

B-type receptor for platelet-derived growth factor mediates a chemotactic response by means of ligand-induced activation of the receptor protein-tyrosine kinase

(endothelial cells/cell motility/platelet-derived growth factor)

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ABSTRACT Porcine aorta endothelial cells are devoid of receptors for platelet-derived growth factor (PDGF). We have transfected such cells with cDNA for the PDGF B-type receptor, both the wild-type receptor and a mutant form of the receptor (K634A), in which the putative nucleotide-binding lysine of the protein-tyrosine domain has been changed to alanine. Immunoprecipitation studies of metabolically labeled cells showed that both types of receptors were synthesized and processed to the mature form of M_r 190,000. In cells expressing the wild-type receptor, PDGF-BB, the natural ligand for the B-type receptor, induced membrane ruffling and reorganization of actin. Such a response has previously been seen in cells expressing the natural PDGF B-type receptor in response to PDGF-BB. No such effect was induced in nontransfected cells or in cells expressing the K634A mutant receptor. PDGF was also shown to be chemotactic for cells expressing the wild-type receptor, whereas no chemotactic response was elicited in control cells or in cells expressing the K634A mutant receptor. Our study thus provides formal evidence that the PDGF B-type receptor mediates a motility response including actin reorganization and chemotaxis. Furthermore, the results establish a role for the receptor-associated protein-tyrosine kinase in the transduction of the chemotactic signal.

Chemotaxis is the directed locomotion of cells along a chemical gradient. In eukaryotes, chemoattractants are generally believed to have an important function in mediating cell trafficking in development, inflammation, and tissue repair. In mammalian species, chemotaxis of neutrophil granulocytes has been particularly well studied (for reviews, see refs. 1–3), and a number of chemoattractants have been identified, the prototype substance being fMet-Leu-Phe (4). Chemotaxis of fibroblasts and vascular smooth muscle cells has been found to be induced by a variety of functionally different substances. Thus, fibronectin, (5) collagen peptides (6), and growth factors, such as platelet-derived growth factor (PDGF) (7–9), have been found to be chemoattractants.

Detailed studies on the molecular mechanism of chemotaxis in mammalian cells have been hampered by difficulties in the identification, characterization, and molecular cloning of receptors for chemoattractants. The finding that both recombinant PDGF (A.S., A. Hammacher, B.W., and C.-H.H., unpublished data) and PDGF purified from platelet lysates (7–9) are chemotactic at physiological concentrations, suggests that a chemotactic response is mediated by PDGF receptors.

PDGF is made up as dimers of covalently bound A and B polypeptide chains (10) and exists as three isoforms, PDGF-AA, PDGF-AB, and PDGF-BB (11–13). Recent studies have

shown the presence of two types of PDGF receptors that display differences in ligand-binding specificity (14, 15). The A-type receptor binds all three isoforms with high affinity, whereas the B-type receptor binds PDGF-BB with high affinity, PDGF-AB with lower affinity, and does not bind PDGF-AA. The B-type receptor is identical to the previously identified PDGF receptor that was found to possess a ligand-stimulatable protein-tyrosine kinase activity (16). cDNAs for the A-type (17, 18) and B-type (19–21) PDGF receptors have been cloned; their nucleotide sequences predict proteins with similar overall structural organization. Each receptor has an extracellular part that is composed of five immunoglobulin-like domains, a single transmembrane segment, and an intracellular protein-tyrosine kinase domain.

We have previously shown that a motility response in fibroblasts—i.e., actin reorganization and chemotaxis—is induced by PDGF-AB and PDGF-BB, but not by PDGF-AA (22–24) and is, therefore, probably mediated by the B-type receptor in these cells. In the present investigation we have expressed human recombinant B-type receptors in porcine aortic endothelial (PAE) cells that lack endogenous receptors. Addition of PDGF-BB to such cells was found to induce actin reorganization and chemotaxis. No such response was elicited in control cells or in cells expressing a recombinant receptor, made nonfunctional with regard to its kinase activity by a point mutation in the ATP-binding site. Our results thus provide formal evidence that the PDGF B-type receptor mediates a motility response, including chemotaxis, and that this effect is functionally linked to the protein-tyrosine kinase activity of the receptor.

