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Mutation of Proline-1003 to Glycine in the Epidermal Growth Factor (EGF) Receptor Enhances Responsiveness to EGF

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We have shown previously that the epidermal growth factor (EGF) receptor is phosphorylated at Ser-1002 and that this phosphorylation is associated with desensitization of the EGF receptor. Ser-1002 is followed immediately by Pro-1003, a residue that may promote the adoption of a specific conformation at this site or serve as a recognition element for the interaction of the EGF receptor with other proteins. To examine these possibilities, we have mutated Pro-1003 of the EGF receptor to a Gly residue and have analyzed the effect of this mutation on EGF-stimulated signaling. Cells expressing the P1003G EGF receptors exhibited higher EGF-stimulated autophosphorylation and synthetic peptide phosphorylation compared to cells expressing wild-type EGF receptors. In addition, the ability of EGF to stimulate PI 3-kinase activity and mitogen-activated protein kinase activity was enhanced in cells expressing the P1003G EGF receptor. Cells expressing P1003G receptors also demonstrated an increased ability to form colonies in soft agar in response to EGF. These results indicate that mutation of Pro-1003 leads to a potentiation of the biological effects of EGF. The findings are consistent with the hypothesis that Pro-1003 plays a role in a form of regulation that normally suppresses EGF receptor function.

INTRODUCTION

The epidermal growth factor (EGF)[1] receptor is a 170-kDa transmembrane glycoprotein with intrinsic tyrosine protein kinase activity (Cohen *et al.*, 1980; Ushiro and Cohen, 1980; Lin *et al.*, 1984; Ullrich *et al.*, 1984). Binding of EGF to the extracellular domain of the receptor triggers a series of events that culminates in DNA synthesis and mitosis (Carpenter, 1979). Early responses stimulated by EGF include EGF receptor dimerization (Yarden and Schlessinger, 1987a,b; Cochet *et al.*, 1988), tyrosine protein kinase activation, and autophosphorylation (Carpenter *et al.*, 1978; Cohen *et al.*, 1980; Ushiro and Cohen, 1980). Autophosphorylation occurs at tyrosine residues within the cytoplasmic domain of the EGF receptor (Downward *et al.*, 1985; Hsuan *et al.*, 1989; Walton *et al.*, 1990) and provides sites for the binding of SH2-containing proteins. Many of the effects of EGF appear to be mediated by the interaction of the receptor with SH2-containing proteins such as Grb2 (Buday and Downward, 1993; Gale *et al.*, 1993; Li *et al.*, 1993; Rozakis-Adcock *et al.*, 1993), PI 3-kinase (Bjorge *et al.*, 1990), and phospholipase C-γ (Margolis *et al.*, 1990; Vega *et al.*, 1992).

The activity of the EGF receptor is regulated by a variety of mechanisms. Receptor down regulation occurs after prolonged treatment of cells with EGF and is due to the internalization and degradation of cell surface EGF receptors. The resulting loss of EGF receptors leads to a decrease in the ability of cells to respond to EGF. EGF receptor function is also modulated through receptor desensitization. Desensitization refers to a situation in which the number of cell surface receptors remains the same, but their ability to transduce a signal is reduced. EGF receptor desensitization appears to be

[1] Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EGF, epidermal growth factor; HBSS, Hanks-buffered saline solution; PBS, phosphate-buffered saline, PMSF, phenylmethylsulfonyl fluoride.

the result of phosphorylation of the receptor on serine or threonine residues. For example, protein kinase C phosphorylates the EGF receptor at Thr-654 (Cochet *et al.*, 1984; Friedman *et al.*, 1984; Hunter *et al.*, 1984; Davis and Czech, 1985; Downward *et al.*, 1985). This has been shown to decrease the affinity of the receptor for EGF (Cochet *et al.*, 1984; Friedman *et al.*, 1984; Downward *et al.*, 1985), decrease receptor tyrosine kinase activity (Cochet *et al.*, 1984; Friedman *et al.*, 1984; Downward *et al.*, 1985), and inhibit receptor internalization (Lund *et al.*, 1990). More recently, Countaway *et al.* (1992) have shown that EGF receptors isolated from desensitized cells were phosphorylated at Ser-1046, Ser-1047. Mutation of these residues to alanines blocked receptor desensitization. Subsequent studies by this group demonstrated that the Ala-1046, Ala-1047 mutation led to an inhibition of EGF receptor internalization and down regulation and was associated with a potentiation of the biological effects of EGF (Theroux *et al.*, 1992).

