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Role of Tyrosine Kinase and Membrane-Spanning Domains in Signal Transduction by the Platelet-Derived Growth Factor Receptor

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Three types of mutations were introduced into the platelet-derived growth factor (PDGF) receptor to cause a loss of PDGF-stimulated tyrosine kinase activity: (i) a point mutation of the ATP-binding site, (ii) a deletion of the carboxyl-terminal region, and (iii) replacement of the membrane-spanning sequences by analogous transmembrane sequences of other receptors. Transfectants expressing mutated receptors bind, ^{125}I-labeled PDGF with a high affinity but had no PDGF-sensitive tyrosine kinase activity, phosphatidylinositol turnover, increase in the intracellular calcium concentration, change in cellular pH, or stimulation of DNA synthesis. However, PDGF-induced receptor down regulation was normal in the mutant cells. These results indicate that the transmembrane sequence has a specific signal-transducing function other than merely serving as a membrane anchor and that the receptor kinase activity is necessary for most responses to PDGF but is not required for receptor down regulation.

The interaction of platelet-derived growth factor (PDGF) with its receptor causes a rapid activation of the receptor tyrosine kinase, stimulation of several second messenger pathways, internalization of the receptor, and initiation of DNA replication. Little is known about the mechanism by which binding of the ligand to the extracellular domain causes an activation of the intracellular kinase domain. Whether the single transmembrane sequence plays a specific role, for example, by propagating a conformational change across the membrane, or merely serves an anchoring function to facilitate other receptor reactions such as receptor clustering has not been made clear.

The roles of the receptor tyrosine kinase activity and receptor internalization have also not been determined. There is considerable evidence that tyrosine kinase plays an essential role in cell proliferation and transformation mediated by growth factors or oncogenes. Studies of temperature-sensitive mutants of the *src* and *abl* oncogenes have established a correlation of tyrosine kinase activity with cell transformation (17, 21). Mutagenesis experiments in which the tyrosine kinase of the v-*src* transforming protein was inactivated by altering the ATP-binding site of the kinase domain have shown a similar dependence of transforming potential on tyrosine kinase activity (15, 22). The ATP-binding sites of the tyrosine kinase domains of the insulin and epidermal growth factor (EGF) receptors have been similarly modified (3–5, 13). These mutated receptors lost the ability to mediate ligand-stimulated DNA synthesis, suggesting a crucial role for tyrosine kinase in this response. The mutated insulin and EGF receptors with inactivated ATP-binding sites also failed to undergo ligand-induced down regulation (4, 13), the phenomenon of receptor loss caused by ligand-enhanced metabolic processing of the receptor. On the basis of these findings, it was proposed that tyrosine kinase activity is essential for growth factor-stimulated receptor down regulation as well as growth factor-induced DNA synthesis (3, 4). However, the mechanism by which tyrosine phosphorylation might be involved in this process is not understood.

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A 180-kilodalton (kDa) receptor mediates PDGF-stimulated DNA synthesis as well as the rapid cellular responses to PDGF, including tyrosine kinase activity (6, 10), phosphatidylinositol (PI) hydrolysis (2), and change in intracellular pH (14). Recent experiments have documented that the PDGF receptor undergoes ligand-dependent down regulation and have shown that the half-life of the PDGF receptor is reduced from 3 h to approximately 1 h when ligand is bound to receptor-bearing cells (16). To investigate the role of tyrosine kinase in PDGF-stimulated DNA synthesis, PI hydrolysis, pH change, and receptor down regulation, we constructed mutants of the PDGF receptor that were defective in PDGF-stimulated tyrosine kinase activity. To ensure that the alterations in PDGF responsiveness observed in the mutant receptors could be attributed to the loss of tyrosine kinase activity, and not merely to conformational changes induced by the mutation, we inactivated the kinase with three independent mutations. In the first group, the ATP-binding site was inactivated by a point mutation. In the second group, the carboxyl terminus of the receptor was deleted distal to the kinase sequences. In the third group of mutations, the transmembrane sequence of the PDGF receptor was replaced by corresponding transmembrane sequences from other membrane receptors. The mutated receptors were expressed by transfection of their respective cDNAs into CHO cells that have few, if any, PDGF receptors, and the tyrosine kinase activity and cellular responses of mutant cells were measured. In each of the mutants there was a loss of PDGF-stimulated tyrosine kinase activity and a parallel loss of the ability of PDGF to induce DNA synthesis, PI hydrolysis, and changes in intracellular calcium and pH. However, in these kinase-deficient mutants, PDGF-induced receptor down regulation occurred normally, indicating that receptor down regulation does not require tyrosine kinase activity.

