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Deletion of the Kinase Insert Sequence of the Platelet-Derived Growth Factor β-Receptor Affects Receptor Kinase Activity and Signal Transduction

LIV SEVERINSSON,[1]† BO EK,[1] KARIN MELLSTRÖM,[2]‡ LENA CLAESSON-WELSH,[1] AND CARL-HENRIK HELDIN[1]*

Ludwig Institute for Cancer Research, Biomedical Center, Box 595, S-751 23 Uppsala,[1] and Department of Pathology, University Hospital, S-751 85 Uppsala,[2] Sweden

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A characteristic feature of the platelet-derived growth factor (PDGF) β-receptor is the presence of an insert sequence in the protein tyrosine kinase domain. A receptor mutant which lacks the entire insert of 98 amino acids was expressed in CHO cells, and its functional characteristics were compared with those of the wild-type receptor. The mutant receptor bound PDGF-BB with high affinity and mediated internalization and degradation of the ligand with efficiency similar to that of the wild-type receptor but did not transduce a mitogenic signal. It was found to display a decreased autophosphorylation after ligand stimulation and had a decreased ability to phosphorylate exogenous substrates; phosphofructokinase was not phosphorylated at all, whereas a peptide substrate was phosphorylated, albeit at a lower rate compared with phosphorylation by the wild-type receptor. Furthermore, the mutant receptor did not mediate actin reorganization but mediated an increase in c-*fos* expression. The data indicate that the insert in the kinase domain of the PDGF β-receptor is important for the substrate specificity or catalytic efficiency of the kinase; the deletion of the insert interferes with the transduction of some, but not all, of the signals that arise after activation of the receptor.

Platelet-derived growth factor (PDGF) is a potent mitogen for connective tissue cells (for reviews, see references 22 and 34). Structurally, PDGF is composed of two homologous disulfide-bonded polypeptide chains, denoted A and B. All three dimeric isoforms, PDGF-AA, -AB, and -BB, have been identified and purified from platelets and transformed cells (17, 21, 37). The different PDGF isoforms bind to two distinct receptor types; the α-receptor (also called the A-type receptor) binds all three PDGF isoforms with high affinity, whereas the β-receptor (also called the B-type receptor) binds PDGF-BB with high affinity and PDGF-AB with lower affinity but does not bind PDGF-AA with any appreciable affinity (19, 20, 31).

The two PDGF receptor types have similar structures, as revealed by the amino acid sequences deduced from cDNA clones (5, 6, 16, 25, 43). Both receptors contain extracellular domains with a characteristic spacing of the cysteine residues, suggesting that they each consist of five immunoglobulinlike domains; the amino acid sequence similarity in this region is about 30%. The intracellular parts contain protein tyrosine kinase domains, which in both receptor types have an inserted sequence of about 100 amino acids without homology to other kinase domains. The amino acid sequence similarity is about 80% in the tyrosine kinase domains and about 30% in the inserted sequences and the carboxy-terminal tails. The receptor for colony-stimulating factor 1 (9) and the c-*kit* product, a receptorlike molecule for a still-unidentified ligand (44), display similar structural features and form a subfamily with the two PDGF receptor types among the protein tyrosine kinases.

* Corresponding author.
† Present address: Cytel Corporation, 11099 North Torrey Pines Road, Suite 100, La Jolla, CA 92037.
‡ Present address: KABIGEN AB, S-112 87 Stockholm, Sweden.

Expression of the PDGF β-receptor cDNA in CHO cells leads to the synthesis of functionally active receptors (13, 36). With the aim of elucidating the function of the various domains of the receptor, analysis of the properties of receptor mutants has started. One important question concerns the function of the insert sequence in the kinase domain. The removal of most of the insert was recently reported to lead to the synthesis of a receptor which could transduce the early signals of PDGF, including stimulation of protein tyrosine kinase activity and stimulation of phosphatidylinositol turnover, but which could not transduce a mitogenic signal (14). Here we report data on a receptor mutant from which the entire insert was removed (the Δki-mutant receptor). Similar to the mutant studied by Escobedo and Williams (14), the Δki-mutant receptor did not transduce a mitogenic signal. We noted, however, that the ligand-stimulated autophosphorylation was decreased and the substrate specificity of the receptor kinase was altered. Our data indicate that the kinase insert is important for some but not all of the signals of the PDGF β-receptor.

MATERIALS AND METHODS

Plasmids. The cDNA encoding the full-length human PDGF β-receptor was cloned into the mammalian expression vector pSV7d containing an early simian virus 40 promoter (39) kindly provided by L. Rall, Chiron Corp., Emeryville, Calif.

To obtain a mutant receptor lacking the tyrosine kinase insert (the Δki-mutant receptor), a 2,895-base-pair *Eco*RI fragment (HPDGFR-2A3) (5) was cloned into the *Eco*RI site of M13 mp19. Single-stranded template was prepared for oligonucleotide-directed mutagenesis with an oligomer (48 nucleotides long) that bridged between nucleotide positions 2266 to 2289 and nucleotides 2581 to 2604 in the cDNA clone

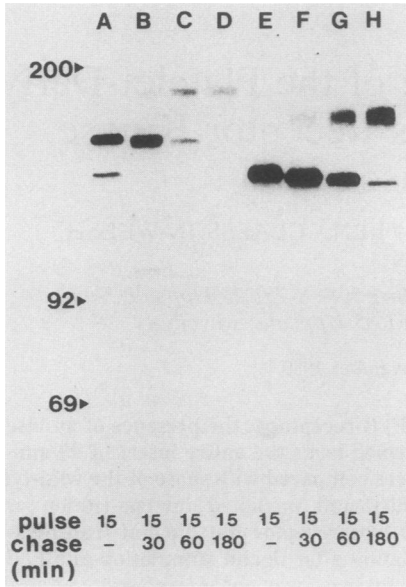

FIG. 1. Pulse-chase analysis of the wild-type PDGF β- and Δki-mutant receptors. CHO cells expressing wild-type (lanes A to D) and Δki-mutant (lanes E to H) PDGF β-receptors were pulse-labeled with [^{35}S]methionine and [^{35}S]cysteine for 15 min (lanes A and E). They were then chased, using a fivefold molar excess of unlabeled methionine and cysteine for 30 min (lanes B and F), 60 min (lanes C and G), or 180 min (lanes D and H). Glycoproteins isolated by Lens culinaris chromatography were immunoprecipitated with the PDGFR-1 serum prior to analysis by SDS-gel electrophoresis and fluorography. The positions of markers of molecular mass (in kilodaltons) are indicated.

HPDGFR-8 (5). This would lead to a deletion of amino acids 701 to 798, corresponding to the kinase insert. The mutagenesis was performed with the oligonucleotide-directed in vitro mutagenesis system (Amersham Corp.).

The mutagenesis reactions were transformed into *Escherichia coli* TG-1 cells, and the resulting plaques were screened by M13 dideoxy sequencing. The replicative form of the mutant M13 vector was prepared, and an *Eco*RI-*Bam*HI fragment was purified by agarose gel electrophoresis. The mutated *Eco*RI-*Bam*HI fragment was ligated to a *Bam*HI-*Bam*HI fragment (nucleotides 2855 to 5220 in HPDGFR-8) and to *Eco*RI-*Bam*HI-cleaved pSV7d. The resulting plasmids were screened for correctly orientated inserts by restriction enzyme mapping.

