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Differential Effects of Carboxy-Terminal Sequence Deletions on Platelet-Derived Growth Factor Receptor Signaling Activities and Interactions with Cellular Substrates

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Chimeric receptors composed of the human epidermal growth factor receptor (EGF-R) extracellular domain fused to wild-type and truncated platelet-derived growth factor receptor (PDGF-R) intracellular sequences were stably expressed in NIH 3T3 cells devoid of endogenous EGF-Rs. This experimental system allowed us to investigate the biological activity of PDGF-R cytoplasmic-domain mutants in PDGF-R-responsive NIH 3T3 cells by activating PDGF-specific signaling pathways with EGF. Deletion of 74 carboxy-terminal amino acids severely impaired the ability of the PDGF-R cytoplasmic domain to associate with cellular substrates in vitro. This deletion also inhibited receptor and substrate phosphorylation, reduced the receptor's mitogenic activity, and completely abolished its oncogenic signaling potential. Surprisingly, removal of only six additional amino acids, including Tyr-989, restored substantial receptor and substrate phosphorylation capacity as well as transforming potential and yielded a receptor with wild-type levels of ligand-induced mitogenic activity. However, the ability of this chimera to bind phospholipase Cγ was severely impaired in comparison with the ability of the wild-type receptor, while the association with other cellular proteins was not affected. Further deletion of 35 residues, including Tyr-977, nearly abolished all PDGF-R cytoplasmic-domain biological signaling activities. None of the three C-terminal truncations completely abolished the mitogenic potential of the receptors or had any influence on ligand binding or receptor down regulation. Together, these data implicate the 80 C-terminal-most residues of the PDGF-R, and possibly Tyr-989, in phospholipase Cγ binding, while receptor sequences upstream from Asp-988 appear to be essential for specific interactions with other cellular polypeptides such as *ras* GTPase-activating protein and phosphotidylinositol 3-kinase. Thus, the mutants described here allow the separation of distinct PDGF-activated signaling pathways and demonstrate that phospholipase Cγ phosphorylation is not required for mitogenesis and transformation.

Platelet-derived growth factor (PDGF) is a potent mitogen for cells of mesenchymal origin. PDGF-A and -B represent two variants of this growth factor, which exist as active AA or BB homodimers and as AB heterodimers. The biological actions of these growth factors are mediated by two structurally homologous, membrane-spanning, cell surface receptors (PDGF-Rα and PDGF-Rβ), which are members of the large family of receptor tyrosine kinases (reviewed in references 26, 67, and 72). As first shown for the structurally related epidermal growth factor receptor (EGF-R) (57), ligand binding to PDGF-Rs induces dimerization and activation of the intrinsic kinase activity, which leads to phosphorylation of cellular substrates and the receptor itself. This process initiates an intracellular signaling cascade that ultimately results in a cell type- and receptor-specific cellular response. The molecular nature of this pleiotropic receptor-generated signal is still poorly understood.

Kinase-inactive receptor mutants of the PDGF-R and all other receptor tyrosine kinases (RTKs) tested are unable to couple to any of the known signaling pathways, which emphasizes the essential role of tyrosine phosphorylation in biological signal transduction by this class of receptors (9, 16, 19, 24, 28, 45, 48, 71). Activation of the receptor kinase leads first to phosphorylation at tyrosine residues within the receptor cytoplasmic domain. It has been shown by several laboratories that these phosphorylated residues mediate the binding of *src* homology 2 (SH2)-containing proteins to the receptor. SH2 domains were found in phospholipase (PLCγ; 60, 61), Cγ GTPase-activating protein (GAP; 64, 70), Nck (40), Crk (43, 44), Vav (33), tensin (13), and phosphotidylinositol 3 (PI3)-kinase-associated p85 (18, 53, 59). Binding and subsequent tyrosine phosphorylation have been shown to be essential for activation of the enzymatic function of PDGF-R (2, 30, 31, 34, 38, 47, 49–52).

Recently, phosphorylated tyrosine residues of several receptors have been identified as representing the primary interaction sites with the SH2 domains of several substrates. The locations of the identified Tyr phosphorylation target residues within the various RTK cytoplasmic domains are different, suggesting that the known substrates interact at specific sites in a receptor-specific manner. While PDGF-R binds to PI3-kinase and GAP (20, 32), the closely related colony-stimulating factor-1 receptor interacts only with PI3-kinase (54, 68), indicating that GAP interacts with a specific sequence motif in the PDGF-R which is not present in colony-stimulating factor-1 receptor. Furthermore, while major bindings sites for PLCγ and p85 are found in the EGF-R C-terminal tail region (55, 59), there is evidence that the kinase insertion sequence mediates the binding of p85 and GAP with the PDGF-R (20).

Information regarding the identities and functions of phos-

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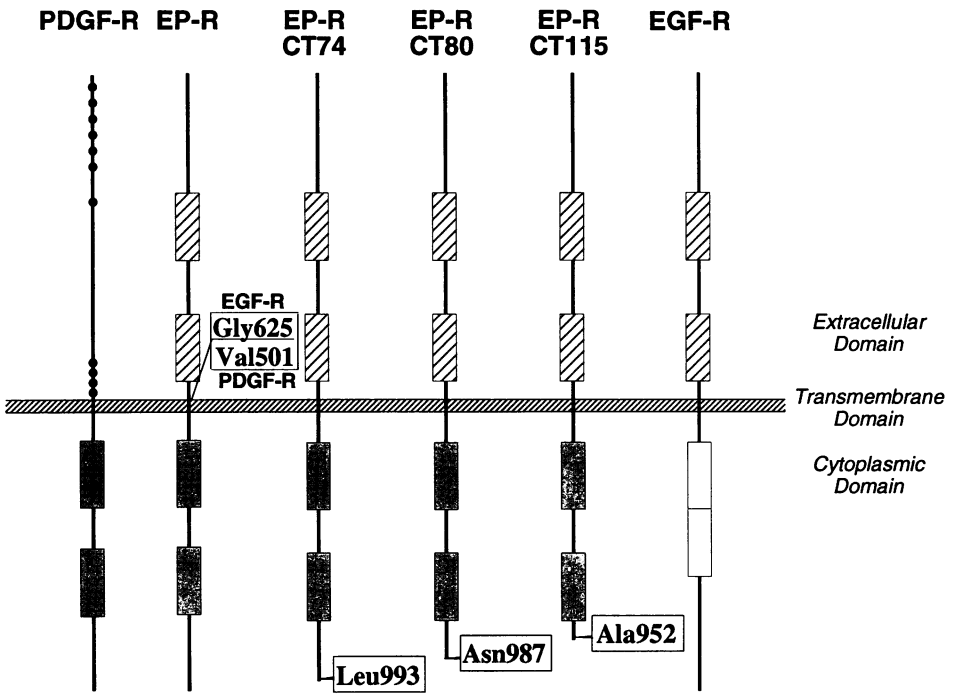