We have recently demonstrated that the EGF receptor in A431 cells can be desensitized and that this desensitization is associated with enhanced in vivo phosphorylation of the receptor on Ser-1002 (Kuppuswamy *et al.*, 1993). This phosphorylation is associated with a decrease in the ability of EGF to stimulate receptor dimerization (Kuppuswamy and Pike, 1991), tyrosine kinase activity (Kuppuswamy *et al.*, 1993), phosphatidylinositol (PI) turnover (Cunningham *et al.*, 1989), and receptor internalization (Kuppuswamy and Pike, 1989). Ser-1002 is followed immediately by Pro-1003, a residue that may be important in determining the conformation of the receptor at this site or for recognition by interacting proteins. To further investigate the role of this Ser-Pro site in the regulation of EGF receptor function, we employed site-directed mutagenesis to construct an EGF receptor in which Pro-1003 was converted to a glycine residue. This alteration should remove any kinks introduced into the structure of the EGF receptor by the proline residue. In addition, the glycine would not be recognized by enzymes requiring proline as part of the recognition sequence. We report here that this point mutation results in the production of an EGF receptor that exhibits enhanced responsiveness to EGF in a variety of in vivo and in vitro assays. These data suggest that Pro-1003 plays an important role in a form of regulation that suppresses EGF receptor function in vivo.

MATERIALS AND METHODS

Materials

Gene Amp kit was from Perkin Elmer Cetus (Norwalk, CT). Sequenase sequencing kit was from United States Biochemical (Cleveland, OH). Reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL). Anti-phosphotyrosine antibodies (4G10) were from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibodies to the EGF receptor (DB-1) were raised against a GST-fusion protein containing residues 672–899 of the EGF receptor. ^{125}I-EGF was prepared using the chloramine T method as described (McFarthing, 1992). All other reagents were from Sigma (St. Louis, MO).

Methods

Mutagenesis and Expression of EGF Receptor. A full length clone of human EGF receptor was kindly provided by Roger Davis (University of Massachusetts). A *Bste*II to *Sac* I fragment containing nucleotides 2889–3291 was subcloned in pBlueScript to give pBS1. Primers were synthesized corresponding to nucleotides 3259–3296 for the sense primer (5'AGCAGCGCCTCCACGTCACGGACTCCCCTCCTGAGCTC 3') containing the Pro to Gly mutation and nucleotides 3258–3218 for the anti-sense primer (5'GAAGAAGCCCTGCTGTGGGATGAGGTACTCGTCGGCATCCA 3'). These primers were used with pBS1 as template to amplify the entire plasmid by polymerase chain reaction. The reaction products were ligated and used to transform *Escherichia coli*. Transformants containing the mutation were identified by DNA sequencing. A *Bgl* II to *Sac* I fragment containing the mutation was transferred into the full length EGF receptor coding sequence in plasmid pBlueScript to yield pBlueScript EGFR-PG. The insert from pBlueScript EGFR-PG was transferred to the mammalian expression vector pJC119 at the *Xho* I cloning site (Sprague *et al.*, 1983) to produce pJC119 EGFR-PG. pJC119 EGFR-PG or pJC119 EGFR-WT (containing the wild-type EGF receptor) were cotransfected with the antibiotic resistance plasmid pSV2NEO into NIH3T3 cells using a standard calcium chloride precipitation method (Gorman *et al.*, 1984). Resistant colonies were selected in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum plus 400 μg/ml G418. Stable clones were screened for expression of the EGF receptor by quantitating the binding of ^{125}I-EGF.

^{125}I-EGF Binding. Cells were preincubated in DMEM containing 0.1% bovine serum albumin (BSA) for 60 min at 37°C. After three washes with Hanks-buffered saline solution (HBSS), the cells were incubated for 2 h at 4°C with 50 pM ^{125}I-EGF in DMEM containing 40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and 0.1% BSA. Subsequently, the monolayers were washed three times with HBSS and solubilized by incubation with 1 ml of 1 N NaOH for 1 h at 37°C. Aliquots were transferred to vials and counted in an LKB gamma counter. Nonspecific binding was determined in parallel incubations carried out in the presence of 500 nM unlabeled EGF. For Scatchard analysis, ^{125}I-EGF binding was carried out in a similar fashion except that increasing concentrations of unlabeled EGF were added to the incubations. Results were analyzed using the LIGAND computer program (Munson and Rodbard, 1980)

EGF Receptor Down Regulation. Cells were plated on 12-well dishes 5 d in advance. The cells were washed once with HBSS and incubated with 1 nM EGF in DMEM containing 0.1% BSA at 37°C for the indicated times. At the end of the incubation, the cells were washed once in ice cold HBSS and twice in 50 mM glycine, 100 mM NaCl pH 3.0 to remove cell surface EGF. After three additional washes with HBSS, the binding of ^{125}I-EGF was measured as described above.