Transfections. The wild-type PDGF β-receptor or the Δki-mutant were introduced into Dhfr$^-$, Pro$^-$, CHO cells (kindly provided by L. Rall, Chiron Corp.) by using the calcium phosphate precipitation technique essentially as described by Wigler et al. (42). The CHO cells lack detectable amounts of endogeneous PDGF β-receptors. To allow selection of transfected cells, cotransfection was performed with the plasmid pVV3 carrying the gene conferring resistance to G418. After 48 h, selection was initiated by the addition of 0.5 mg of G418 per ml to the culture medium. Resistant clones were picked after 2 weeks, and clones expressing the desired products, as assayed by ^{125}I-PDGF-BB binding and immunoprecipitation analysis (see below), were subcloned by limiting dilution.

Cultivation and radioactive labeling of cells. CHO cells were cultured in Ham F12 medium (Flow Laboratories, Inc.)–10% fetal calf serum (FCS)–200 μg of proline per ml–100 U of penicillin per ml–50 μg of streptomycin (Sigma Chemical Co.) per ml or, if transfected, in the same medium supplemented with 0.5 mg of G418 (GIBCO Laboratories and Bethesda Research Laboratories, Inc.) per ml. Labeling was performed in methionine- and cysteine-free Dulbecco modified Eagle medium–10% dialyzed FCS–200 μg of proline per ml supplemented with 100 μCi each of [^{35}S]methionine and [^{35}S]cysteine (Amersham) per ml, as described elsewhere (7). The chase in pulse-chase experiments was performed in complete medium containing a fivefold molar excess of unlabeled methionine and cysteine. The labeling of cells to be stimulated with PDGF-BB was performed in serum-free MCDB 104 medium (26) lacking methionine and cysteine and supplemented with antibiotics, 200 μg of proline per ml, and [^{35}S]methionine and [^{35}S]cysteine.

Antisera. The rabbit antiserum PDGFR-1 was raised against highly purified porcine PDGF β-receptor preparations and recognizes α- and β-receptors, whereas PDGFR-3 was raised in rabbits against a peptide corresponding to amino acids 981 to 995 of the mouse PDGF receptor and recognizes specifically β-receptors (7, 33).

Lysis of cells, immunoprecipitation, and SDS-gel electrophoresis. Lysis of cells, immunoprecipitation of a glycoprotein-enriched fraction, and sodium dodecyl sulfate (SDS)-gel electrophoresis (2) on 5 to 10% polyacrylamide gels were performed as described previously (36).

^{125}I-PDGF-BB binding experiments. PDGF-BB, a recombinant form expressed in the yeast *Saccharomyces cerevisiae* (32), was labeled with ^{125}I to about 65,000 cpm/ng by the procedure described by Bolton and Hunter (3). The binding of ^{125}I-PDGF-BB to transfected cells was performed in 12-well Costar dishes as described elsewhere (36), and the data were evaluated by Scatchard analysis (35). The time course of internalization and degradation of ^{125}I-PDGF-BB was estimated by determining the amount of trichloroacetic acid nonprecipitable radioactivity released from the cells, as described elsewhere (36).

Assay of cell proliferation. For the determination of [^{3}H]thymidine incorporation, assay cells were seeded sparsely in 12-well Costar dishes in Ham F12 medium with 10% FCS. Two days later, when the cells were subconfluent, the plating medium was replaced by serum-free Ham F12 medium. Forty-eight hours later, 1 μCi of [^{3}H]thymidine (Amersham) and 0 or 20 ng of PDGF-BB or 10% FCS was added. After 24 h of incubation, cells were washed twice with ice-cold phosphate-buffered saline (PBS), and high-molecular-weight ^{3}H radioactivity was precipitated with 10% trichloroacetic acid for 30 min at 4°C. After the cells were washed twice in PBS, ^{3}H radioactivity was solubilized in 0.2 M NaOH–1% SDS and determined by liquid scintillation counting. Cell growth was also estimated by determining the increase in cell number. For this assay, cells were serum starved as described above, and 0 or 20 ng of PDGF-BB or 10% FCS was added to the cells. Cell numbers were determined by counting the cells before any additions were made (day 0) and then at 24-h intervals for a period of 2 days after stimulation. Each value represents an average of three cell cultures.

Phosphorylation assay. The phosphorylation assay was performed essentially as described before (11). Briefly, 5 μg of membrane protein was incubated for 10 min at 0°C in a mixture containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.4), 3 mM $MnCl_2$, 0.05% Triton X-100, and 15 μM [γ-^{32}P]ATP (Amersham) (containing 2 μCi of radioactivity) with or without 1.3 μg of PDGF-BB per ml. For the determination of kinase activity against exogenous substrates, phosphofructokinase or a

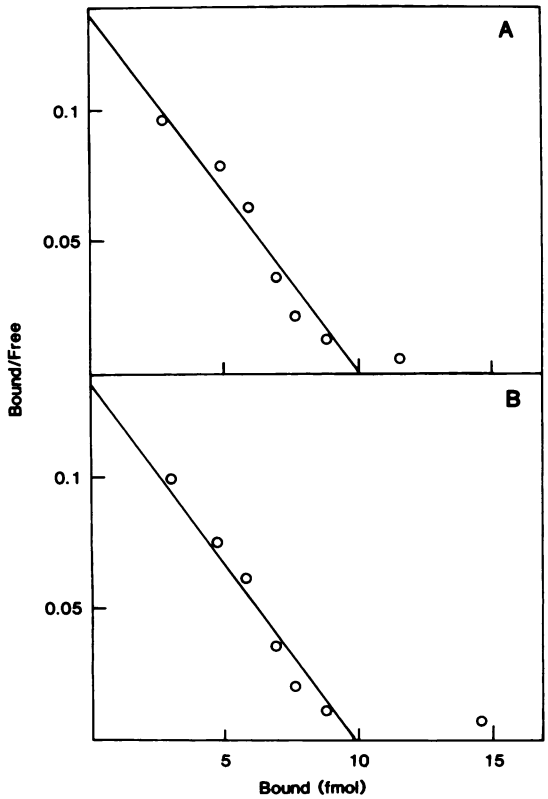

FIG. 2. Scatchard analysis of ^{125}I-PDGF-BB binding to CHO cells expressing wild-type or Δki-mutant PDGF β-receptors. ^{125}I-PDGF-BB binding to CHO cells expressing wild-type (A) or Δki-mutant (B) PDGF β-receptors was determined in the presence of unlabeled PDGF-BB at concentrations ranging from 0 to 300 ng/ml. Cells were incubated at 4°C for 90 min, and binding data were analyzed by the method of Scatchard (35).