MATERIALS AND METHODS

Construction and expression of PDGF receptor mutants. A carboxyl-terminal deletion (Δ*ct*) mutant was prepared by using restriction endonucleases (*Pvu*II and *Hinc*II) (Fig. 1A). A sequence of 297 base pairs encoding 99 amino acids from

the carboxyl terminus of the receptor was deleted. This deletion reduced the length of the carboxyl-terminal region from 145 to 46 amino acids and removed 4 tyrosine residues (Y935, Y939, Y977, and Y989). An ATP-binding site mutant (K602A) was obtained by site-directed mutagenesis (29) by using an oligomer encoding the change of lysine 602 for an alanine residue. Transmembrane mutants were prepared by substituting sequences encoding transmembrane regions of the low-density lipoprotein receptor, the *neu* oncogene, or the c-*neu* counterpart of the *neu* oncogene (Fig. 1B). The DNA fragments for each transmembrane region were chemically synthesized as four overlapping oligomers that were ligated to yield a fragment with a blunt 5′ end and a 3′ *Sac*II site, to allow cloning into the PDGF receptor cDNA sequence. The synthetic transmembrane regions were inserted into a receptor clone in which we introduced a *Stu*I site at the 5′ end of the transmembrane region and a *Sac*II site 3 amino acids after the 3′ end of the transmembrane region. To verify the mutations, all of the mutant cDNA clones were sequenced by using dideoxy nucleotides and the Sequenase sequencing system (20). All of the mutated receptor cDNAs were cloned into an expression vector under the transcriptional control of the simian virus 40 early promoter. Stable transfectants were prepared by cotransfecting plasmids from each receptor mutant with a plasmid that conferred resistance to neomycin (pSV2neo) by the calcium precipitation technique (24). Clones resistant to G418 (an analog of neomycin) were expanded and analyzed for the expression of the receptor with antibodies directed to the extracellular domain of the receptor (rabbit polyclonal, A677). Goat anti-rabbit immunoglobulin G conjugated to fluorescein isothiocyanate was used as an indicator in a fluorescence-activated cell sorter, as described previously (8).

Binding of ^{125}I-labeled PDGF to mutated receptor. Cells were plated in Ham F-12 medium supplemented with 5% bovine serum, penicillin, and streptomycin. After 12 h the cultures were changed to quiescent medium (Ham F-12 medium, 0.1% bovine serum albumin, and 5 μg of transferrin per ml) for 48 h. Cells were collected from confluent cultures by treatment with a solution containing 2 mM EDTA in phosphate-buffered saline. Binding of PDGF to its receptor was performed with cells in suspension. A total of 10^6 cells were incubated with 40,000 cpm of ^{125}I-labeled PDGF (10,000 to 20,000 cpm/ng) (26) for 45 min at 37°C in the presence of increasing concentrations of unlabeled PDGF. Bound PDGF was separated from free PDGF by centrifugation of the cell suspension on a Ficoll gradient (Pharmacia Fine Chemicals, Piscataway, N.J.) (18). The amount of PDGF associated with the cell pellet was determined in a gamma counter.

Expression of PDGF receptor mutants by immunoprecipitation. The level of PDGF receptor expression in the mutant cell lines was determined by immunoprecipitation of ^{35}S-labeled receptor with a receptor antibody. Cells (3×10^5) were incubated with [^{35}S]methionine (0.25 μCi/ml) in methionine-free medium for 2 h. Cell lysates were prepared and immunoprecipitations were performed as described previously (16). Immunoprecipitates were analyzed by sodium dodecyl sulfate [SDS]-polyacrylamide gel electrophoresis. Dried gels were exposed to XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) for 24 h.