Western Blotting. Cells were treated without or with 50 nM EGF and lysed in RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 100 μM sodium orthovanadate. Lysates were incubated on ice for 10 min and clarified by centrifugation at 12 000 × *g* for 10 min at 4°C. Protein was determined by BCA assay, and equal protein samples were subjected to gel electrophoresis and transferred to nitrocellulose. Anti-phosphotyrosine blots were probed with 4G10 antibody and developed using ^{125}I-labeled protein A followed by autoradiography. EGF receptor blots were probed with DB-1 and developed using an alkaline phosphatase detection system.

Arg-Arg Src Peptide Phosphorylation. Cells were incubated in DMEM containing 0.1% BSA for 1 h and then scraped into 1 ml of buffer containing 10 mM tris(hydroxymethyl)aminomethane (Tris)-HCl pH 7.2, 2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N'-N'-tetraacetic acid, 1 mM PMSF, 100 μM vanadate, 1 μg/ml leupeptin, and 1 μg/ml aprotinin. Cells were homogenized with 30 strokes in a Dounce homogenizer and centrifuged at 18 000 rpm for 30 min in an

SS34 rotor. The resulting pellets were resuspended in 500 μl buffer containing 40 mM imidazole, 250 mM NaCl, 10% glycerol pH 7.5. EGF receptor tyrosine protein kinase activity was then measured on a 6-μl aliquot of the resuspended membranes using [γ-^{32}P]ATP and 2 mM Arg-Arg-Src peptide as substrates for 10 min at 30°C as described previously (Pike, 1987). Samples were spotted on Whatman P81 phosphocellulose paper (Clifton, NJ) and washed three times in 75 mM H_3PO_4 and then counted for ^{32}P.

Phosphopeptide Mapping. Cells were incubated overnight in phosphate-free DMEM containing 0.5% calf serum and 700 μCi/ml $^{32}PO_4^{3-}$. After treatment with 100 nM EGF for 20 min, cultures were lysed in RIPA buffer, and EGF receptors were immunoprecipitated using the DB-1 anti-EGF receptor antibody. Immunoprecipitates were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, and the band corresponding to the EGF receptor was identified by autoradiography. The EGF receptor band was excised from the gel, and phosphopeptide mapping was carried out as described by Kuppuswamy *et al.* (1993).

Mitogen-activated Protein (MAP) Kinase Assay. Cells were treated without or with 50 nM EGF for 5 min, then washed twice with phosphate-buffered saline and scraped into 0.6 ml buffer containing 20 mM HEPES, 50 mM β-glycerol phosphate pH 7.2, 1 mM dithiothreitol (DTT), 100 μM sodium orthovanadate, 10 mM benzamidine, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 2 μg/ml pepstatin A, and 1 mM PMSF. The cells were homogenized with 30 strokes in a Dounce homogenizer and centrifuged for 30 min at 18 000 rpm in an SS34 rotor. The supernatants were collected, and a 6-μl aliquot of each sample was mixed with 12 μl of a reaction mix to yield (in final concentrations): 50 mM β-glycerol phosphate, 1 mM DTT, 100 μM vanadate, 10 mM $MgCl_2$, 0.4 mg/ml BSA, 100 μM ATP, 2.4 μCi [γ-^{32}P]ATP, and 0.33 mg/ml myelin basic protein. Reactions were incubated for 15 min at 30°C and terminated by the addition of trichloroacetic acid to a final concentration of 2.5%. Samples were spotted and counted as stated above for the Arg-Arg-Src peptide assay.

PI-3 Kinase Assay. Before the assay, cells were incubated in DMEM containing 0.1% BSA for 1.5 h. For the assay, cells were treated without or with 50 nM EGF for the times indicated then washed and lysed in PI 3-kinase solubilization buffer (137 mM NaCl, 20 mM Tris pH 8.0, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 1% Triton X-100, 1 mM DTT, and 400 μM sodium orthovanadate plus protease inhibitors as above). Lysates were clarified by centrifugation for 10 min at 12 000 × *g*. The supernatants were collected, and protein concentrations were determined by BCA assay. Equal amounts of cell protein were incubated with anti-phosphotyrosine antibody 4G10 for 2 h on ice, and immune complexes were precipitated by addition of protein A Sepharose. Assay of PI 3-kinase activity in the immunoprecipitates was carried out as described by Auger *et al.* (1990).