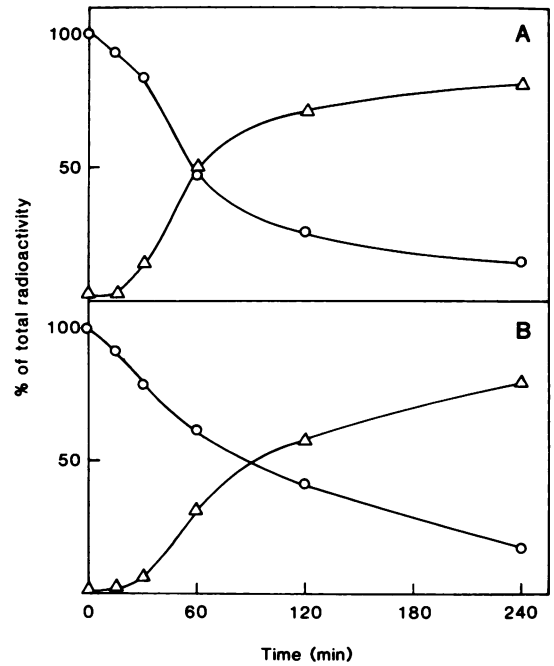

FIG. 3. Internalization and degradation of ^{125}I-PDGF-BB by wild-type and Δki-mutant receptors. CHO cells expressing wild-type (A) or Δki-mutant (B) PDGF β-receptors were incubated with ^{125}I-PDGF-BB (~100,000 cpm per well) for 90 min at 4°C. After being washed, cells were incubated at 37°C in Ham F12 medium containing 1 mg of bovine serum albumin per ml for various time periods. The medium was removed and subjected to trichloroacetic acid precipitation; the amount of trichloroacetic acid nonprecipitable radioactivity was taken as a measure of the degradation of ^{125}I-PDGF-BB (△). Cell-associated radioactivity (○) was collected in a Triton X-100 lysate.

peptide corresponding to amino acids 849 to 861 of the PDGF β-receptor was added to the phosphorylation assay at a concentration of 0.1 mg/ml and 2.5 mM, respectively. Phosphorylation of exogenous substrates was performed at 30°C for 5 min.

For the analysis of phosphorylation events in intact cells, confluent cell cultures in 25-cm^2 cell culture flasks were incubated in methionine- and cysteine-free MCDB 104 medium supplemented with 1 mg of bovine serum albumin and 0.35 mCi each of [^{35}S]methionine and [^{35}S]cysteine (Amersham) in a volume of 1 ml. After 4 h of labeling at 37°C, 100 ng of PDGF-BB per ml was added and incubation continued for an additional 10 min; cells were then chilled, washed, and lysed in 0.1% SDS–1% Triton X-100–1% deoxycholate–150 mM NaCl–50 mM Tris (pH 8.8)–10 mM EDTA–1 mM phenylmethylsulfonyl fluoride–1% Trasylol. Immunoprecipitation with antiphosphotyrosine antibodies was performed as described earlier (10), except that immunoprecipitated proteins were released from immunocomplexes by incubation with 50 mM phenylphosphate.

Samples were analyzed by SDS-gel electrophoresis and then autoradiographed or fluorographed.

Fluorescence microscopy of PDGF-BB-induced actin reorganization. CHO cells were seeded sparsely on glass coverslips in 35-mm petri dishes and were allowed to grow for 2 days in Ham F-12 medium supplemented with 10% FCS. The medium was then changed to serum-free Ham F-12 medium, and the experiment was performed after 2 to 4 days of serum starvation. PDGF-BB or serum was added, and the cells were incubated at 37°C for 10 min in a humidified atmosphere and then fixed for 20 min at room temperature with 3% paraformaldehyde freshly made in PBS followed by 100% acetone at 20°C for 5 min. The fixative was removed by repeated washings with PBS, and the cells were stained with 50 μl of rhodamine-conjugated phalloidin (~10 μg/ml) (a generous gift from T. Wieland, Heidelberg, Federal Republic of Germany) in a humidified atmosphere for 30 min. The coverslips were rinsed three times in PBS and once in distilled water and finally mounted in a 1:1 solution of glycerol and PBS. The specimens were analyzed in a Leitz photomicroscope equipped for epifluorescence. Micrographs were taken with a 63× immersion objective, using Kodak Tri-X film.

Analysis of c-*fos* expression. Subconfluent CHO cells expressing either the wild-type β-receptor or the Δki-mutant receptor were maintained in Ham F12 medium without FCS for 4 days. The medium was then changed to Ham F12 containing 0.1 mg of bovine serum albumin per ml and 20 ng of PDGF-BB per ml. After incubation for 0, 0.5, 1, 2, and 8 h at 37°C, RNA was prepared from the cells by the method of Chirgwin et al. (4). After electrophoresis of 20-μg portions of RNA in the presence of formaldehyde and transfer to nitrocellulose, hybridization was performed, using 10^6 cpm/ml of a ^{32}P-random-priming-labeled human c-*fos* cDNA probe (a 2.8-kilobase-pair *Nco*I-*Xho*I fragment of pc-*fos* [human]-1, obtained from R. Müller). Hybridization was also

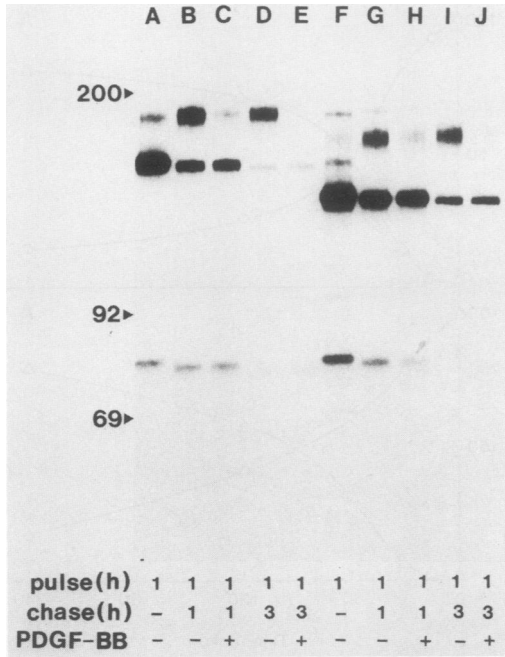

FIG. 4. Degradation of wild-type and Δki-mutant receptors after ligand stimulation. CHO cells expressing wild-type (lanes A to E) or Δki-mutant (lanes F to J) PDGF β-receptor were pulse-labeled for 1 h in serum-free MCDB 104 medium and then chased in the same medium containing a fivefold molar excess of unlabeled methionine and cysteine in the absence (−) (lanes B, D, G, and I) or presence (+) (lanes C, E, H, and J) of PDGF-BB (50 ng/ml) for 1 h (lanes B, C, G, and H) or 3 h (lanes D, E, I, and J). Glycoprotein fractions were immunoprecipitated with the PDGFR-3 serum prior to analysis by SDS-gel electrophoresis and fluorography. The positions of markers of molecular mass (in kilodaltons) are indicated.