Receptor tyrosine kinase activity in intact cells. The pattern of PDGF-induced tyrosine phosphorylation in the different receptor mutants and control cells was determined by immunoprecipitation with phosphotyrosine antibody (25). ^{35}S-labeled lysates from 3×10^5 cells that were incubated in the presence and in the absence of 10 nM PDGF for 3 h at 4°C were immunoprecipitated as described previously (16). Labeled immunoprecipitates were analyzed on SDS-polyacrylamide gels. The receptor was detected by exposure of the dried gel to XAR-5 film (Kodak) for 24 h.

Early cellular responses. Conversion of phosphoinositides into inositol phosphates was measured as follows. Confluent cultures were grown in 35 mm plates and were placed in quiescent medium for 24 h in the presence of 20 μM [*myo*-^{3}H]inositol. After 24 h cells were stimulated with PDGF (10 nM) for 30 min at 37°C, and the water-soluble inositol phosphates were separated by ion-exchange chromatography with 1-ml columns of AG 108 X8 (Bio-Rad Laboratories, Richmond, Calif.) (2).

Receptor down regulation. Cells were plated in 35-mm-diameter wells. Confluent cultures of CHO transfectants were incubated with [^{35}S]methionine as described above. After treatment with PDGF (10 nM) for 0, 30, and 120 min at 37°C, the cells were lysed. Receptors were immunoprecipitated with an antibody directed against the intracellular domain of the PDGF receptor. The labeled immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis. The gel was dried and exposed as described above. The amount of down-regulated receptor was determined by densitometric analysis of the autoradiogram.

Rate of thymidine incorporation into DNA. Cells were seeded in Ham F-12 medium containing 5% fetal bovine serum. Twenty-four hours later the medium was replaced with quiescent medium. After 48 h confluent cultures were incubated with or without PDGF for 16 h. Following PDGF treatment, the cells were incubated with 0.75 μCi of [^{3}H]thymidine (25 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) for 45 min at 37°C. Following incubation with thymidine, cells were rinsed with cold phosphate-buffered saline and lysed in 1% SDS. DNA from the solubilized samples was precipitated with 10% trichloroacetic acid in the cold, as described previously (7). Radioactivity in the samples was determined by scintillation counting.

RESULTS

Expression of PDGF receptor mutants. The PDGF receptor cDNA was mutated in the following ways. The codon for the lysine at amino acid position 602, a site that corresponds to known ATP-binding sites of other tyrosine kinases (28), was converted to an alanine codon (K602A mutant); the coding sequence for the carboxyl-terminal 145 amino acids (amino acids 922 to 1067) was deleted, thus removing most of the sequences 3′ to the kinase domain (amino acids 922 to 1020) (Δ*ct* mutant) (Fig. 1A); and the sequences that encoded the receptor membrane spanning region were replaced by the corresponding membrane sequences for the LDL receptor, the *neu* oncogene, and the hydrophobic region from the normal cellular counterpart (c-*neu*) of the *neu* oncogene (transmembrane mutants) (Fig. 1B). Wild-type and mutant receptor cDNA sequences were expressed under the transcriptional control of the simian virus 40 early promoter by transfection of CHO fibroblasts, and stable transfectants were selected. The transfectants expressed a surface receptor protein that was recognized by an antibody to the external domain of the PDGF receptor (16) in a fluorescent-activated cell sorter.

Analysis of the cell lines by immunoprecipitation of ^{35}S-labeled receptor with receptor antibody directed against a cytoplasmic domain (16) showed that the mutant cell lines expressed receptor in levels comparable to those of wild-