Growth in Soft Agar. Cells were plated at 5 × 10^4 cells per 35-mm well in DMEM plus 10% calf serum and 0.3% agar on a bottom layer of 0.6% agar. EGF was included in the plating medium at concentrations ranging from 0 to 20 nM. Cells were fed every 4 d with 1 ml fresh medium containing 0.3% agar and the appropriate concentration of EGF. Colonies containing >20 cells were scored 7 d after plating.

RESULTS

^{125}I-EGF Binding and Receptor Down Regulation

Wild-type and P1003G EGF receptors were expressed in NIH-3T3 cells that possess negligible levels of endogenous EGF receptors. Several clones expressing each type of receptor were analyzed for their ability to bind ^{125}I-EGF. Figure 1A presents Scatchard plots of these binding data for a clone expressing wild-type EGF receptors (WT1) and a clone expressing the P1003G EGF receptor (PG6). As can be seen from the figure, both wild-type and P1003G EGF receptors exhibited high

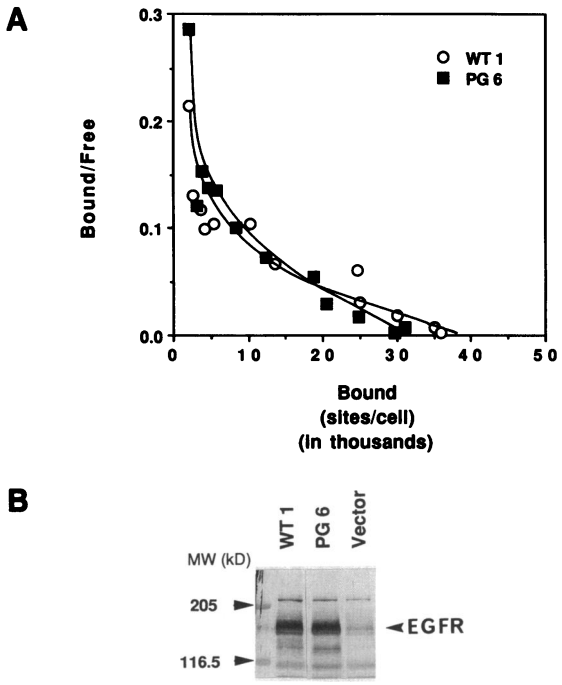

Figure 1. EGF receptor levels in cells expressing wild-type and P1003G EGF receptors. (A) Cells were grown to confluence in 12-well multidishes. ^{125}I-EGF binding was carried out as described in MATERIALS AND METHODS. Results were analyzed using the LIGAND computer program and are presented as Scatchard plots. Results represent the mean of triplicate determinations. (B) WT1 cells, PG6 cells, and 3T3 cells transfected with vector alone were grown to confluence on D-100 plates. Lysates were prepared as outlined in MATERIALS AND METHODS, and aliquots containing equal amounts of protein were run on a 10% SDS polyacrylamide gel. The proteins were transferred to nitrocellulose and blotted with the polyclonal anti-EGF receptor antibody, DB-1.

and low affinity ^{125}I-EGF binding sites. Mutation of Pro-1003 to Gly did not significantly alter the affinity of EGF for either class of sites. ^{125}I-EGF binding affinities were 10–20 pM for the high affinity site and ~700 pM for the low affinity site in cells expressing either the wild-type or the P1003G EGF receptor. Similar results were obtained when other clones expressing wild-type and P1003G EGF receptor were examined. These data indicate that mutation of Pro-1003 does not lead to major changes in the ligand-binding properties of the receptor. These data were corroborated by Western blot analysis of the EGF receptors present in WT1 cells, PG6 cells, and cells transfected with vector alone. As can be seen from the data in Figure 1B, WT1 and PG6 cells expressed a similar number of EGF receptors, whereas the cells transfected with vector alone expressed no detectable EGF receptors. By Scatchard analysis, cells transfected with vector alone had fewer than 2000 EGF receptors per cell. Because WT1 cells (~40 000 receptors/cell) and PG6 cells (~32 000 receptors/cell) expressed similar numbers of EGF receptors, these clones were used in most further analyses.

Several clones expressing wild-type or P1003G EGF receptors were analyzed for their ability to undergo EGF-induced receptor down regulation. Three clones expressing wild-type and three clones expressing P1003G EGF receptors were incubated with 1 nM EGF for various lengths of time and washed. The remaining cell surface receptors were then quantitated by ^{125}I-EGF binding. As shown in Figure 2, both wild-type (left) and P1003G (right) EGF receptors were lost from the cell surface after prolonged incubation with EGF. However, for all three clones expressing the P1003G EGF receptor, down regulation was more rapid and more extensive than in the three clones expressing the wild-type receptor. These data suggest that the P1003G mutation is associated with an increase in the rate and extent of receptor down regulation.