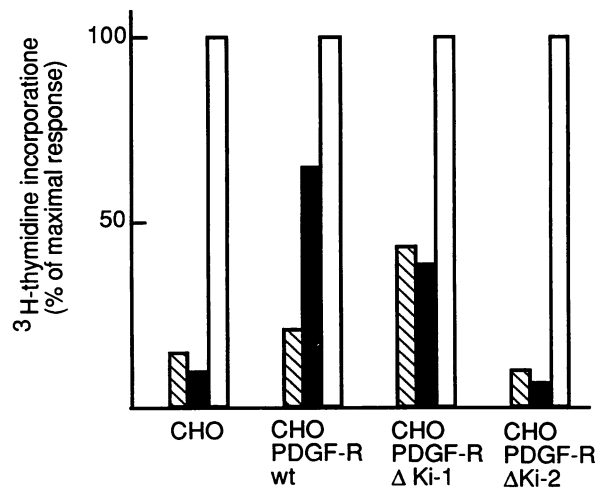

FIG. 5. Effect of PDGF-BB on [^{3}H]thymidine incorporation in cells expressing wild-type or Δki-mutant receptors. [^{3}H]thymidine incorporation was determined in untransfected CHO cells, CHO cells expressing the wild-type PDGF β-receptor (PDGF-R wt) and two different clones expressing the Δki-mutant receptor (PDGF-R Δki-1 and -2), with (■) or without (▨) 20 ng of PDGF-BB per ml or with 10% FCS (□). Trichloroacetic acid-precipitable radioactivity was determined and expressed as the percentage of the value obtained after stimulation with 10% FCS for each cell line.

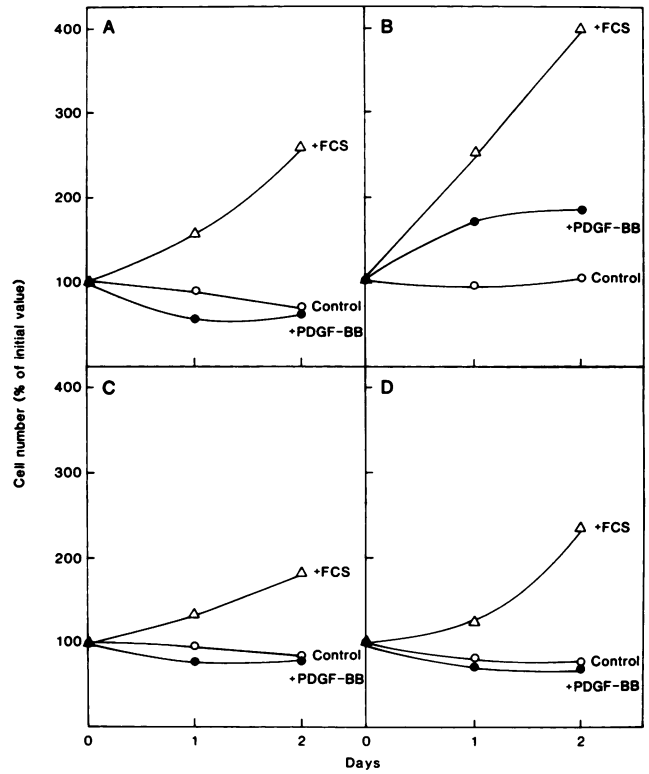

FIG. 6. Effect of PDGF-BB on cell number of CHO cells expressing wild-type or Δki-mutant receptors. Control CHO cells (A), cells expressing the wild-type PDGF β-receptor (B), and two independent CHO cell lines expressing the Δki-mutant receptor (C and D) were serum starved as described in Materials and Methods. Two days later (day 0), cells were treated with PDGF-BB (+PDGF-BB) (20 ng/ml), 10% FCS (+FCS), or nothing (control). Cell numbers were determined by counting the cells at 24-h intervals for 2 days after stimulation. Each value represents an average of three cell cultures.

performed with a ^{32}P-labeled cDNA corresponding to glyceraldehyde-3-phosphate-dehydrogenase (40) (obtained from R. Wu) in order to assess the amount of RNA loaded in each well.

RESULTS

Expression of a Δki-mutant of the PDGF β-type receptor in CHO cells. A PDGF β-receptor mutant lacking the kinase insert was constructed by removing the nucleotides corresponding to amino acids 701 to 798 (numbering according to reference 5) from the cDNA. The number of remaining amino acids in this part of the kinase domain is thus the same as that in the epidermal growth factor receptor (18). The Δki-mutant receptor and the wild-type receptor were then expressed in CHO cells under the control of a simian virus 40 promoter. Stable cell lines were isolated, and the expression of receptors was analyzed by immunoprecipitation of cells pulse-labeled with [^{35}S]methionine and [^{35}S]cysteine for 15 min. From a cell line expressing the Δki-mutant, a 150-kilodalton (kDa) component was precipitated by PDGFR-1, an antiserum made against purified β-receptor (33); upon prolonged chase, this component was converted to a 180-kDa species (Fig. 1). The wild-type receptor was synthesized as a 160-kDa component and chased, with the same kinetics

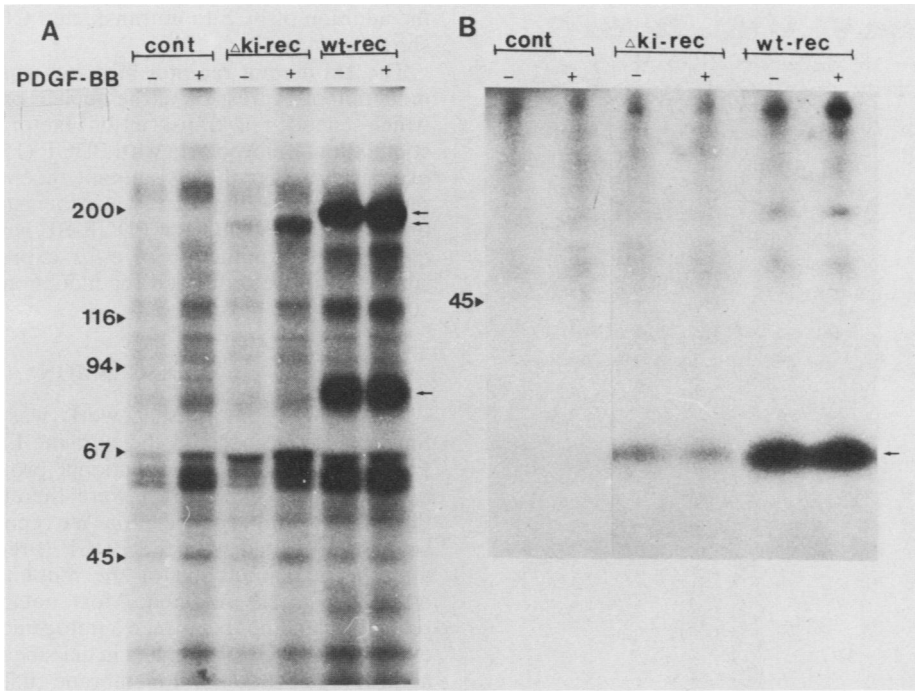

FIG. 7. Phosphorylation induced by PDGF-BB in membranes prepared from CHO cells expressing wild-type or Δki-mutant receptors. Membranes prepared from CHO cells expressing the wild-type (wt-rec), Δki-mutant (Δki-rec) PDGF β-receptor, or nontransfected CHO cells (cont) were subjected to the phosphorylation assay as described in Materials and Methods. (A) Phosphofructokinase was added to the assay as an exogenous substrate; samples were analyzed by SDS-gel electrophoresis on 7 to 12% polyacrylamide gels and then autoradiographed. Arrows on the right indicate (top to bottom) the wild-type receptor, the Δki-mutant receptor, and phosphofructokinase. (B) A peptide (corresponding to amino acids 849 to 861 of the PDGF β-receptor) was added as exogenous substrate; samples were analyzed by SDS-gel electrophoresis on 7 to 22% polyacrylamide gels and then autoradiographed. The position of the peptide substrate in the gel is indicated by the arrow on the right. The positions of markers of molecular mass (in kilodaltons) are indicated.

as the Δki-mutant, to a 190-kDa species (Fig. 1). Treatment with endoglycosidase H led to a degradation of the 150- and 160-kDa precursor forms to 130 and 140 kDa, respectively, while the mature forms, as expected, were resistant (data not shown). Thus, the molecular mass of the Δki-mutant was about 10 kDa smaller than that of the wild-type receptor, which is the size difference expected from the deletion of 98 amino acids.