FIG. 1. Schematic structures of chimeric receptors. Single cysteine residues (filled circles) and cysteine-rich regions (hatched boxes) are indicated in parental PDGF-R and EGF-R extracellular domains, respectively. Shaded boxes in the PDGF-R and open boxes in the EGF-R intracellular domains represent tyrosine kinase domains. The C-terminal amino acids in EP-R truncation mutants and the residues flanking the fusion point between PDGF-R and EGF-R are indicated.

phorylation sites within the PDGF-Rβ is still rather limited. Kazlauskas and Cooper (34) identified the kinase insertion sequence residues Tyr-719 and Tyr-825 as phosphorylation target sites, and Fantl et al. (21) demonstrated that a Phe-825 mutation causes a decrease in PDGF-R kinase activity. Alteration of Tyr-719, furthermore, abolished association of PI3′ kinase activity with the receptor. More recently, Kashishian et al. (32) and Fantl et al. (20) identified Tyr-771 of the human PDGF-Rβ and Tyr-739 of the mouse PDGF-Rβ, respectively, as the interaction sites for GAP.

To further map the locations of PDGF-Rβ (for simplicity, designated below as PDGF-R) substrate interaction sites, we constructed a series of C-terminal deletion mutants and expressed them in the form of chimeric receptors which consist of the extracellular EGF-R ligand-binding domain fused to PDGF-R cytoplasmic domains (EP-Rs). All constructs were expressed on the surface of NIH 3T3 cells devoid of endogenous EGF-Rs, where they formed high-affinity binding sites. Progressive C-terminal truncations of PDGF-R cytoplasmic sequences had differential effects on receptor phosphorylation, cellular substrate interactions, and signal generation. Our findings indicate a crucial role for the C tail in PDGF-R interaction with PLCγ and suggest Tyr-989 and Tyr-977 as major interaction sites. Furthermore, they demonstrate a lack of C-terminal sequence involvement and PLCγ phosphorylation in mitogenic signaling and transformation.

MATERIALS AND METHODS

Expression plasmid construction. Chimeric receptor expression plasmids were constructed as follows. For EP-R, human EGF-R cDNA sequences (65) coding for the ligand-binding domain (amino acids −24 to 625) were fused to PDGF-R transmembrane and cytoplasmic-domain sequences (amino acids 501 to 1067; 73). A *Hin*dIII-*Bst*xI fragment (amino acids −24 to 625) coding for the entire EGF-R extracellular ligand-binding domain was connected to a *Bgl*I-*Kpn*I mouse PDGF-R (73) fragment coding for the transmembrane and cytoplasmic domains by using a synthetic DNA. The combined EGF-R–PDGF-R sequence was then cloned into a cytomegalovirus promoter-enhancer-driven expression vector (15).

For EP-Rs CT74, CT80, and CT115, the PDGF-R cDNA *Hin*cII restriction fragment (amino acids 633 to 1020) was subcloned into the *Hin*cII restriction site of pUC18. The plasmid was linearized by cutting it with *Hin*dIII, and the 3′ end of the insert was digested with *Bal* 31 for various times, cut with *Eco*RI (at the 5′ end), and recloned into the *Eco*RI and *Hin*cII restriction sites of pUC18. The 3′ deletions were sequenced, and three (amino acids 943, 987, and 993) were selected (Fig. 1). A synthetic fragment containing stop codons in three reading frames and the restriction site for *Nhe*I were cloned 3′ of the insert into the *Pst*I and *Hin*dIII sites of pUC18. After being cut with *Apa*I (amino acid 643) and *Nhe*I, the three 3′ deletions were cloned into the *Apa*I-*Nhe*I restriction sites of the PDGF-R cDNA. To generate EP-R chimeric receptor mutants, the EGF-R ligand-binding domain was fused to the C-terminal PDGF-R truncation by exchanging the *Cla*I (amino acid −31)-to-*Dra*III (amino acid 867) PDGF-R C-terminal fragment with the *Cla*I (amino acid −24, EGF-R cDNA)-to-*Dra*III (amino acid 807, PDGF-R cDNA) EP-R cDNA fragment.

Generation of cell lines. Stably transfected NIH 3T3 (clone 2.2) cell lines were selected by using media supplemented with 400 μg of Geneticin (GIBCO Laboratories) per ml, starting 2 days after transfection. Two weeks later, G418-

resistant cell lines were treated with appropriate concentrations of methotrexate (GIBCO) to select for amplified cDNA expression. Final methotrexate concentration was 250 nM for all cell lines. Confluent cell monolayers in six-well dishes were washed twice with phosphate-buffered saline (PBS) and were labeled in 2 ml of methionine-free medium prepared from a minimum essential medium (MEM) kit (GIBCO) supplemented with 100 μCi of L-[^{35}S]methionine (1,000 Ci/mmol; Amersham) per ml. Seventeen hours later, cells were washed with PBS before being lysed and immunoprecipitated (37) by using a monoclonal antibody against the human EGF-R extracellular domain (monoclonal antibody 108.1; 3) or a polyclonal antibody obtained after immunization of rabbits with a synthetic peptide directed against the C-terminal portion of the PDGF-R. Proteins were separated on a 7.5% sodium dodecyl sulfate (SDS)–polyacrylamide gel (39), treated with Amplify (Amersham), dried, and exposed to X-ray film.