Tyrosine Kinase Activity and Phosphorylation of Wild-Type and P1003G EGF Receptors

Despite the fact that the P1003G EGF receptor was more rapidly down regulated, cells expressing this mutated receptor showed enhanced responsiveness to EGF. The data in Figure 3A demonstrate that autophosphorylation of the EGF receptor was markedly increased both in intensity and duration in PG6 cells as compared to WT1 cells. Quantitation of the phosphotyrosine content of the EGF receptor indicated a two- to threefold increase in receptor autophosphorylation in PG6 cells as compared to WT1 cells. No EGF-stimulated autophosphorylation could be detected in cells transfected with vector alone (Figure 3A, vector lanes). Assay of membranes derived from WT1 and PG6 cells for tyrosine kinase activity using the Arg-Arg-Src peptide substrate demonstrated that basal and EGF-stimulated peptide phosphorylation was twofold greater in PG6 cells than in WT1 cells (Figure 3B). Cells transfected with vector alone showed no EGF-stimulated peptide phosphorylation. Thus, both EGF receptor autophosphorylation and phosphorylation of exogenous substrates were enhanced in cells expressing the P1003G EGF receptor.

To further examine the effect of the P1003G mutation on EGF receptor phosphorylation, WT1 and PG6 cells were labeled overnight with $^{32}PO_4^{3-}$. The cells were treated with EGF for 20 min, and then ^{32}P-labeled EGF receptors were subjected to two-dimensional (2-D) tryptic phosphopeptide mapping. Figure 4 shows the peptide maps of EGF receptors derived from cells expressing wild-type (WT1) or P1003G (PG6) EGF receptors. Although the pattern of phosphopeptides is similar for the wild-type and P1003G receptors, the phosphorylation of two peptides, 3 and 4, was markedly increased in the P1003G receptors as compared to the wild-type receptors. Phosphoamino acid analyses indicated that peptide 3 contained exclusively phosphoserine, whereas peptide 4 contained roughly equivalent amounts of phosphoserine and phosphothreonine. Our inability to obtain sufficient amounts of material prevented further analyses of individual phosphopeptides.

Comparison of the Biological Response to EGF in WT1 and PG6 Cells

The ability of EGF to stimulate PI 3-kinase activity in WT1 cells and PG6 cells was next compared (Figure 5). In WT1 cells, EGF stimulated a twofold increase in PI 3-kinase activity. The response peaked at 2 min and declined by 5 min to a level ~50% higher than control. By contrast, in PG6 cells, EGF stimulated a four- to fivefold increase in PI 3-kinase activity. The response in PG6 cells also peaked at 2 min and while PI 3-kinase activity declined thereafter, a two- to threefold increase in activity was maintained for ≥15 min. These data demonstrate that the ability of EGF to stimulate PI 3-kinase activity was significantly enhanced in PG6 cells.

The effect of EGF on the stimulation of MAP kinase activity was also potentiated in PG6 cells as compared to WT1 cells. As shown in Figure 6, EGF rapidly stimulated MAP kinase activity in both cell lines, but the maximal level of EGF-stimulated MAP kinase activity was nearly twofold greater in cells expressing the P1003G EGF receptor than in cells expressing the wild-type receptor.

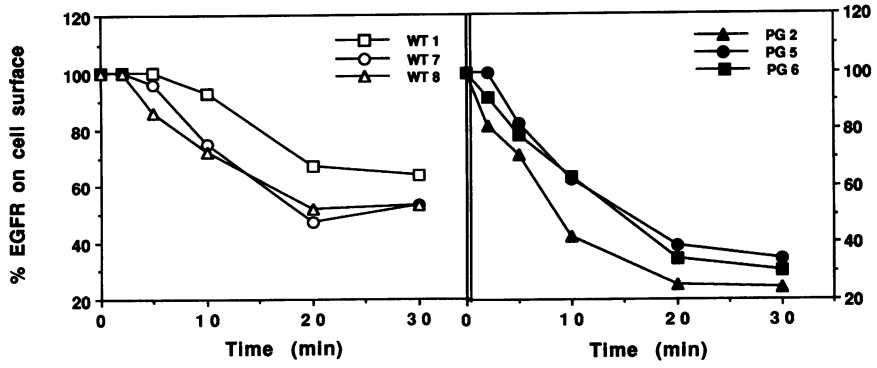

Figure 2. Down regulation of wild-type and P1003G EGF receptors. Cells expressing wild-type (left) or P1003G (right) EGF receptors were grown to 80% confluency in 12-well multidishes and treated with 1 nM EGF at 37°C for the times indicated. After a wash with low pH buffer, 50 pM ^{125}I-EGF was added to the cultures for 2 h at 4°C, and ^{125}I-EGF binding was determined as described in MATERIALS AND METHODS. Results represent the mean of triplicate determinations from a representative experiment.