The Δki-mutant receptor binds and mediates degradation of ligand. The binding of ^{125}I-PDGF-BB to CHO cells expressing the Δki-mutant or the wild-type receptor was determined, and the data were subjected to Scatchard analysis. Straight lines indicating K_ds of about 0.2 nM were observed for both the mutant and the wild-type receptors; the two cell lines also had similar numbers of receptors, 25,000 and 30,000 per cell, respectively (Fig. 2). Furthermore, the mutant receptor was found to mediate internalization and degradation of the ligand, measured as release of low-M_r ^{125}I radioactivity into the medium after incubation at 37°C, with efficiency similar to that of the wild-type receptor (Fig. 3).

The Δki-mutant receptor is degraded after ligand binding. The binding of PDGF-BB to its receptor induces internalization of the ligand-receptor complex, followed by degradation of the ligand as well as the receptor. The 180-kDa Δki-mutant receptor was found to be degraded after ligand binding, with efficiency similar to that of the 190-kDa wild-type receptor (Fig. 4). Consistent with this observation, the down regulation of cell surface receptors, observed after incubation with various concentrations of PDGF-BB for 60 min at 37°C, was similar for the mutant and wild-type receptors (data not shown).

The Δki-mutant receptor does not transduce a mitogenic signal. The ability of the Δki-mutant receptor to transduce a mitogenic signal was investigated by using two different types of assays, stimulation of [^{3}H]thymidine incorporation (Fig. 5) and increase of cell number (Fig. 6). In both assays, two different cell lines expressing the Δki-mutant receptor were found not to respond to stimulation by PDGF-BB but responded to stimulation by 10% FCS. A cell line expressing the wild-type receptor did respond to PDGF-BB in the two assays used (Fig. 5 and 6).

The Δki-mutant receptor has a lower kinase activity than the wild-type receptor. Earlier studies have shown that PDGF rapidly induces autophosphorylation of its own receptor (11). We investigated whether the wild-type PDGF β-receptor and Δki-mutant receptor expressed in CHO cells also underwent ligand-stimulated autophosphorylation. A protein of 190 kDa was labeled when membranes prepared from CHO cells transfected with the wild-type β-receptor were incubated with [^{32}P]ATP and PDGF-BB (Fig. 7). When membranes prepared from a cell line expressing the Δki-mutant receptor were subjected to the autophosphorylation assay, a faint band of the expected size (180 kDa) was seen. The incorporation of phosphate into the Δki-mutant receptor was estimated at about 10% of that of the wild-type receptor. The phosphorylation of exogenous substrates was also examined by using this assay; it was found that the Δki-mutant receptor did not phosphorylate phosphofructokinase at all and phosphorylated a peptide substrate at only about 20% of the efficiency of the wild-type receptor (Fig. 7).

Analysis of the autophosphorylation of the wild-type and Δki-mutant receptors in intact cells also revealed that re-

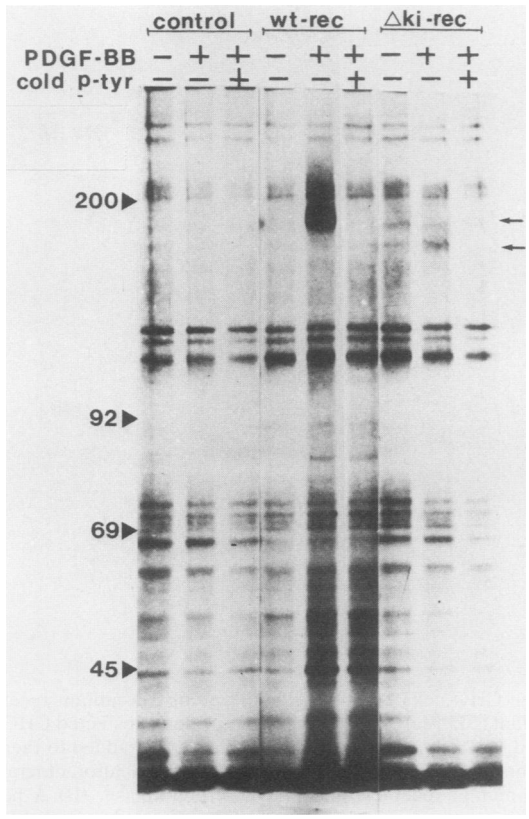

FIG. 8. Protein tyrosine phosphorylation induced by PDGF-BB in untransfected CHO cells and in CHO cells expressing wild-type or Δki-mutant receptors. Untransfected CHO cells (control) and CHO cells expressing the wild-type (wt-rec) or Δki-mutant (Δki-rec) PDGF β-receptor were labeled with [^{35}S]methionine and [^{35}S]cysteine, treated with PDGF-BB, and subjected to immunoprecipitation, using an antiphosphotyrosine serum in the absence (−) or presence (+) of phosphotyrosine (cold p-tyr) to block the specific immunoreaction. Samples were analyzed by SDS-gel electrophoresis and fluorography. Arrows on the right indicate the wild-type and Δki-mutant receptors. The positions of markers of molecular mass (in kilodaltons) are indicated.

moval of the kinase insert decreased the autophosphorylation to about 10% of that of the wild-type receptor (Fig. 8). It is possible that the lower autophosphorylation of the Δki-mutant receptor is due both to a lowered kinase activity and to the loss of autophosphorylation site(s); it was recently reported that one of two major autophosphorylation sites in the PDGF β-receptor is located in the insert region (23).

The Δki-mutant receptor does not mediate actin reorganization. The stimulation of human fibroblasts with PDGF purified from human platelets induces a rapid and transient reorganization of actin filaments and increases the membrane-ruffling activity (28, 29). A similar response was seen in CHO cells expressing the wild-type PDGF β-type receptor; stimulation for 10 min with 10 ng of PDGF-BB per ml induced outgrowth of lamellae and an intense membrane-ruffling activity located predominantly at the edges of the cells (Fig. 9D). In contrast, untransfected CHO cells showed no such response (Fig. 9B). The stimulation of CHO cells expressing the Δki-mutant receptor with PDGF-BB yielded no response (Fig. 9F). However, these cells did respond to the addition of 20% FCS with the formation of lamellae at the edges of the cells, a response similar to that observed after the addition of FCS to untransfected CHO cells (results not shown).

The Δki-mutant receptor mediates expression of c-*fos*. The induction of expression of the nuclear proto-oncogene c-*fos*, which encodes a transcription factor, is seen after the stimulation of fibroblasts with PDGF (15, 24, 30). CHO cells expressing either the Δki-mutant receptor or the wild-type receptor were serum starved and then stimulated for various time periods with 20 ng of PDGF-BB per ml. Both receptors could mediate induction of c-*fos* expression with kinetics similar to those described for induction in fibroblasts (Fig. 10).