Phosphorylation analysis. Confluent six-well tissue culture dishes of stably transfected cells were incubated for 18 h in Dulbecco MEM (DMEM)-F12 medium (GIBCO) containing 0.5% fetal calf serum. The medium was replaced by DMEM-F12 containing phenylarsenoxide (35 μM), sodium vanadate (250 μM), and growth factor (human EGF or human PDGF at 100 ng/ml). After incubation for 10 min at 37°C, cells were washed once with PBS and lysed in 0.4 ml of SDS-Laemmli buffer. Polypeptides, separated by SDS-polyacrylamide gel electrophoresis (PAGE) on a 7.5% gel, were transferred by Western blotting (immunoblotting) to nitrocellulose (Schleicher & Schuell; BA85) (63). The filter was coated by incubation in PBS–2% milk powder–0.02% Tween 20–1 mM EDTA for 1 h. The antibody reaction (antiphosphotyrosine antibody 5E2 diluted 1:1,000) was carried out overnight at 4°C and was followed by three washes with PBS containing 0.02% Tween 20. *Staphylococcus aureus* protein A (0.5 μCi of ^{125}I-labeled protein A [Amersham, Braunschweig Germany] per ml) was added for 3 h in PBS containing 2% milk powder and 0.02% Tween 20, and then the washing procedure described above was repeated. Filters were exposed to X-ray film with an intensifier screen.

Thymidine incorporation assay. Cells (10^5 per well) were seeded into 24-well dishes pretreated with 0.2% gelatin (Difco). Cells were grown for 3 days in 10% calf serum and then starved for 48 h in 0.5% calf serum. EGF or PDGF was added, and the cells were incubated for 18 h. [^{3}H]thymidine (Amersham) was added, and after 4 h, cells were washed three times with PBS, incubated with ice-cold 10% trichloroacetic acid for 30 min, and washed twice with the same solution. The trichloroacetic acid precipitate was solubilized in 0.2 M NaOH–1% SDS, neutralized, and counted in a scintillation counter.

Growth in soft agar. Subconfluent, stably transfected cells were trypsinized and plated (10^5 cells per 6-cm dish) in the presence or absence of 10 nM EGF (Collaborative Research) or 1 nM PDGF (PDGF, Inc.) in 4 ml of MEM, each plate containing 8% fetal bovine serum (GIBCO) and 0.2% agar (Difco; Noble agar) above a bottom layer of 0.4% agar in 5 ml of MEM–8% fetal bovine serum. Colonies were photographed after 14 days.

Ligand-induced receptor degradation. Cells were labeled for 18 h with [^{35}S]methionine (Amersham), and receptor degradation was subsequently measured by a chase in normal culture medium in the presence of PDGF (50 ng/ml) or EGF (100 ng/ml) for various times. Receptors were immunoprecipitated with a monoclonal antibody directed against the extracellular domain of EGF-R (monoclonal antibody 108.1) or a polyclonal rabbit antibody directed against the C-terminal portion of PDGF-R, respectively, and analyzed on a 7.5% polyacrylamide gel. The gel was treated with Amplify (Amersham), dried, and exposed to X-ray film.

Immunoprecipitations. Confluent stably transfected cells were incubated in DMEM-F12 medium (GIBCO) containing 0.5% fetal calf serum for 18 h. The medium was replaced by DMEM-F12 containing phenylarsenoxide (35 μM) and sodium orthovanadate (250 μM) in the presence or absence of EGF (100 ng/ml; Amgen). After incubation for 10 min at 37°C, cells were washed once with PBS and lysed in HNTG buffer containing H-Triton X-100, 1 mM phenylmethylsulfonyl fluoride, aprotinin (1 mg/ml), sodium vanadate (250 μM), leupeptin (20 μM), ATP (100 μM), GTP (10 μM), $MgCl_2$ (1 mM), and $MnCl_2$ (100 μM). Lysates were cleared by centrifugation (10 min at 12,000 rpm and 4°C) and diluted 1:5 in the same buffer containing 0.1% Triton X-100 (washing buffer). Receptors were immunoprecipitated with 5 μl of antibody 108.1 and 20 μl of protein A-Sepharose in the presence or absence of EGF (10 ng/ml) for 3 h at 4°C. Immunoprecipitates were washed three times with 13 ml of washing buffer containing no growth factor.

In parallel, 3T3 clone 2.2 cells were labeled overnight with [^{35}S]methionine and lysed as described above. The soluble protein extracts were diluted 1:5 in washing buffer and preincubated with 10 μl of protein A-Sepharose for 30 min at 4°C to eliminate nonspecific binding. Protein A-Sepharose was pelleted by centrifugation for 30 s at 2,000 rpm (Heraeus Varifuge F), and the supernatant was added to the immunoprecipitated receptors. Adsorption of [^{35}S]methionine-labeled proteins to immunoprecipitated receptors was carried out at 4°C for 3 h in the presence or absence of growth factor (10 ng/ml). The immunoprecipitates were washed three times with 15 ml of washing buffer each within minutes and solubilized in 100 μl of SDS lysis buffer. Proteins were analyzed on 7, 8.5, or 10% SDS–polyacrylamide gels (39).

RESULTS

To analyze cytoplasmic-domain mutants of the PDGF-R in cells that are normally responsive to PDGF stimulation, we constructed chimeric receptors consisting of the human EGF-R extracellular-domain sequences linked to mouse PDGF-Rβ transmembrane and cytoplasmic portions. In addition to a parental construct containing the complete EP-R, we generated three C-terminal deletions of 74, 80, and 115 amino acids, which were designated EP-Rs CT74, CT80, and CT115, respectively (Fig. 1). By expression in NIH 3T3 cells, which are devoid of EGF-Rs but which express about 10^4 endogenous PDGF-Rs, these chimeric receptors permitted us to analyze the role of C-terminal sequences in PDGF signaling events within a compatible cellular background (58).