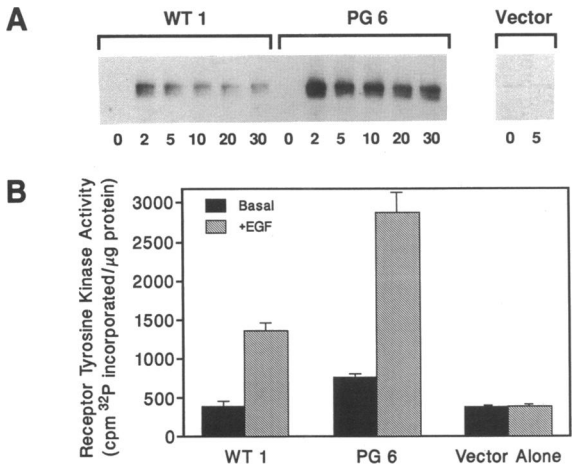

Figure 3. Kinase activity of wild-type and P1003G EGF receptors. (A) Autophosphorylation of wild-type and P1003G EGF receptors. WT1 cells and PG6 cells were treated with 50 nM EGF at 37°C for the times indicated. Cells transfected with vector alone were treated with 50 nM EGF at 37°C for 5 min. Cells were lysed and aliquots containing equal amounts of protein were run on a 10% SDS polyacrylamide gel. After electrophoresis and transfer to nitrocellulose, the proteins were analyzed by Western blot using anti-phosphotyrosine antibodies. (B) Phosphorylation of a synthetic peptide by membranes derived from WT1 cells, PG6 cells, and cells transfected with vector alone. Cells were treated with 50 nM EGF for 5 min at 37°C. Membranes were prepared and assayed for their ability to phosphorylate the Arg-Arg-Src peptide as outlined in MATERIALS AND METHODS. Results represent the mean ± SD of triplicate determinations from a representative experiment.

To determine whether enhanced biological responsiveness in short term assays such as PI 3-kinase or MAP kinase activation translated into enhanced transforming potential, the ability of EGF to stimulate the growth in soft agar of NIH 3T3 cells expressing wild-type or P1003G EGF receptors was compared. The data are shown in Figure 7. In the absence of EGF, WT1 cells and PG6 cells formed a similar, low number of colonies in soft agar. Both cell lines formed progressively more colonies as the concentration of EGF in the medium increased. However, at any given concentration of EGF, cells expressing the P1003G EGF receptor formed a substantially greater number of colonies than did cells expressing the wild-type receptor. Cells transfected with vector alone failed to grow in soft agar either in the presence or absence of EGF. These data indicate that mutation of Pro-1003 results in the production of an EGF receptor with enhanced transforming ability.

DISCUSSION

Our data indicate that EGF receptors mutated at Pro-1003 display enhanced responsiveness to EGF both in vivo and in vitro. The P1003G EGF receptor was more highly autophosphorylated in vivo in response to EGF, and the in vitro tyrosine kinase activity of the P1003G EGF receptor was also increased relative to that of wild-type EGF receptor. Several downstream indicators of EGF receptor activity were also affected by this mutation. EGF-stimulated PI 3-kinase activity, MAP kinase activity, and soft agar growth were all potentiated in cells expressing the P1003G EGF receptor. These results are similar to the findings of Theroux *et al.* (1992) who observed a potentiation of EGF effects after mutation of Ser-1046 and Ser-1047 to alanine. The phosphorylation of Ser-1046 and Ser-1047 had been shown to be involved in a calcium-dependent desensitization of the EGF receptor (Countaway *et al.*, 1992). Removal of these sites of phosphorylation was postulated to release the receptor from this inhibitory input thereby increasing responsiveness to EGF. Likewise, conversion of Thr-654 to alanine resulted in the production of a receptor that showed an enhanced ability to stimulate phosphatidylinositol turnover (Decker *et al.*, 1990). Thus, alteration of the EGF receptor at sites involved in receptor desensitization appears to sensitize the receptor to EGF.