DISCUSSION

The aim of the present work was to investigate the function of the insert in the tyrosine kinase domain of the PDGF β-receptor. The functional properties of a mutant receptor lacking the insert were therefore compared with those of the wild-type receptor. We report that certain of the signals associated with the PDGF β-receptor are not seen after ligand stimulation of the mutant receptor, whereas other signals are retained. Most notably, the Δki-mutant receptor could not transduce a mitogenic signal. Of the early events that PDGF stimulates in cells bearing the β-receptor, actin reorganization and membrane ruffling were not stimulated by the mutant receptor. Furthermore, the kinase activity of the mutant receptor was altered; the ligand-stimulated receptor autophosphorylation and the phosphorylation of an exogenous peptide substrate were about 10 and 20%, respectively, of that of the wild-type receptor. In contrast, the mutant receptor did not phosphorylate phosphofructokinase at all. This suggests that the kinase insert may be important for the substrate specificity or catalytic efficiency of the receptor kinase.

The kinase activity of the PDGF β-receptor has been found to be essential for most of its functional properties. Thus, receptors in which the ATP-binding lysine residue has been mutated cannot mediate stimulation of kinase activity, phosphatidylinositol turnover, increase in cytoplasmic Ca^{2+} or pH, mitogenicity (12), chemotaxis, or actin reorganization (B. Westermark, A. Siegbahn, C.-H. Heldin, and L. Claesson-Welsh, Proc. Natl. Acad. Sci. USA, in press). In addition, there is recent evidence suggesting that the effect of PDGF on phosphatidylinositol turnover is mediated via tyrosine phosphorylation. Thus, phospholipase C-γ is phosphorylated on tyrosine residues in PDGF-stimulated cells (27, 41). Furthermore, there are indications that a phosphatidylinositol kinase is associated with the receptor and possibly activated by tyrosine phosphorylation (1, 8). The inability of the Δki-mutant to stimulate actin reorganization and mitogenicity might thus be due to a loss of the ability to phosphorylate key substrates of the receptor kinase that are involved in the regulation of these pathways. Such a selective loss of phosphorylation of certain substrates might be due to the kinase insert forming a domain that is needed for an efficient interaction between these substrates and the receptor. Alternatively, removal of the insert sequence leads to a conformational change in other parts of the receptor molecule that may affect its catalytic properties. Interestingly, in contrast to the results with the PDGF β-receptor, the insert in the kinase domain of the colony-stimulating factor 1 receptor has been found to be dispensable for its kinase activity and mitogenic effect (38).

The Δki-mutant receptor bound and mediated degradation of ligand with efficiency similar to that of the wild-type

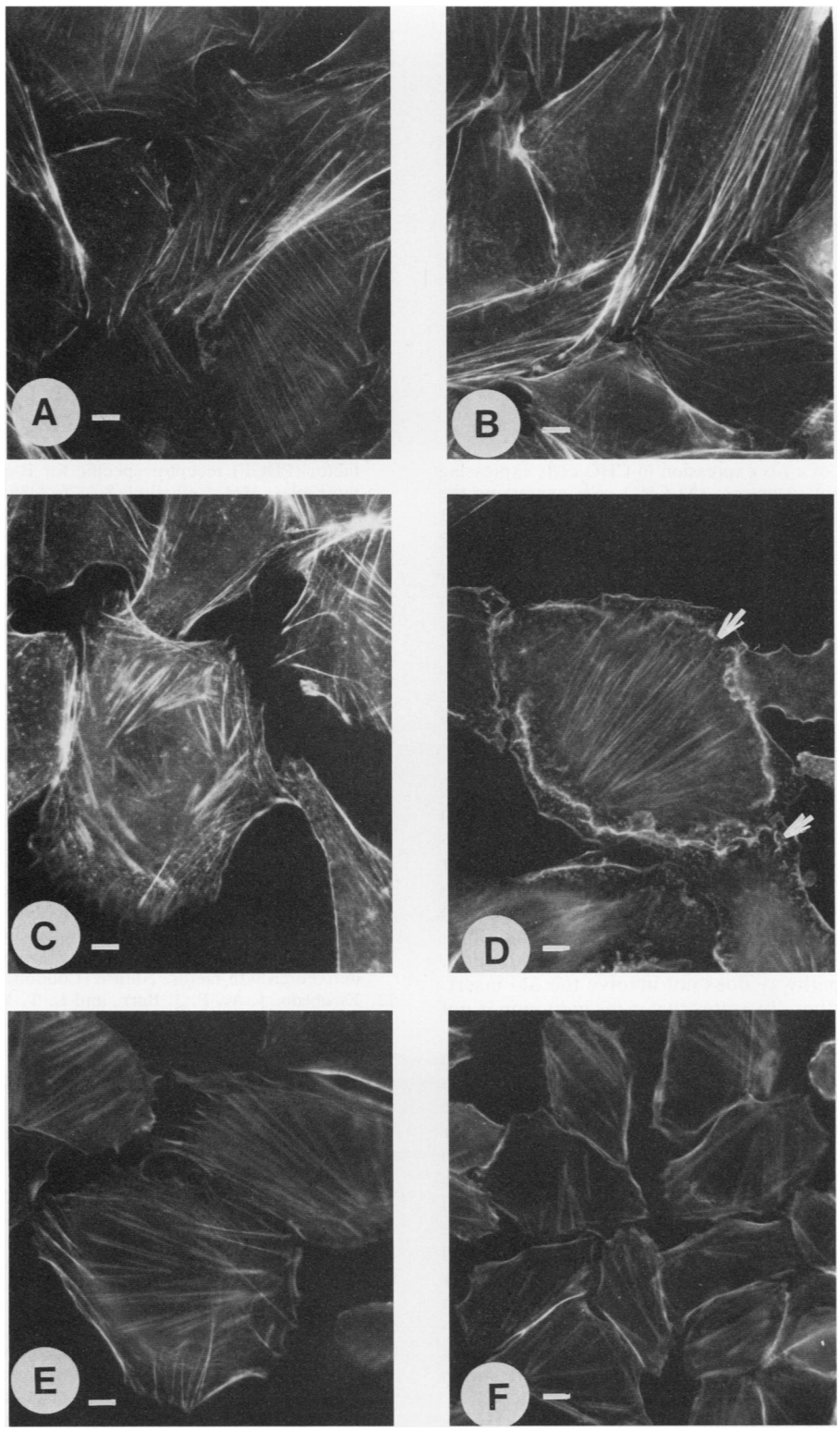

FIG. 9. Effect of PDGF-BB on actin filament organization in untransfected CHO cells and in CHO cells expressing wild-type or Δki-mutant receptors. The organization of actin filaments was visualized before (A, C, and E) and after (B, D, and F) 10 min of exposure to 10 ng of PDGF-BB per ml in untransfected CHO cells (A and B), CHO cells expressing the wild-type PDGF β-receptor (C and D), and CHO cells expressing the Δki-mutant receptor (E and F) by staining with rhodamine-conjugated phalloidin. The arrows in panel D indicate membrane ruffles. The bars indicate 10 μm.