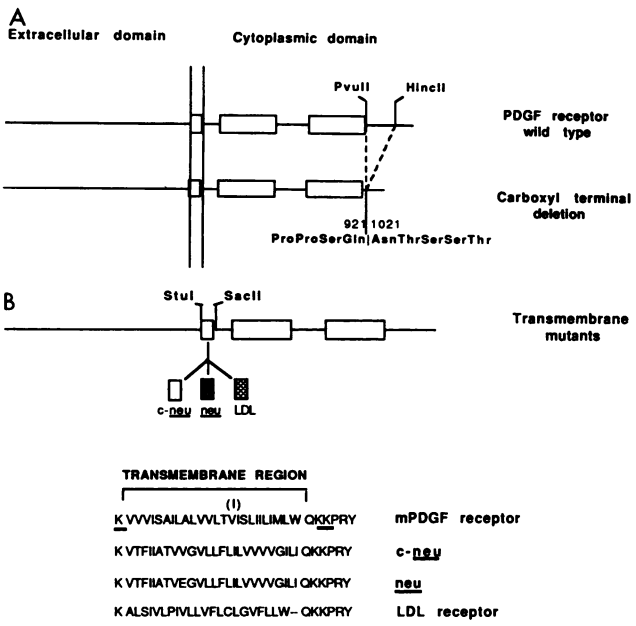

FIG. 1. PDGF receptor mutants. (A) In the carboxyl-terminal deletion mutant (Δ*ct*), the coding sequences for amino acids residues 922 to 1020 were deleted from the native receptor with the endonucleases *Pvu*II and *Hin*cII. (B) Transmembrane mutants. The coding sequence for the transmembrane region of the PDGF receptor was substituted by the synthesized transmembrane sequences of the c-*neu* gene, the *neu* oncogene, and the LDL receptor. Substitution of these sequences was possible because of the presence of an *Stu*I restriction site at the 5′ end of the transmembrane region and a *Sac*II restriction site at the 3′ end of the transmembrane region. Both restriction sites were generated by site-directed mutagenesis. The amino acid sequences of the transmembrane region of the mouse and human PDGF receptors, c-*neu*, *neu*, and the LDL receptor are shown for comparison. The positions of the *Stu*I and *Sac*II cleavage sites are underlined.

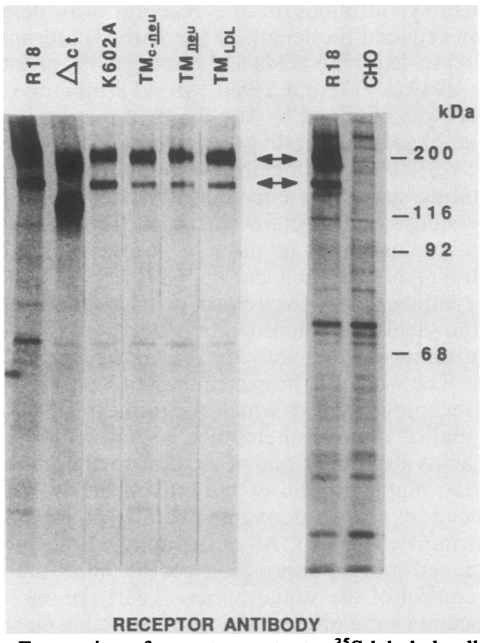

FIG. 2. Expression of receptor mutants. ^{35}S-labeled cell lysates from CHO transfectants that expressed wild-type PDGF receptor (R18), the Δct mutant, the ATP-binding site mutant (K602A), or the transmembrane (TM) mutants ($TM_{c\text{-}neu}$, TM_{neu}, TM_{LDL}) were analyzed by immunoprecipitation with a receptor antibody directed against amino acids 738 to 760 in the intracellular domain (14). CHO cells that lacked PDGF receptors were included as a control. The upper arrow indicates the PDGF receptor protein, and the lower arrow indicates the precursor of the receptor.

type R18 transfectants (Fig. 2 and Table 1). The Δ*ct* mutant cells expressed abnormally high levels of a receptor precursor (J. A. Escobedo and L. T. Williams, submitted for publication), suggesting an abnormality in receptor processing (Fig. 2). The binding of ^{125}I-labeled PDGF to each of the cell lines was of high affinity (Table 1). Half-maximal inhibition of binding by unlabeled PDGF occurred at 0.05 to 0.25 nM, which is the same concentration required to half maximally inhibit ^{125}I-labeled PDGF binding in BALB/c 3T3 cells that have native PDGF receptors. Approximately 5,000 to 10,000 receptor sites per cell were expressed in the mutant and wild-type receptor transfectants (Table 1).