The EP-R chimera and its C-terminal deletion mutant derivatives were expressed in mouse NIH 3T3 fibroblasts (clone 2.2) (27, 29) by using an expression vector with cytomegalovirus promoter elements and a simian virus 40 promoter-driven dihydrofolate reductase gene for the selection of transfected cell clones with methotrexate (1). Stable transfectants were isolated; lines expressing comparable receptor numbers were identified and used for all of the following experiments to ensure that the observed biological effects were not due to variability in receptor numbers. The relative numbers of ^{125}I-EGF binding sites (EP-R, 1.2×10^5 per cell; EP-R CT74, 1.5×10^5 per cell; EP-R CT80, 1×10^5 per cell; EP-R CT115, 1.6×10^5 per cell) correlated well with

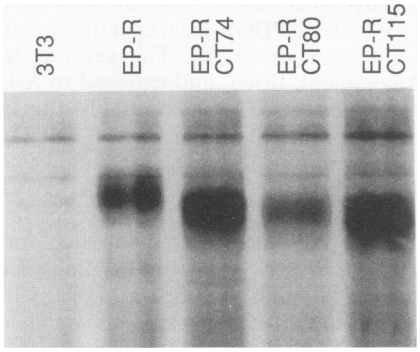

FIG. 2. Biosynthesis of chimeric receptors in transfected NIH 3T3 cells. Metabolically labeled chimeric receptors were immunoprecipitated with antibody directed against the extracellular domain of EGF-R (antibody 108.1; 3). 3T3 represents an NIH 3T3 (subclone 2.2) line that overexpresses the PDGF-R but is devoid of endogenous EGF receptors.

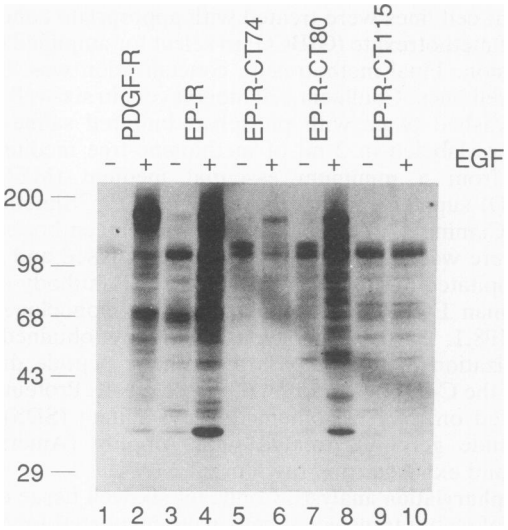

FIG. 3. Ligand-stimulated tyrosine phosphorylation of cellular polypeptides. Confluent cell monolayers of NIH 3T3 clone 2.2 cells and transfected clonal derivatives expressing EP-R, EP-R CT74, EP-R CT80, and EP-R CT115 were starved for 18 h in medium containing 0.5% serum. Growth factors were added for 10 min as indicated (PDGF in lane 2 and EGF in lanes 4, 6, 8, and 10), and aliquots of total cell extracts representing equal numbers of cell surface receptors (determined by ^{125}I-ligand binding) were separated on a 7.5% SDS–polyacrylamide gel. After transfer to nitrocellulose, proteins phosphorylated on tyrosine residues were visualized by incubation with antiphosphotyrosine antibody and ^{125}I-protein A and subsequent exposure to X-ray film for 24 h.

receptor chimera expression levels as determined by [^{35}S]Met labeling and immunoprecipitation (Fig. 2). In this analysis, EP-R was determined to be 180 kDa, while EP-R CT74, EP-R CT80, and EP-R CT115 migrated at positions consistent with their decreased sizes of 173, 172, and 160 kDa, respectively.

Biological activities of EP-R truncation mutants. We have shown previously that the EP-R chimera behaves like the endogenous wild-type PDGF-R with respect to receptor and substrate phosphorylation, induction of thymidine incorporation into DNA, Ca^{2+} influx, pH changes, and the abilities of transfected NIH 3T3 cells to grow in soft agar (58). To examine the functional consequences of PDGF-R C-terminal truncations, we first investigated receptor and substrate phosphorylation activities. Confluent monolayers of cells expressing EP-R, EP-R CT74, EP-R CT80, or EP-R CT115 were starved in DMEM containing 0.5% fetal calf serum for 18 h prior to ligand stimulation and subsequent addition of SDS-lysis buffer. Polypeptides containing phosphorylated tyrosine residues were analyzed by SDS-PAGE and immunoblotting with the antiphosphotyrosine antibody 5E2 (22).

As shown in Fig. 3, EGF stimulation of EP-R-expressing cells led to increased tyrosine phosphorylation of the chimeric receptor (180 kDa; see also Fig. 6) and a number of other, not-yet-identified proteins. Deletion of 74 amino acids of the PDGF-R C terminus (EP-R CT74) resulted in a marked decrease (~90%) in both receptor and substrate phosphorylation. Only the 173-kDa mutant receptor acquired detectable phosphotyrosine upon ligand stimulation (see also Fig. 6). Surprisingly, removal of only six additional amino acids, including Tyr-989 (EP-R CT80), restored both receptor phosphorylation and substrate phosphorylation to levels comparable to that observed for the parental EP-R chimera. Interestingly, the ligand-stimulated substrate phosphorylation pattern obtained with the CT80 mutant was slightly altered from that of the untruncated chimera. Two polypeptides of 40 kDa each were phosphorylated by EP-R CT80 and not by EP-R, while a 38-kDa protein was phosphorylated only by EP-R. Whether these and other differences between the EP-R and PDGF-R substrate phosphorylation patterns represent specific characteristics of the mutants or clonal differences between the different cell lines remains to be investigated. Further deletion of 35 amino acids (EP-R CT115), including Tyr-989 and Tyr-977, completely abolished tyrosine phosphorylation of substrates as well as receptor phosphorylation.

To assess the potential of the EP-R-derived mutants to generate mitogenic signals, we measured EGF-stimulated [^{3}H]thymidine incorporation into the DNAs of quiescent, serum-starved cells. While EGF-R-deficient control NIH 3T3 (clone 2.2) cells do not respond to EGF, EP-R-expressing cells exhibit a mitogenic response equivalent to that mediated by endogenous PDGF-Rs upon PDGF stimulation (58). Consistent with the results obtained in the receptor and substrate phosphorylation experiments, EP-R CT74 was impaired in its ability to generate a mitogenic signal, as indicated by an ~30% reduction in [^{3}H]thymidine incorporation in EGF-stimulated EP-R CT74/3T3 cells. Surprisingly, however, the lower mitogenic potential of EP-R CT74 did not reflect the more-pronounced defect in its kinase activity (see above). Further sequence deletions in EP-R CT80 and EP-R CT115 resulted in either full restoration or nearly complete loss of this biological activity, respectively (Fig. 4).

