excluded. The ability of a substrate, such as *ras*GAP, to bind to the receptor *in vivo* depends not only on its relative affinity for each phosphotyrosine site, but also on the extent of phosphorylation of each tyrosine site in the cell receptor population.

The results of this study and other observations indicate that the EGF receptor autophosphorylation sites that are necessary

TABLE III
Association of rasGAP with EGF receptor mutants

Cells expressing the indicated EGF receptor constructs were tested for *ras*GAP association as described in Fig. 8. The amount of *ras*GAP associated with immunoprecipitated EGF receptors was corrected for the amount of immunoprecipitated receptors. Data are expressed as percent of *ras*GAP associated with EGF receptor in cells expressing wild-type (wt) EGF receptor and correspond to the average ± S.E. of at least three independent experiments. Since the amount of receptor-associated *ras*GAP is very low, the estimated error inherent in these experiments is expectedly high and is reflected in the values obtained from different experiments.

EGF receptor	*ras*GAP per EGF receptor immunoprecipitate
	% of wt
wt	100
1173F	83 ± 32
1148F	62 ± 19
1086F	89 ± 37
1068F	72 ± 15
992F	76 ± 27
F2	35 ± 9
F3	18 ± 12
F5	6 ± 9
Dc63	38 ± 10
Dc123	0
Dc214	0

for *ras*GAP binding may also be involved in PLC-γ1[3] (63, 64) and PI 3-kinase binding[3] (65), suggesting the existence of shared binding sites for different SH2-containing substrates. We found that mutation or deletion of tyrosines 1173 and 1148 (F2, Dc63 EGF receptor mutants) significantly decreased *in vivo* *ras*GAP association with the EGF receptor (Table III). Previously, data have shown that *in vitro* binding of a PLCγ-1 SH2 fusion protein to a mutant EGF receptor lacking tyrosine residues 1173 and 1148 exhibited a lower affinity compared to recognition of wild-type receptor (63). Also, quantitative binding studies have shown that the SH2 domains from p85 and *ras*GAP bind to equivalent or overlapping sites on the EGF receptor (65). By contrast, the existence of specific and non-overlapping binding sites in the PDGF β-receptor for these three SH2-containing substrates has been described (43–45). Specific sites for PLC-γ1 recognition have also been identified in the fibroblast growth factor (39, 40) and *trk* receptors (41), although *ras*GAP binding sites have not been reported. Therefore, *ras*GAP binding to EGF receptor clearly differs from the PDGF β-receptor model.

Similar to the receptor association studies, our data did not reveal any particular autophosphorylation site(s) specifically required for *ras*GAP tyrosine phosphorylation by the EGF receptor (Table II). In the context of the full-length EGF receptor, *ras*GAP phosphorylation is maximal only in the presence of all autophosphorylation sites. Interestingly, however, the truncated EGF receptor Dc214, which does not associate with detectable amounts of *ras*GAP, phosphorylates this substrate to the same extent as the wild-type receptor (Figs. 5 and 6). Thus, our data indicate that the interaction of *ras*GAP SH2 domains with EGF receptor autophosphorylation sites is not obligatory for EGF-induced *ras*GAP phosphorylation. Taken together the data suggest that phosphorylation of multiple autophosphorylation sites or their deletion by carboxyl-terminal truncation of receptor provides a proper conformation of the kinase domain for efficient *ras*GAP phosphorylation. This hypothesis would be consistent with an inhibitory role of the non-phosphorylated carboxyl terminus, as has been suggested previously for the regulation of kinase activity (6). Tyrosine phosphorylation of a 120-kDa protein by the mutant receptor Dc214 was previously analyzed by another group (66). Their results

[3] C. Soler and G. Carpenter, manuscript in preparation.