Tyrosine phosphorylation measurements. When PDGF binds to its receptor, the receptor tyrosine kinase is stimulated. The major substrate of the receptor kinase that is detectable with phosphotyrosine antibody is the receptor itself (8, 10). PDGF-stimulated tyrosine kinase activity of the receptor was measured by anti-phosphotyrosine antibody immunoprecipitation of lysates of ^{35}S-labeled, PDGF-stimulated cells. No tyrosine-phosphorylated receptor was detected in the ATP-binding site mutant, the carboxyl-terminal deletion mutant, or the transmembrane mutants (Fig. 3). In vitro measurements of the tyrosine kinase activity in these receptors mutants confirmed the tyrosine kinase data from intact cells (W. J. Fantl, unpublished data).

Early cellular responses to PDGF. PDGF is a very potent activator of the hydrolysis of membrane PI and stimulates a change in cellular calcium that may be linked to the PI metabolic pathway. PDGF failed to stimulate PI hydrolysis in any of the cell lines expressing kinase-defective mutants (Fig. 4A). Similarly, the kinase-deficient receptor did not mediate PDGF-stimulated changes in intracellular calcium levels or changes in pH (data not shown). However, these responses to PDGF could be easily measured in the wild-type cells (14).

PDGF-stimulated mitogenesis. The mitogenic effect of PDGF on the receptor mutants was measured by determining the rate of [^{3}H]thymidine incorporation into DNA after PDGF treatment. There was no mitogenic response observed in any of the kinase-defective mutants, including the ATP-binding site mutant, the carboxyl-terminal deletion mutant, or the transmembrane mutants (Fig. 4B). Cells

TABLE 1. Expression of receptors in different cell types

Cell type	Estimated receptor no.[a]	Apparent K_d (nM)[b]
R18	8,000	0.08
Δ*ct*	10,000	0.25
K602A	7,000	0.05
$TM_{c\text{-}neu}$[c]	4,500	0.06
TM_{neu}	5,000	0.09
TM_{LDL}	5,500	0.08

[a] Relative receptor abundance values were determined by scanning densitometry of radioautograms of ^{35}S-labeled receptor immunoprecipitates. R18 cells contained 8,000 receptors, as determined by Scatchard analysis.

[b] Apparent K_ds were determined from binding competition curves, as described previously (27).

[c] TM, Transmembrane mutant.

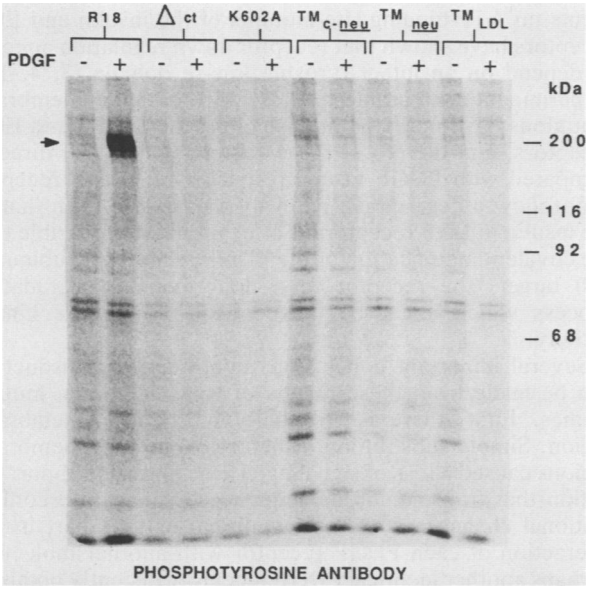

FIG. 3. PDGF-induced tyrosine phosphorylation in intact cells. ^{35}S-labeled lysates from CHO cells expressing the wild-type receptor (R18), the Δct mutant, or the ATP-binding site mutant (K602A) and transmembrane (TM) mutants ($TM_{c\text{-}neu}$, TM_{neu}, and TM_{LDL}) incubated in the presence or absence of PDGF were immunoprecipitated by using a phosphotyrosine antibody. The phosphorylated receptor band is indicated by the arrow.