Comparable results were obtained in a cell transformation assay, in which we examined the abilities of control and transfected NIH 3T3 cells to form colonies in soft agar upon ligand stimulation. In the presence of 10 μM EGF, EP-R/3T3 and EP-R CT80/3T3 cells displayed transformed phenotypes and efficiently formed colonies in soft agar, analogous to PDGF-treated control NIH 3T3 cells. In contrast, EP-R CT74/3T3 and EP-R CT115/3T3 cells had no transformed characteristics (Fig. 5).

Association of truncated receptor chimeras with cellular factors. Having shown that C-terminal truncations of increasing lengths have differential effects on receptor and substrate phosphorylation as well as on signal generation,

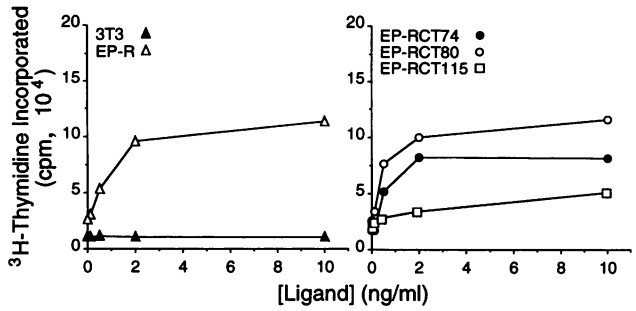

FIG. 4. Mitogenic activities of chimeric receptors. [^{3}H]thymidine incorporation into DNA was determined as described in Materials and Methods. Equal numbers of cells were incubated for 18 h in the presence of increasing concentrations of EGF. [^{3}H]thymidine was added for 4 h, and trichloroacetic acid-precipitable radioactivity was determined. The average of two independent experiments is shown.

we next examined the capacities of the truncated receptors to interact with cellular factors which may be involved in signal transmission. Quiescent cells expressing EP-R, EP-R CT74, EP-R CT80, or EP-R CT115 were kept for 18 h in 0.5% fetal calf serum prior to EGF stimulation. Cells were subsequently lysed, and chimeric receptors were immunoprecipitated with an anti-EGF-R extracellular-domain antibody. In parallel, NIH 3T3 (clone 2.2) cells were metabolically labeled overnight with [^{35}S]methionine and lysed, and Triton X-100-soluble polypeptides from these cells were added to immunoprecipitated receptors. This protocol permitted us to detect association between receptors and potential substrates or other cellular factors. As shown in Fig. 6A, under these stringent experimental conditions, the EP-R reproducibly bound four major polypeptides of 145, 110, 105, and 85 kDa and weakly associated with three cellular components that were detected as faint 120-, 90-, and 88-kDa bands; the polypeptides of 120, 110, and 85 kDa are likely to represent GAP and the catalytic and noncatalytic subunits of PI3′ kinase, respectively. Binding of each of these polypeptides to EP-R CT74 was strongly reduced and therefore under our experimental conditions barely detectable. Interestingly, the deletion of only six additional amino acids in EP-R CT80 fully restored the potential to associate with the 110-, 105-, and 85-kDa polypeptides to a level similar to or even above that of EP-R. Interaction with the 145-kDa protein, however, was severely and selectively impaired, as indicated by the faintness of the corresponding band (Fig. 6A). No association with cellular factors was detected for the EP-R CT115 chimeric receptor mutant.

Western blot analysis of the association mixtures (Fig. 6B) identified the 145-kDa band in the EP-R lanes as PLCγ and revealed that within the limits of the detection method, equal quantities of chimeric receptors were present in all relevant lanes. Furthermore, phosphotyrosine antibody analysis of the Western blot confirmed the differences in receptor phos-