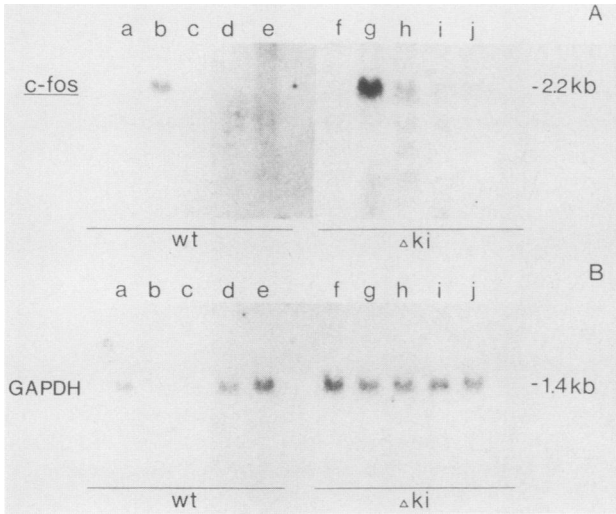

FIG. 10. Induction of c-*fos* expression in CHO cells expressing wild-type or Δki-mutant receptors. (A) CHO cells expressing the wild-type PDGF β-receptor (wt) (lanes a to e) or the Δki-mutant receptor (Δki) (lanes f to j) were serum starved and then exposed to PDGF-BB for 0.5 h (lanes b and g), 1 h (lanes c and h), 4 h (lanes d and i), and 8 h (lanes e and j). A Northern (RNA) blot of 20 μg of RNA for each time point was prepared from unstimulated (lanes a and f) and stimulated (lanes b to e and g to j) cells. The blot was hybridized with a c-*fos* cDNA probe corresponding to a 2.2-kilobase (kb) transcript. (B) To assess the amount of RNA loaded in each lane, hybridization was also performed with a glyceraldehyde-3-phosphatedehydrogenase (GAPDH) cDNA probe corresponding to a 1.4-kilobase (kb) transcript.

receptor. Likewise, ligand binding induced down regulation of mutant and wild-type receptors with equal efficiency. This indicates that the kinase insert is without function for receptor internalization and trafficking. In contrast, a receptor mutant that lacked almost the entire intracellular domain was found to escape degradation after ligand binding (36).

The Δki-mutant receptor also mediated c-*fos* induction, indicating that this pathway does not involve the Δki insert. This observation further illustrates that c-*fos* induction is not sufficient for initiation of DNA synthesis.

Escobedo and Williams (14) recently reported that a mouse PDGF β-receptor mutant lacking 82 amino acids (numbered 716 to 797 according to reference 5) of the kinase insert transduced the early signals of the wild-type receptor but did not induce a mitogenic signal. Our data on a human receptor mutant that lacks the entire 98-amino-acid insert are largely consistent with and extend the data of Escobedo and Williams. A major difference, however, is that whereas the Δki-mutant receptor of Escobedo and Williams retained full kinase activity, our Δki-mutant receptor had a considerably decreased kinase activity measured as autophosphorylation and phosphorylation of at least certain exogenous substrates. The reason for the difference is not known but may be related to differences in the species of the receptors and in the number and location of the amino acids deleted.

Our data are consistent with the possibility that the kinase insert has a role for the substrate specificity or catalytic properties of the PDGF β-receptor kinase. A careful analysis of the substrate specificities of the Δki-mutant and wild-type receptors might help to identify substrates in intact cells that are involved in the pathways leading to initiation of DNA synthesis and actin reorganization.

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LITERATURE CITED

1. **Auger, K. R., L. A. Serunian, S. P. Soltoff, P. Libby, and L. C. Cantley.** 1989. PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. Cell **57**:167–175.
2. **Blobel, G., and B. Dobberstein.** 1975. Transfer of proteins across membranes. II. Reconstitution of functional rough microsomes from heterologous components. J. Cell Biol. **67**:837–851.
3. **Bolton, A. E., and W. M. Hunter.** 1973. The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. Biochem. J. **133**:529–539.
4. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
5. **Claesson-Welsh, L., A. Eriksson, A. Morén, L. Severinsson, B. Ek, A. Östman, C. Betsholtz, and C.-H. Heldin.** 1988. cDNA cloning and expression of a human platelet-derived growth factor (PDGF) receptor specific for B-chain-containing PDGF molecules. Mol. Cell. Biol. **8**:3476–3486.
6. **Claesson-Welsh, L., A. Eriksson, B. Westermark, and C.-H. Heldin.** 1989. cDNA cloning and expression of the human A-type PDGF receptor establishes structural similarity to the B-type receptor. Proc. Natl. Acad. Sci. USA **86**:4917–4921.
7. **Claesson-Welsh, L., A. Hammacher, B. Westermark, C.-H. Heldin, and M. Nistér.** 1989. Identification and structural analysis of the A-type receptor for PDGF: similarities with B-type receptor. J. Biol. Chem. **264**:1742–1747.
8. **Coughlin, S. R., J. A. Escobedo, and L. T. Williams.** 1989. Role of phosphatidylinositol kinase in PDGF receptor signal transduction. Science **243**:1191–1194.
9. **Coussens, L., C. Van Beveren, D. Smith, E. Chen, R. L. Mitchell, C. M. Isacke, I. M. Verma, and A. Ullrich.** 1986. Structural alteration of viral homologue of receptor protooncogene fms at carboxyl terminus. Nature (London) **320**:277–280.
10. **Ek, B., and C.-H. Heldin.** 1984. Use of an antiserum against phosphotyrosine for the identification of phosphorylated components in human fibroblasts stimulated by platelet-derived growth factor. J. Biol. Chem. **259**:11145–11152.
11. **Ek, B., B. Westermark, Å. Wasteson, and C.-H. Heldin.** 1982. Stimulation of tyrosine-specific phosphorylation by platelet-derived growth factor. Nature (London) **295**:419–420.
12. **Escobedo, J. A., P. J. Barr, and L. T. Williams.** 1988. Role of tyrosine kinase and membrane-spanning domains in signal transduction by the platelet-derived growth factor receptor. Mol. Cell. Biol. **8**:5126–5131.
13. **Escobedo, J. A., M. T. Keating, H. E. Ives, and L. T. Williams.** 1988. Platelet-derived growth factor receptors expressed by cDNA transfection couple to a diverse group of cellular responses associated with cell proliferation. J. Biol. Chem. **263**: 1482–1487.
14. **Escobedo, J. A., and L. T. Williams.** 1988. A PDGF receptor domain essential for mitogenesis but not for many other responses to PDGF. Nature (London) **335**:85–87.
15. **Greenberg, M. E., and E. B. Ziff.** 1984. Stimulation of 3T3 cells induces transcription of the c-*fos* proto-oncogene. Nature (London) **311**:433–438.
16. **Gronwald, R. G. K., F. J. Grant, B. A. Haldeman, C. E. Hart, P. J. O'Hara, F. S. Hagen, R. Ross, D. F. Bowen-Pope, and M. J. Murray.** 1988. Cloning and expression of a cDNA coding for the human platelet-derived growth factor receptor: evidence for more than one receptor class. Proc. Natl. Acad. Sci. USA **85**:3435–3439.
17. **Hammacher, A., U. Hellman, A. Johnsson, K. Gunnarsson, A. Östman, B. Westermark, Å. Wasteson, and C.-H. Heldin.** 1988. A major part of PDGF purified from human platelets is a heterodimer of one A chain and one B chain. J. Biol. Chem. **263**:16493–16498.
18. **Hanks, S. K., A. M. Quinn, and T. Hunter.** 1988. The protein

kinase family: conserved features and deduced phylogeny of the catalytic domains. Science **241**:42–52.