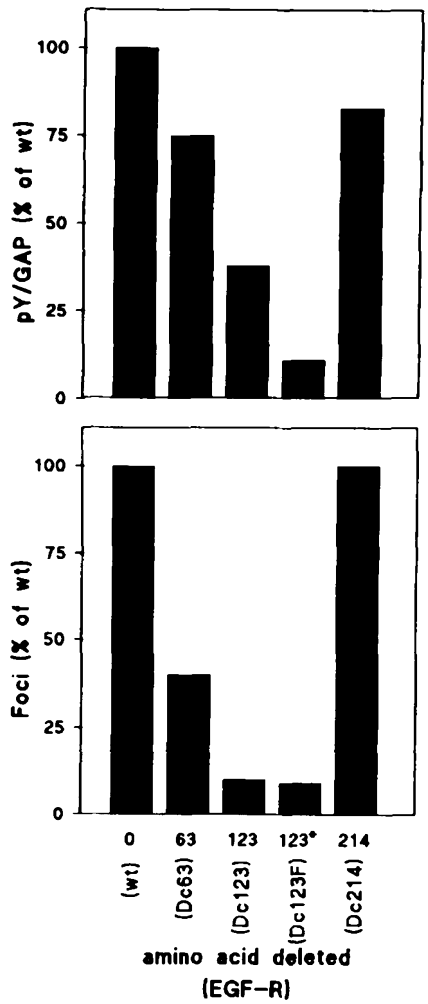

FIG. 9. **Comparison of EGF-induced focal transformation and tyrosine phosphorylation of *ras*GAP by different EGF receptor mutants.** EGF-induced focal transformation and *ras*GAP tyrosine phosphorylation by different EGF receptors with increasing carboxyl-terminal deletions were compared. *Upper panel*, amount of phosphotyrosine (*pY*) in *ras*GAP in EGF-treated cells. Quantitation of EGF-induced tyrosine phosphorylation of *ras*GAP by different EGF receptor mutants is indicated in Table I. *Bottom panel*, focal transformation induced by cells expressing EGF receptor mutants. Foci were counted 2 weeks after transfection of EGF receptors. Cells were cultured in the presence of 20 ng/ml EGF. The wild-type EGF receptor give rise to approximately 1600 foci/μg DNA (=100). No foci were observed in the absence of EGF in any of the transfected cell lines, except Dc214. Focal transformation produced by Dc214 in the absence of EGF was 20% of that produced by Dc214 or wild-type receptors in the presence of EGF. Results are the average of more than five independent transfections. *In addition to 123 amino acid residues deletion, Tyr-992 was mutated to phenylalanine.

showed that in response to EGF several tyrosine phosphorylated proteins, including pp120, were recovered in *ras*GAP immunoprecipitates from cells transfected with this EGF receptor mutant. However, the authors concluded that pp120 did not correspond to *ras*GAP as it was not recovered by reimmunoprecipitation of dissociated complexes.

PLC-γ1 behaves differently from *ras*GAP (Figs. 5 and 6) and other unidentified cellular tyrosine phosphorylated proteins (Fig. 7). Compared to the wild-type EGF receptor, phosphorylation of PLC-γ1 by the truncated EGF receptor Dc214 is significantly reduced. This is consistent with previous results demonstrating that EGF-induced tyrosine phosphorylation of PLC-γ1 was positively regulated by receptor autophosphorylation (53, 56). Although another group reported that the truncated EGF receptor Dc214 phosphorylates PLC-γ1 to the same

extent as the wild-type receptor (67), in that study phosphorylation levels were not corrected for the significantly different (10-fold) levels of EGF receptor expression. Also, in that prior study, data were obtained at one time point, *i.e.* 5 min during a 37 °C incubation in the presence of EGF. We have found that PLC-γ1 phosphorylation at 37 °C has a sharp optimum, being maximal at 1 min and then rapidly decreased. Since the truncated EGF receptor Dc214 does not bind PLC-γ1 (Fig. 8) (67), we suggest that, unlike *ras*GAP, PLC-γ1 binding to the EGF receptor carboxyl terminus is obligatory for a significant level of tyrosine phosphorylation.

Our data also suggest that phosphorylation of *ras*GAP and/or *ras*GAP-associated proteins, unlike PLC-γ1 phosphorylation, may be necessary for the EGF mitogenic pathway. The receptor mutant Dc214, which does produce a wild-type level of EGF-induced transforming activity (Fig. 9) (68, 69), fails to phosphorylate PLC-γ1, but does phosphorylate *ras*GAP and *ras*GAP-associated proteins to the same extent as the wild-type receptor (Fig. 6). Several groups have recently found that phosphorylation of PLCγ-1 by fibroblast growth factor and PDGF-β receptors is dispensable for mitogenesis (37, 39, 40). Also, PDGF-induced *ras*GAP association with the receptor was described as not essential for mitogenesis, at least in TRMP and NMuMG cells transfected with PDGF receptors (43, 44). Consistent with our results, it has been reported that PDGF stimulation of *ras*GAP tyrosine phosphorylation in NIH 3T3 cells does correlate with mitogenic signaling (70). Also, tyrosine phosphorylation of *ras*GAP has been shown to correlate with the transforming activity of $p56^{lck}$ (71). Finally, v-*src* mutants that fail to phosphorylate *ras*GAP and pp62 demonstrate poor transforming activity (22). Therefore, the phosphorylation of *ras*GAP and *ras*GAP-associated proteins may be biologically important, however, it is not known whether these phosphorylations are essential to achieve mitogenesis.

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