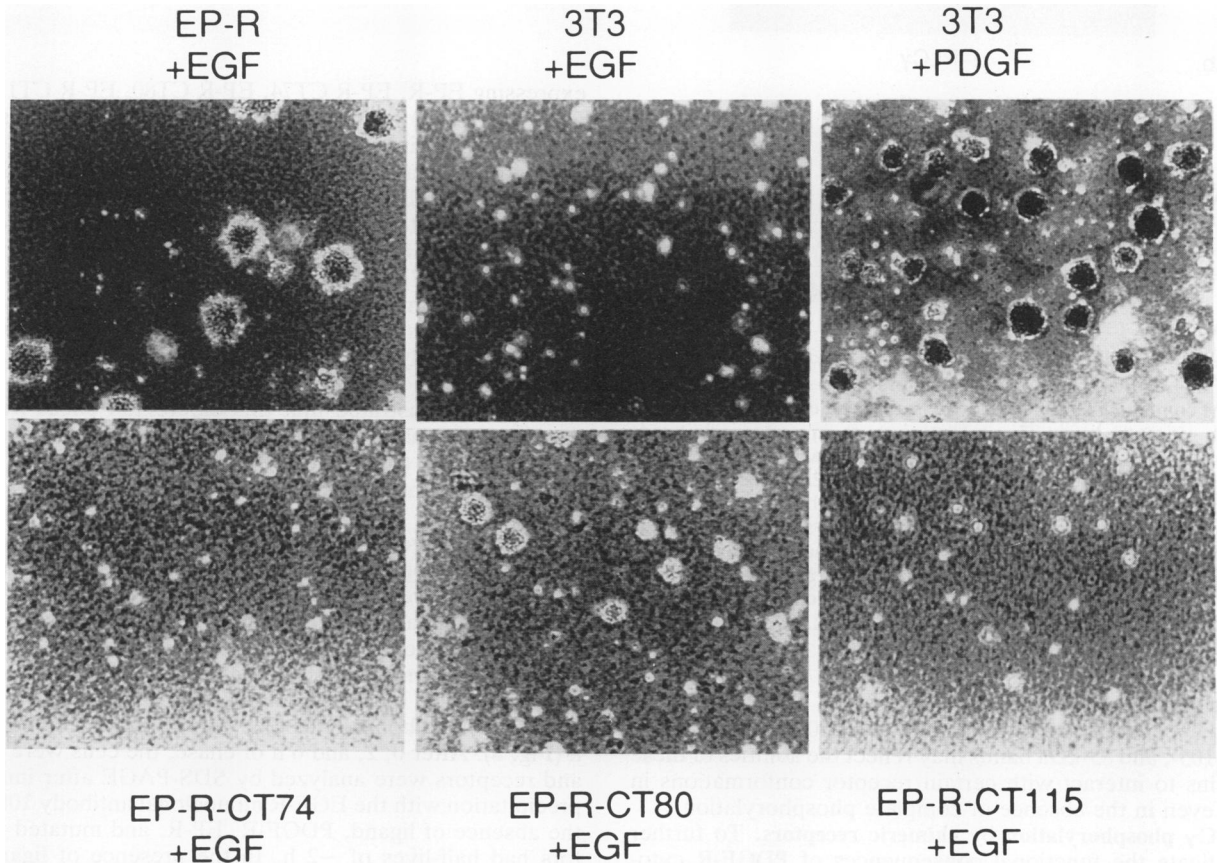

FIG. 5. Anchorage-independent cell growth in soft agar. Cells were suspended in DMEM containing 8% fetal bovine serum and 0.2% agar and grown in the presence of ligand for 14 days.

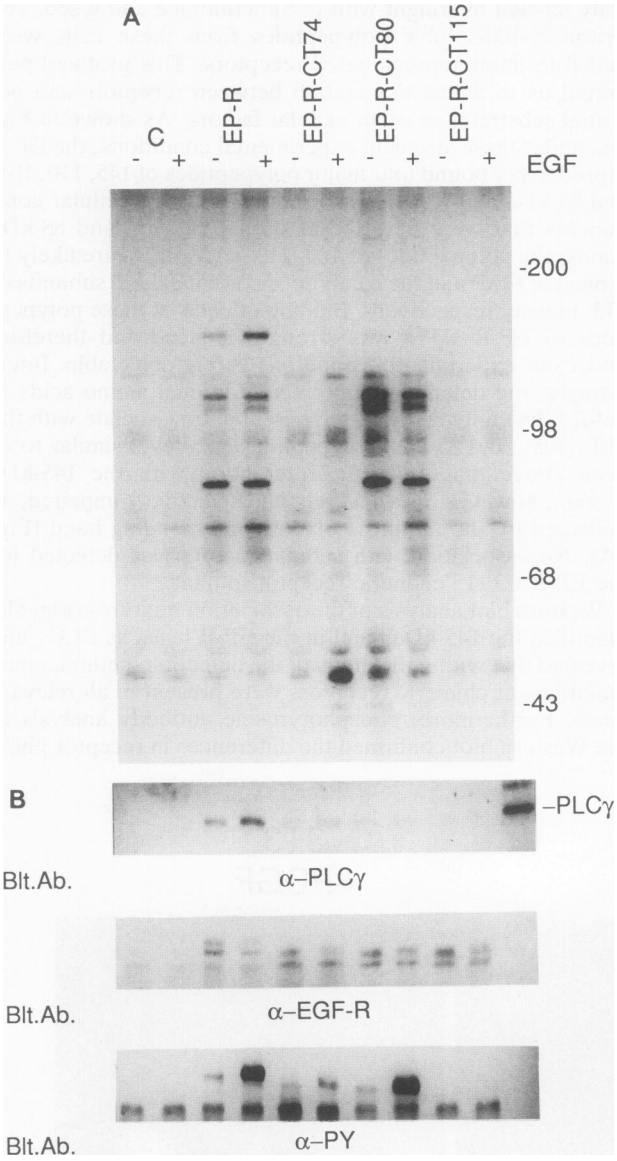

FIG. 6. Association of cellular polypeptides with chimeric receptors. Cells expressing chimeric receptors were starved for 18 h in DMEM containing 0.5% fetal bovine serum and exposed to EGF for 10 min prior to lysis. The receptors were immunoprecipitated, washed, and incubated with Triton X-100 extracts from metabolically labeled ([^{35}S]methionine) NIH 3T3 (clone 2.2) cells. Receptor-protein complexes were washed and analyzed by SDS-PAGE and autoradiography (A) and by Western blot analysis (B). The immunoblots were probed in parallel with polyclonal rabbit (Blt. Ab) anti-PLCγ-, anti-EGF-R extracellular domain-, and antiphosphotyrosine-specific 5E2 antibodies. The αPY panel demonstrates ligand-induced tyrosine phosphorylation of the EGF-R. C, no receptors.

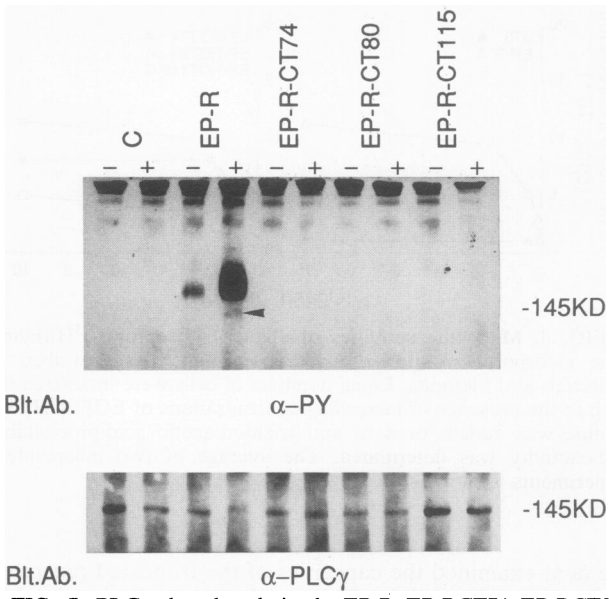

FIG. 7. PLCγ phosphorylation by EP-R, EP-RCT74, EP-RCT80, and EP-RCT115. Stably expressing NIH 3T3 cells were starved for 24 h, and after addition of EGF (100 ng/ml) for 10 min, PLCγ was immunoprecipitated, separated by SDS-PAGE, and transferred to nitrocellulose. The immunoblot was probed with antiphosphotyrosine antibody (5E2) and anti-PLCγ antiserum as indicated. Protein bands were detected by using a horseradish peroxidase-coupled second antibody and the ECL (Amersham) detection assay. A 10-s exposure is shown. The arrow shows PLCγ phosphorylated on tyrosine. C, no receptor.

phorylation activity shown in Fig. 3. The apparent lack of ligand dependence for EP-R and EP-R CT80 association with 110-, 105-, and 85-kDa bands may reflect the abilities of these proteins to interact with certain receptor conformations in vitro even in the absence of complete phosphorylation.