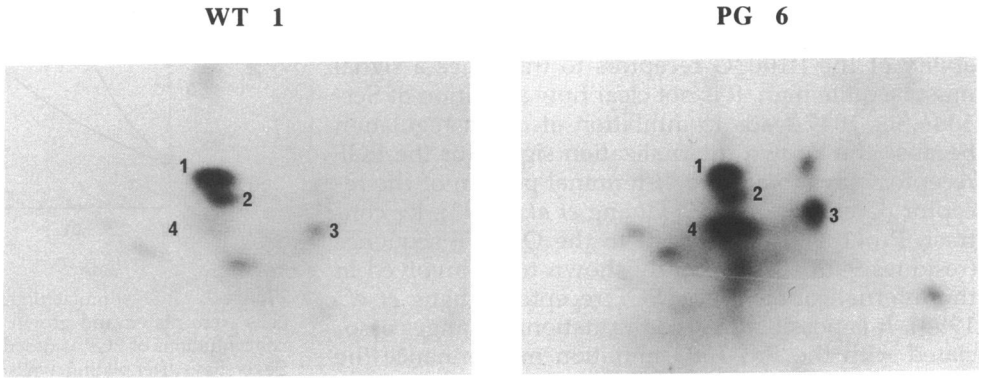

Figure 4. 2-D tryptic phosphopeptide maps of in vivo ^{32}P-labeled wild-type and P1003G EGF receptors. WT1 or PG6 cells were grown on D150 plates and labeled for 18 h with 0.7 mCi/ml $^{32}PO_4^{3-}$ in phosphate-free DMEM containing 0.5% calf serum. At the end of the labeling period 100 nM EGF was added for 20 min and the EGF receptors were isolated by immunoprecipitation with an anti-EGF receptor antibody followed by SDS polyacrylamide gel electrophoresis as described in MATERIALS AND METHODS. Receptors were digested with trypsin and separated by 2-D thin layer chromatography. ^{32}P-labeled phosphopeptides were visualized by autoradiography.

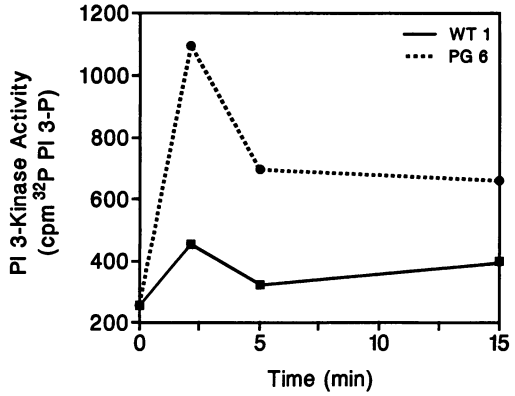

Figure 5. EGF-stimulated PI 3-kinase activity in cells expressing wild-type or P1003G EGF receptors. WT1 and PG6 cells were treated without or with 50 nM EGF for the indicated times and then lysed in Triton X-100–containing buffer. Supernatants were immunoprecipitated with anti-phosphotyrosine antibodies, and the immunoprecipitates were assayed for PI 3-kinase activity as described in MATERIALS AND METHODS. Under the same conditions, cells transfected with vector alone showed a basal PI 3-kinase activity of 47 ± 3 cpm and 99 ± 9 cpm after a 2-min stimulation with EGF.

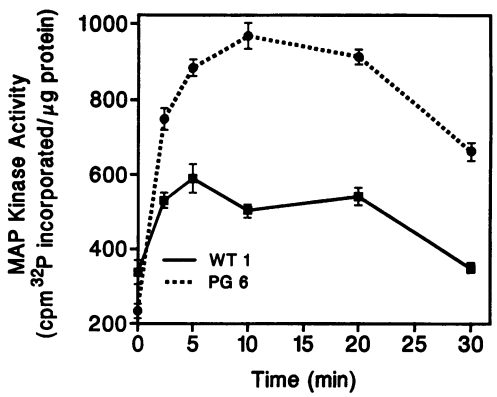

Figure 6. EGF-stimulated MAP kinase activity in cells expressing wild-type or P1003G EGF receptors. WT1 and PG6 cells were treated without or with 50 nM EGF for the times indicated, then washed, and cell cytosols prepared. The cytosols were assayed for MAP kinase activity using myelin basic protein as substrate as outlined in MATERIALS AND METHODS. Results represent the mean ± SD of triplicate determinations from a representative experiment.

Our data demonstrating that mutation of Pro-1003 leads to a potentiation of the effects of EGF are therefore consistent with the hypothesis that Pro-1003 is involved in a regulatory process, such as receptor desensitization, that normally leads to the suppression of EGF receptor signal transducing activity.