19. **Hart, C. E., J. W. Forstrom, J. D. Kelly, R. A. Smith, R. Ross, M. J. Murray, and D. F. Bowen-Pope.** 1988. Two classes of PDGF receptor recognize different isoforms of PDGF. Science **240**:1529–1531.
20. **Heldin, C.-H., G. Bäckström, A. Östman, A. Hammacher, L. Rönnstrand, K. Rubin, and B. Westermark.** 1988. Binding of different dimeric forms of PDGF to human fibroblasts: evidence for two separate receptor types. EMBO J. **7**:1387–1393.
21. **Heldin, C.-H., A. Johnsson, S. Wennergren, C. Wernstedt, C. Betsholtz, and B. Westermark.** 1986. A human osteosarcoma cell line secretes a growth factor structurally related to a homodimer of PDGF A chains. Nature (London) **319**:511–514.
22. **Heldin, C.-H., and B. Westermark.** 1989. Platelet-derived growth factor: three isoforms and two receptor types. Trends Genet. **5**:108–111.
23. **Kazlauskas, A., and J. A. Cooper.** 1989. Autophosphorylation of the PDGF receptor in the kinase insert region regulates interactions with cell proteins. Cell **58**:1121–1133.
24. **Kruijer, W., J. A. Cooper, T. Hunter, and I. Verma.** 1984. Platelet-derived growth factor induces rapid but transient expression of the c-fos gene and protein. Nature (London) **312**:711–716.
25. **Matsui, T., M. Heideran, T. Miki, N. Popescu, W. La Rochelle, M. Kraus, J. Pierce, and S. A. Aaronson.** 1989. Isolation of a novel receptor cDNA establishes the existence of two PDGF receptor genes. Science **243**:800–804.
26. **McKeehan, W. L., K. A. McKeehan, S. L. Hammond, and R. G. Ham.** 1977. Improved medium for clonal growth of human diploid fibroblasts at low concentrations of serum protein. In Vitro (Rockville) **13**:399–416.
27. **Meisenhelder, J., P.-G. Suh, S. G. Rhee, and T. Hunter.** 1989. Phospholipase C-γ is a substrate for the PDGF and EGF receptor protein-tyrosine kinases in vivo and in vitro. Cell **57**:1109–1122.
28. **Mellström, K., C.-H. Heldin, and B. Westermark.** 1988. Induction of circular membrane ruffling on human fibroblasts by platelet-derived growth factor. Exp. Cell Res. **177**:347–359.
29. **Mellström, K., A.-S. Höglund, M. Nistér, C.-H. Heldin, B. Westermark, and U. Lindberg.** 1983. The effect of platelet-derived growth factor on morphology and motility of human glial cells. J. Muscle Res. Cell Motil. **4**:589–609.
30. **Müller, R., R. Bravo, J. Bruckhart, and T. Curran.** 1984. Induction of c-fos gene and protein by growth factors precedes activation of c-myc. Nature (London) **312**:716–720.
31. **Nistér, M., A. Hammacher, K. Mellström, A. Siegbahn, L. Rönnstrand, B. Westermark, and C.-H. Heldin.** 1988. A glioma derived A chain homodimer has different functional activities than a PDGF AB heterodimer purified from human platelets. Cell **52**:791–799.
32. **Östman, A., G. Bäckström, N. Fong, C. Betsholtz, C. Wernstedt, U. Hellman, B. Westermark, P. Valenzuela, and C.-H. Heldin.** 1989. Expression of three recombinant homodimeric isoforms of PDGF in *Saccharomyces cerevisiae*. Evidence for differences in receptor binding and functional activities. Growth Factors **1**: 271–281.
33. **Rönnstrand, L., M. P. Beckmann, B. Faulders, A. Östman, B. Ek, and C.-H. Heldin.** 1987. Purification of the receptor for platelet-derived growth factor from porcine uterus. J. Biol. Chem. **262**:2929–2932.
34. **Ross, R., E. W. Raines, and D. F. Bowen-Pope.** 1986. The biology of platelet-derived growth factor. Cell **46**:155–169.
35. **Scatchard, G.** 1949. The attraction of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. **51**:660–672.
36. **Severinsson, L., L. Claesson-Welsh, and C.-H. Heldin.** 1989. A PDGF B type receptor lacking most of the intracellular domain escapes degradation after ligand binding. Eur. J. Biochem. **182**:679–686.
37. **Stroobant, P., and M. D. Waterfield.** 1984. Purification and properties of porcine platelet-derived growth factor. EMBO J. **3**:2963–2967.
38. **Taylor, G. R., M. Reedijk, V. Rothwell, L. Rohrschneider, and T. Pawson.** 1989. The unique insert of cellular and viral *fms* protein tyrosine kinase domains is dispensable for enzymatic and transforming activities. EMBO J. **8**:2029–2037.
39. **Truett, M. A., R. Blacher, R. L. Burke, D. Caput, C. Chu, D. Dina, K. Hartog, C. H. Kuo, F. R. Masiarz, J. P. Merryweather, R. Najarian, C. Pachl, S. J. Potter, J. Puma, M. Quiroga, L. B. Rall, A. Randolph, M. S. Ureda, P. Valenzuela, H. H. Dahl, J. Favalaro, J. Hansen, O. Nordfang, and M. Ezban.** 1985. Characterization of the polypeptide composition of human factor VIII:C and the nucleotide sequence and expression of the human kidney cDNA. DNA **4**:333–349.
40. **Tso, J. Y., X. H. Sun, T. Kao, K. S. Reece, and R. Wu.** 1985. Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs; genomic complexity and molecular evolution of the gene. Nucleic Acids Res. **13**:2485–2502.
41. **Wahl, M. I., N. E. Olashaw, S. Nishibe, S. G. Rhee, W. J. Pledger, and G. Carpenter.** 1989. Platelet-derived growth factor induces rapid and sustained tyrosine phosphorylation of phospholipase C-γ in quiescent BALB/c 3T3 cells. Mol. Cell. Biol. **9**:2934–2943.
42. **Wigler, M., R. Sweet, G. K. Sim, B. Wold, A. Pellicier, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel.** 1979. Transformation of mammalian cells with genes from procaryotes and eucaryotes. Cell **16**:777–785.
43. **Yarden, Y., J. A. Escobedo, W.-J. Kuang, T. L. Yang-Feng, T. O. Daniel, P. M. Tremble, E. Y. Chen, M. E. Ando, R. N. Harkins, U. Francke, V. A. Friend, A. Ullrich, and L. T. Williams.** 1986. Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factor receptors. Nature (London) **323**:226–232.
44. **Yarden, Y., W.-J. Kuang, T. Yang-Feng, L. Coussens, S. Munemitsu, T. J. Dull, E. Chen, J. Schlessinger, U. Francke, and A. Ullrich.** 1987. Human proto-oncogene c-kit: a new cell surface receptor tyrosine kinase for an unidentified ligand. EMBO J. **6**:3341–3351.