PLCγ phosphorylation by chimeric receptors. To further investigate the functional consequences of PDGF-R cytoplasmic-domain truncations, we examined the phosphorylation of PLCγ in intact cells. Quiescent NIH 3T3 cells expressing EP-R, EP-R CT74, EP-R CT80, EP-R CT115, or no receptors (C) were kept for 18 h in 0.5% fetal calf serum prior to EGF stimulation. Cells were lysed, and PLCγ was immunoprecipitated with PLCγ-specific antibodies. Subsequent SDS-PAGE and immunoblot analyses with antiphosphotyrosine antibodies demonstrated phosphorylation of PLCγ by the EP-R but not by the C-terminally truncated receptors (Fig. 7). Immunoprecipitation with PLCγ antibody also led to coimmunoprecipitation of EP-R, while, consistent with the results shown in Fig. 6, none of the deletion mutants associated tightly enough with the substrate to be coimmunoprecipitated. Probing the immunoblot with anti-PLCγ antibody demonstrated that this differential effect was not due to variations in PLCγ expression levels in the various stable cell lines (Fig. 7, lower panel).

Mutated receptors undergo normal down regulation. Upon ligand binding, some receptor tyrosine kinases, such as EGF-R, are targeted to lysosomes by a process that requires functional cytoplasmic-domain determinants (67) and is termed down regulation. Unlike EGF-R, PDGF-R down regulation appears to be independent of kinase activity (72). To address whether this characteristic property was preserved in our chimeric EP-R constructs, we determined the lifetime of each of the receptors after transfected NIH 3T3 cells were metabolically labeled with [^{35}S]methionine for 20 h (Fig. 8). After 0, 2, and 6 h of chase, the cells were lysed, and receptors were analyzed by SDS-PAGE after immunoprecipitation with the EGF-R monoclonal antibody 108.1. In the absence of ligand, PDGF-R, EP-R, and mutated receptors had half-lives of ~2 h. In the presence of ligand, all receptors were almost completely degraded within that period (Fig. 8). Mutant receptors EP-R CT74, EP-R CT80,

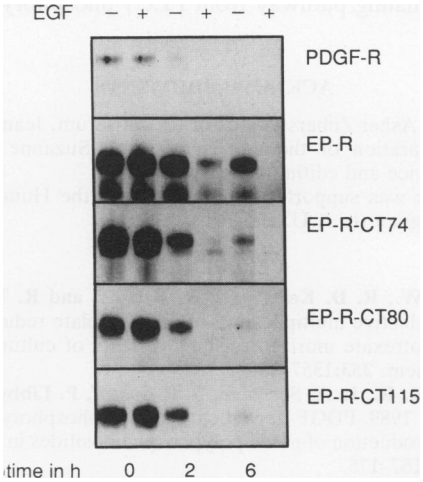

FIG. 8. Ligand-dependent receptor degradation. Cells were labeled for 18 h with [^{35}S]methionine, and receptor degradation was subsequently measured by a chase with cold methionine-containing culture medium in the presence or absence of PDGF (PDGF-R) and EGF after 2 and 6 h.

and EP-R CT115 all behaved like the wild-type receptor, indicating that C-terminal sequences and the phosphorylation state of the receptor are not essential for ligand-induced down regulation.

DISCUSSION

The great diversity of growth and differentiation factors that induce their distinct biological functions through the activation of cell surface RTKs in combination with recent insights into the complexity of the molecular signals generated by this event emphasizes the importance of RTK-specific signal definition and the structures involved in this process. The availability of cloned cDNAs and primary sequence information for a large number of RTKs provided opportunities to study the functional significance of specific receptor subdomains (75). The high degree of sequence diversity found in the C-terminal tail regions of RTKs led to the proposal that this domain contained structural determinants which define receptor-characteristic functions (11, 12, 66). Interestingly, RTK-derived dominant oncogene products, such as v-*erbB*, v-*fms*, and v-*kit*, harbor deletions of various sizes within their C-tail sequences compared with their proto-oncogene counterparts (7, 11, 56, 65, 74). Remarkably, the pathogenic potentials of different avian erythroblastosis virus strains appear to correlate with the nature of the C-terminal structure alterations in corresponding v-*erbB* oncogenes (23), which suggests a key role in signal definition for this region. Moreover, each one of the C-terminal truncations found in viral oncogenes included a tyrosine residue, which in the corresponding proto-oncogene product, EGF-R, represented a major receptor phosphorylation site (14, 65).

Functional analyses of receptors mutated within this structural domain have yielded contradictory results. Tyrosine residue point mutations or deletions altered EGF-R mitogenic or transforming activities only slightly or had no effect that could be detected by the methods employed (4–6, 27, 29, 35, 42, 69). Furthermore, extensive deletions found in v-*erbB* oncogenes support the conclusion that C-terminal EGF-R sequences are not essential for transformation and growth and may to some extent even negatively regulate the mitogenic and transforming signals generated by this receptor. Similarly, replacement of tyrosine phosphorylation sites with phenylalanine residues has been shown to decrease the biological activity of pp60$^{c\text{-}src}$ (8, 36) and pp120$^{gag\text{-}fps}$ (25), and analogous structural alterations in the insulin receptor led to a reduced ability to regulate glucose uptake but caused an increase in the mitogenic signaling potential of this receptor (17, 45, 62). Together with recent findings which demonstrate that the C-terminal 200 amino acids of the EGF-R contain binding sites for PLCγ, GAP, and the p85 subunit of PI3-kinase (41, 59), these observations implicate this RTK region in substrate interaction and phosphorylation but suggest the existence of additional receptor structures that may regulate other aspects of a pleiotropic signal which is generated as a result of the interaction of RTK cytoplasmic domains with multiple signal transducer molecules.