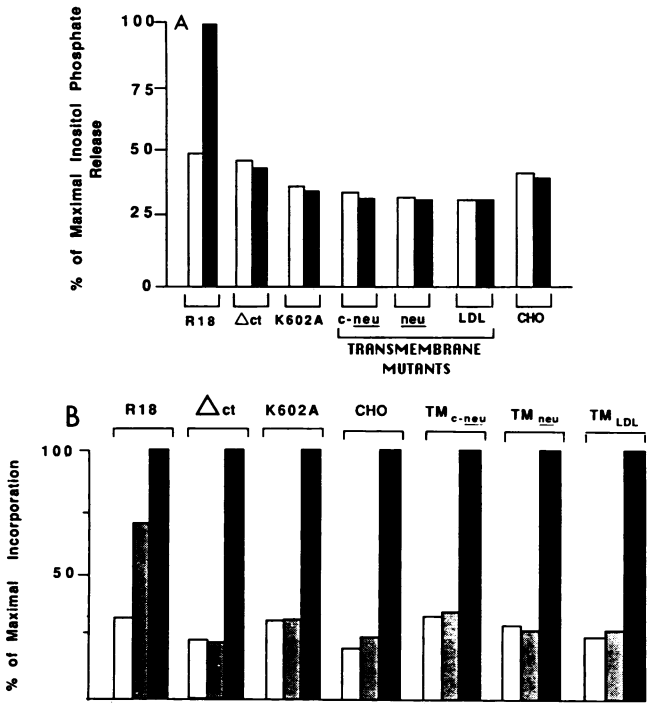

FIG. 4. (A) PDGF-stimulated hydrolysis of PI. CHO cells and transfected CHO cells were labeled with [*myo*-^{3}H]inositol and exposed to PDGF, as indicated. The release of inositol phosphates was measured as described in the text. The values correspond to the average of three independent measurements and are representative of two independent experiments. The results are expressed as the percentage of maximal release of inositol phosphates. Symbols: □, control; ■, PDGF. (B) PDGF-stimulated DNA synthesis. Changes in the rate of thymidine incorporation in response to PDGF treatment was studied in cells expressing the receptor mutants. Each value represents the average of three independent measurements. The standard error was less than 15%. The results are expressed as the percentage of maximal thymidine incorporation stimulated by 10% fetal bovine serum. Symbols: □, control; ▨, PDGF; ■, fetal bovine serum.

expressing the wild-type form of the receptor showed the characteristic increase in DNA synthesis in response to PDGF.

PDGF-mediated receptor down regulation. Treatment of wild-type cells with PDGF induces receptor internalization (12) and the loss of receptor protein, a phenomenon termed down regulation. To test wild-type and mutant cell lines for receptor down regulation, cells were treated with PDGF at 37°C for 0, 30, and 120 min. The amount of PDGF binding to the receptor following down regulation by PDGF was determined by measuring the amount of ^{125}I-labeled PDGF bound to the PDGF-pretreated cells. All mutants cell lines showed between 15 and 48% of maximal binding after 120 min of PDGF treatment (data not shown). A deletion mutant of the receptor (ΔSal) lacking sequences encoding most of the cytoplasmic domain (amino acid residues 603 through 1067) was used as a control and failed to down regulate after PDGF treatment (data not shown). The ability of the wild-type and mutant cells to degrade the receptor in response to PDGF treatment was determined by receptor-immunoprecipitation analysis of ^{35}S-labeled cells treated with PDGF for 0, 30, and 120 min at 37°C. Analysis of the immunoprecipitates by scanning densitometry showed that the receptor mutants degraded the receptor to an extent comparable to the down regulation seen in the wild-type cells (Fig. 5A). In separate experiments, all these mutants down regulated their receptor (80 to 90%) after 3 h of PDGF treatment (Fig. 5B and C).

DISCUSSION

In this study, mutants of the PDGF receptor were designed to investigate the roles of the receptor tyrosine kinase and the receptor transmembrane domains in signal transduction. The tyrosine kinase activity of the receptor was eliminated by three independent types of mutations. The only obvious common feature of these mutants was the loss of PDGF-sensitive tyrosine kinase activity. Thus, it seems likely that the loss of response in the kinase-deficient mutants was directly related to the loss of tyrosine kinase activity.