Of interest is the finding that the ability of EGF to down regulate its receptor was also potentiated by the P1003G mutation. This finding contrasts with the results obtained using EGF receptors in which Ser-1046,Ser-1047 had been converted to alanine residues. Mutation of those residues led to an inhibition of ligand-induced receptor internalization and down regulation (Lund *et al.*, 1990; Countaway *et al.*, 1992). We have shown previously that receptor internalization is inhibited in desensitized A431 cells, thus a potentiation of down regulation after the abrogation of receptor desensitization was anticipated. Because down regulation limits the responsiveness of cells to EGF, the observation that other responses to EGF were still potentiated in the face of increased down regulation suggests that the intrinsic ability of the P1003G receptors to transduce a signal must be quite high. It is not clear how alteration of Ser-1046,Ser-1047 leads to inhibition of down regulation because the known internalization signal for the EGF receptor maps to a more N-terminal portion of the receptor (Chen *et al.*, 1989; Chang *et al.*, 1993). By contrast, Pro-1003 lies adjacent to the QQGFF sequence (residues 996–1000) recently shown to be involved in the internalization of the EGF receptor (Chang *et al.*, 1993). It is possible that conformational changes associated with the Pro-1003 mutation may enhance the ability of the QQGFF signal to mediate receptor internalization, may promote receptor degradation, or may block receptor recycling. Alternatively, if mutation of Pro-1003 blocks phosphorylation of Ser-1002, it is possible that this could ablate a negative effect of phosphorylation on one or more of these processes.

The mechanism by which the P1003G mutation leads to enhanced responsiveness to EGF is not clear. If mutation of Pro-1003 prevented EGF receptor desensitization by blocking phosphorylation of the EGF receptor

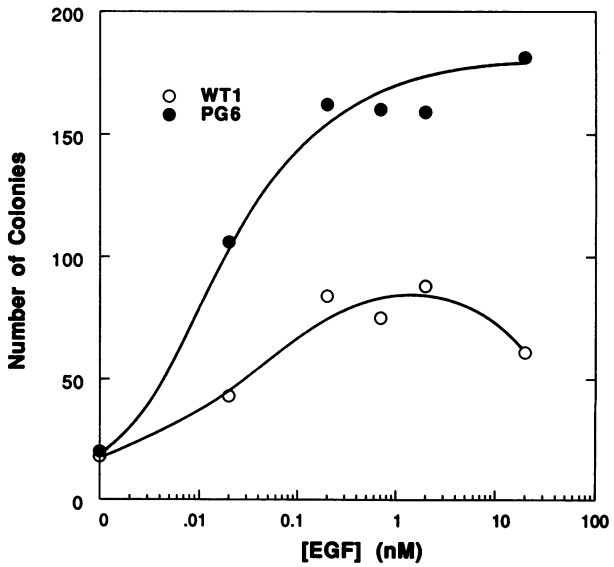

Figure 7. EGF-stimulated growth of cells in soft agar. WT1 or PG6 cells were plated and grown in soft agar containing the indicated concentrations of EGF as described in MATERIALS AND METHODS. Seven days after plating, wells were scored for the number of colonies containing >20 cells. The results represent the total number of colonies from 10 separate fields.

at Ser-1002, then the P1003G EGF receptor would be expected to lack the phosphopeptide that contains that site. Phosphopeptide mapping of the wild-type and P1003G receptors failed to identify a peptide that was present in the wild-type receptor map but absent from the P1003G receptor map. Thus, these data do not provide direct evidence for a change in the phosphorylation state of the P1003G receptors. However, changes in the phosphorylation of Ser-1002 cannot be unequivocally ruled out because the peptide containing Ser-1002 contains multiple phosphorylation sites that could mask the loss of one site and cause splitting of the peptide into multiple spots on our maps. Interestingly, the phosphopeptide maps indicated an increase in the phosphorylation of phosphoserine- and phosphothreonine-containing peptides in the P1003G EGF receptor. Whether this is a result of the enhanced activation of downstream serine/threonine protein kinases or is because of a conformational change in the receptor that increases its ability to serve as a substrate is not known. Zhou *et al.* (1994) have recently reported that de-N-acetyl-G_{M3} enhances serine phosphorylation of the EGF receptor and increases its tyrosine protein kinase activity. Thus, the increased serine phosphorylation of the P1003G EGF receptors may play a causative role in the enhanced responsiveness of these receptors. An alternative possibility for the enhanced responsiveness of the P1003G EGF receptor to EGF is that mutation of Pro-1003 leads to a conformational change in the EGF receptor that directly increases its efficiency of signal transduction. Additional experiments will be required to distinguish between these two possibilities.

The results presented here demonstrate that this region of the receptor, and Pro-1003 in particular, is important in the regulation of EGF receptor function. Because this region lies between the kinase core domain and the COOH-terminal tail, it is interesting to speculate that changes in this region might modulate interactions between the kinase and the regulatory COOH-terminal domain leading to an alteration in receptor function.

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