Addressing this point, Coughlin et al. (10) examined the ability of PDGF-R mutants lacking part of the kinase insertion sequence (Δki mutant) to associate with PI3-kinase activity. These workers found that the kinase insertion domain is essential for binding an 85-kDa protein that was believed to represent PI3-kinase. Subsequently, Kazlauskas and Cooper (34) showed that conversion of Tyr-719 within the kinase insertion sequence to Phe or Gly abolished interaction of the PDGF-R with 120-, 85-, and 72-kDa polypeptides but did not alter the receptor's tyrosine kinase activity.

To investigate in detail the functional significance of PDGF-R carboxy-terminal sequences, we employed chimeric receptors composed of a human EGF-R ligand-binding domain linked to wild-type or mutated mouse PDGF-R cytoplasmic sequences. The chimerae were expressed in host NIH 3T3 cells which lack EGF receptors but are fully responsive to PDGF. Therefore, in these cells, EGF-stimulated effects are the consequences of EP-R chimeric receptor activation and represent PDGF-characteristic responses (58).

We have previously shown that the addition of EGF to EP-R-expressing NIH 3T3 cells leads to receptor and substrate phosphorylation, induction of DNA synthesis, mediation of growth in soft agar, activation of Ca^{2+} release from intracellular stores, and pH increases similar to those mediated by PDGF stimulation of endogenous wild-type PDGF-Rβs (58). In this report, we demonstrate that C-terminal PDGF-R sequence truncations of increasing lengths have pronounced but differential effects on receptor and substrate phosphorylation, substrate binding, and overall biological activity in PDGF-responsive cells but have no effect on ligand binding and receptor down regulation.

Deletion of 74 amino acids from the C terminus of the PDGF-R cytoplasmic domain led to a significant decrease of receptor and substrate phosphorylation and to a loss of the receptor's ability to bind cellular factors in vitro, while, surprisingly, the mitogenic signaling potential was reduced only to approximately 70% of that of the EP-R control.

Remarkably, deletion of only six more amino acids, including Tyr-989, generated a receptor, EP-R CT80, that had nearly wild-type biological activity. In vitro association experiments demonstrated that the EP-R CT80 deletion mutant exhibited binding affinity for polypeptides of 85, 105, and 110 kDa that are equal to EP-R containing an intact PDGF-R cytoplasmic domain, while PLCγ (145 kDa) bound with high affinity only to EP-R. Anti-PLCγ antibody immunoprecipitation from EGF-stimulated NIH 3T3 cells stably expressing the various receptors confirmed this observation

by coimmunoprecipitation of EP-R but not EP-R CT80 with endogenous PLCγ. Immunoblot analysis of these samples with antiphosphotyrosine antibodies also demonstrated that under the conditions existing in the intact cell, EP-R is able to phosphorylate PLCγ on tyrosine residues, while the kinase-competent EP-R CT80 deletion mutant has either lost this particular function or become severely impaired because of the truncation of critical sequences. Our observations with the EP-R CT80 chimera clearly demonstrate the location of a major binding site for PLCγ in the C-tail region of PDGF-R and confirm the existence of additional distinct interaction sites for other substrates at different locations within the cytoplasmic domain.

Further deletion of 35 amino acids, including Tyr-977, almost completely abolished all biological activity: EP-R CT115 had no detectable auto- or substrate phosphorylation activity in NIH 3T3 cells and displayed no detectable in vitro association with cellular substrates, similar to the phenotype reported for a 97-amino-acid PDGF-R deletion mutant (72). Surprisingly, however, EP-R CT115 was able to stimulate a weak (20%) mitogenic response by transfected NIH 3T3 cells.

One must certainly consider the possibility that the observed differential effects on receptor-PLCγ association and phosphorylation were caused by qualitative and/or quantitative changes in phosphorylation efficiency of specific tyrosine residue targets within the PDGF-R cytoplasmic domain by the different deletion mutants. High-pressure liquid chromatography analysis of tryptic digests from activated EP-R and EP-R CT80 indicated the existence of three phosphotyrosine-containing peptides, of which one had changed elution characteristics in the truncation mutant profile (not shown). This suggested that the 80-amino-acid deletion involving Tyr-989 either truncated a tryptic peptide containing a phosphotyrosine, i.e., Tyr-977, or led to de novo phosphorylation of this or other tyrosine residues after the loss of Tyr-989. Further experiments will be needed to resolve whether this Tyr residue is indeed regulating kinase activity or whether, in comparison with EP-R CT74, the additional deletion of six amino acids simply alters the kinase domain conformation in such a way that receptor and substrate phosphorylation are facilitated.

Taken together, C-terminal truncations of PDGF-R cytoplasmic-domain sequences have no effect on ligand binding or receptor internalization and degradation, which indicates that these functions are independent of C-terminal sequences, the phosphorylation state of the receptor, and substrate phosphorylation. C-terminal sequences, on the other hand, play an important role in the interaction with specific substrates and may be crucial for the formation of a functional conformation of the cytoplasmic PDGF-R domain. Deletions involving Tyr-989 and Tyr-977, corresponding to Tyr-1021 and Tyr-1009, respectively, of the human PDGF-Rβ, result in abrogation of the receptor's ability to interact and phosphorylate PLCγ. This, in spite of the absence of sequence motif homologies with PLCγ docking sites in EGF-R and fibroblast growth factor receptor (46, 55), implicates these residues in PLCγ binding to PDGF-Rβ. Most interestingly, the CT80 deletion has no effect on the receptor's ability to mediate a mitogenic and oncogenic response of NIH 3T3 cells and only marginally alters the substrate phosphorylation capacity of the receptor. Thus, the mutants analyzed in this study allow the separation and individual dissection of distinct PDGF-R signaling pathways and further demonstrate complete independence of the mitogenic signaling pathway from PLCγ phosphorylation and activation.

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