Our finding that the kinase-deficient mutants cannot stimulate PI hydrolysis shows for the first time a direct link between receptor-mediated PI hydrolysis and tyrosine kinase. A similar link between the kinase activity and changes in calcium and pH can also be established from these findings. It seems likely that the PDGF receptor either phosphorylates an activator of these responses, for example, phospholipase *c*, or that the autophosphorylation reaction alters the receptor so that it can interact with another molecule which in turn mediates these early responses to PDGF. The effect of kinase inactivation on PDGF-induced mitogenesis was expected in the context of recently reported studies on the ATP-binding site mutants of the EGF and insulin receptors (3–5, 13). Our finding that the three independent types of mutations cause a loss of receptor-mediated mitogenesis makes a compelling case for the role of the kinase in growth factor-stimulated cell proliferation.

An unexpected finding was that PDGF induced down regulation of the receptor in each of the three kinase-deficient mutants. By contrast, recently published experi-

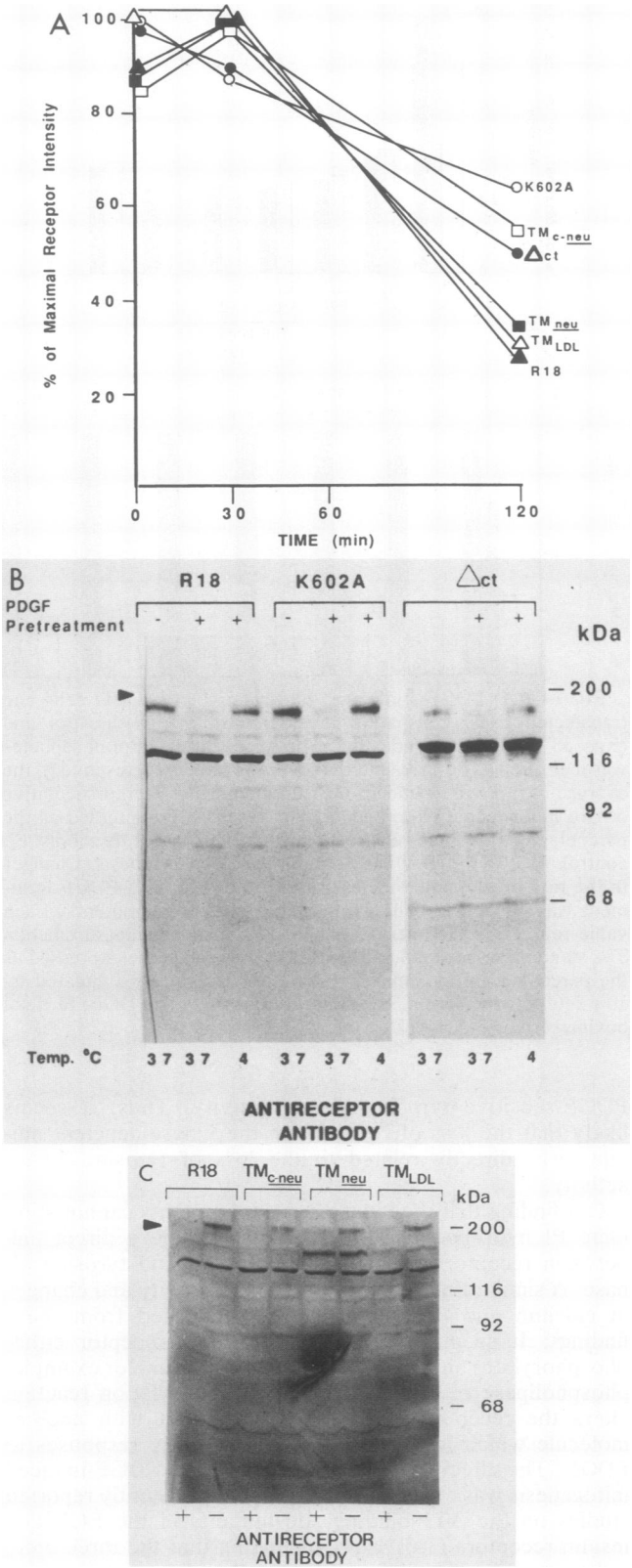

FIG. 5. (A) Time course of PDGF-induced receptor down regulation. [^{35}S]methionine-labeled transfected CHO cells expressing the wild-type receptor, the carboxyl-terminal deletion mutant, the ATP-binding site mutant, or the transmembrane mutants were incubated with a saturating concentration of PDGF for 0, 30, and 120 min. ^{35}S-labeled lysates were immunoprecipitated with receptor antibody as described above. Immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis. The levels of PDGF receptor were determined by scanning densitometry of the radioautogram. The level of down regulation is expressed as the percentage of maximal receptor intensity following PDGF treatment. The absolutes values of the amount of receptor at time zero in arbitrary (densitometric) units for each of the transfectants were as follows: R18, 3.0; Δct, 20.0; K602A, 8.5; $TM_{c\text{-}neu}$, 2.5; TM_{neu}, 4.0; and TM_{LDL}, 11.0 (where TM indicates transmembrane mutants). (B and C) Long-term PDGF-induced down regulation. Cells expressing Δct and K602A mutated forms of the PDGF receptor (B) were treated with a saturating concentration of PDGF for 4 h at 37 or 4°C, as indicated. Cells expressing transmembrane (TM) mutants ($TM_{c\text{-}neu}$, TM_{neu}, and TM_{LDL}) were subjected to similar treatment at 37°C (C). The level of PDGF receptor in cell lysates was determined by immunoblotting with a receptor antibody as described previously (8). Cells expressing the wild-type receptor (R18) were used as a control. In panel C, the plus and minus signs indicate whether there was pretreatment with PDGF.

ments on ATP-binding site mutants of the insulin and EGF receptors have shown that receptor down regulation appears to depend on an intact tyrosine kinase domain (3, 4, 19). Experiments comparable to those with our transmembrane mutations have not been published for the insulin and EGF receptors, and therefore, these systems cannot be directly compared with PDGF in this respect. The PDGF receptor may follow a degradative pathway that differs from that of the insulin in EGF receptors. For example, it is possible that the covalent modification of the PDGF receptor by ubiquitin (28) targets the receptor to a nonlysosomal degradative process which might be independent of tyrosine kinase activity.

Several important conclusions about signal transduction can be made from these results of transmembrane mutant studies. First, there is specificity in the transmembrane region. Simple substitution of heterologous transmembrane regions caused a loss of signaling. These findings support the notion that transmembrane sequences propagate a conformational change across the membrane or take part in the interaction of each PDGF receptor with another molecule, perhaps another identical PDGF receptor. Recently obtained sequence information on the human PDGF receptor (9, 11; L. T. Williams, J. A. Escobedo, M. T. Keating, and S. R. Coughlin, submitted for publication) has shown that there is an extremely high degree of amino acid sequence conservation (96%) between it and the mouse receptor in the transmembrane region, giving further support to the finding that there is specificity in these sequences. Taken together, these data suggest that the transmembrane region provides a definite function other than simply serving as a membrane anchor.

A second conclusion that can be derived from experiments with the transmembrane mutants concerns the activating mutation of the *neu* oncogene. The transforming activity of this oncogene is caused by a single point mutation that converts a valine in the transmembrane region of the c-*neu* gene to glutamic acid, which is found in the corresponding position of the *neu* oncogene (1). Our data show that this mutation does not cause activation of the PDGF receptor kinase domain in the chimeric molecules that we generated. Thus, this mutation is not always a transforming mutation for receptor kinase molecules.

Finally, the transmembrane mutants demonstrate that the binding of ligand does not depend on the transmembrane sequences, unlike the binding of catecholamines to the

β-adrenergic receptor which requires specific transmembrane sequences (23).

In summary, we designed a series of mutants to probe the roles of the PDGF receptor tyrosine kinase and transmembrane domains in mediating signal transduction. Tyrosine kinase activity was essential for a mitogenic response. Three independent mutations that eliminated tyrosine kinase activity also caused a loss of PDGF-stimulated PI hydrolysis. Despite the loss of kinase activity, receptor down regulation occurred normally, thus dissociating this response from other kinase-dependent responses. These experiments have shown that the transmembrane sequences serve a specific function in signal transduction for all of the measured responses except for receptor down regulation.

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