MATERIALS AND METHODS

Tissue Culture and Transfection. The PAE cell line (25) was cultured in F-12 medium supplemented with 10% fetal calf serum (GIBCO) and 100 units of penicillin per ml. The cDNA clone for the human PDGF B-type receptor (21) was cloned into the expression vector pZipNeo (26). Transfection was performed through electroporation, by using an apparatus built by Nordelectronic (Uppsala), and following a described procedure (21). Selection of transfected cells was initiated after 48 hr by adding Geneticin (G418 sulfate; GIBCO/Bethesda Research Laboratories) at 0.5 mg/ml to the culture medium.

PDGF. Recombinant PDGF-BB was purified to apparent homogeneity from supernatants of yeast cells transfected with a PDGF B-chain DNA construct (27).

Radioactive Labeling and Immunoprecipitation. Transfected and control (untransfected) PAE cells were labeled in serum-free MCDB 104 medium, devoid of unlabeled methio-

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Abbreviations: PDGF, platelet-derived growth factor; PAE, porcine aortic endothelial.

nine and cysteine, and supplemented with [³⁵S]methionine and [³⁵S]cysteine, each at 100 μ Ci/ml (1 Ci = 37 GBq; Amersham) for 3 hr at 37°C. Labeled cells were lysed and prepared for immunoprecipitation by enrichment of the glycoprotein fraction by affinity chromatography on *Lens culinaris* lectin-Sepharose 4B columns, as described (28).

PDGF-BB was added at the end of the 3-hr labeling period, to a final concentration of 100 ng/ml, together with bovine serum albumin (Sigma) at a final concentration of 1 mg/ml, when cells were to be examined for PDGF-induced phosphorylation (see Fig. 1). After 5-min incubation in the presence of PDGF, cells were washed with ice-cold phosphate-buffered saline, and immediately processed for immunoprecipitation. In this case the lysis buffer was supplemented with 25 μ M Na₃VO₄.

Antibodies. The rabbit antiserum PDGFR-3 was raised against a synthetic peptide corresponding to amino acids 981–994 of the murine PDGF B-type receptor sequence (19). This antiserum reacts specifically with the PDGF B-type receptor (29). The phosphotyrosine antiserum used in this study has been described (30).

Electrophoresis. SDS/PAGE was run on 5–10% gradient slab gels according to Blobel and Dobberstein (31), with chemicals from Bio-Rad. Gels were prepared for fluorography by soaking in Amplify (Amersham), dried, and exposed to Hyperfilm MP (Amersham).

Fluorescence Microscopy of PDGF-Induced Actin Reorganization. Cells were seeded in routine medium on 24 \times 24-mm glass coverslips contained in 35-mm Petri dishes with an inoculum of $\approx 1 \times 10^4$ cells per dish. After 3-day incubation, medium was changed to F-12/1% fetal calf serum. Incubation with PDGF and staining with rhodamine-conjugated phalloidin were performed 1 day later, as described (22). Briefly, cells were incubated for 30 min with 20 ng of PDGF-BB per ml at 4°C and then for 15 min at 37°C. The cells were fixed in 3% paraformaldehyde in phosphate-buffered saline for 20 min at room temperature followed by 1-min fixation in -20°C acetone. Rhodamine-conjugated phalloidin (50 μ g per ml in phosphate-buffered saline, provided by Th. Wieland, Max-Planck-Institut für Medizinische Forschung, Heidelberg) was added. After 20-min incubation at room temperature, the coverslips were washed and mounted in phosphate-buffered saline/glycerol (1:1) and viewed in a Leitz microscope, equipped for epifluorescence. Photographs were taken on Kodak Tri-X film.

Assay of Chemotaxis. The chemotactic response of endothelial cells was assayed by the leading-front technique with

the use of a modified Boyden chamber (32, 33). Micropore filters (8- μ m-pore size) were soaked in a solution of type-I collagen (100 μ g/ml, provided by K. Rubin, University of Uppsala) at room temperature overnight. The filters were air dried for 30 min immediately before they were used. Cells were detached by trypsinization, washed in Hanks' balanced salt solution and suspended in Eagle's minimum essential medium, supplemented with 10% newborn calf serum (GIBCO) to a final concentration of 2×10^5 cells per ml. The chemotaxis assay was performed as described for fibroblasts (24). Experiments were performed with two filters for each concentration of PDGF-BB. For each set of experiments, the migration distance of cells incubated in the assay medium and with the same medium below the filter served as controls and is referred to as 100% migration.

RESULTS

Transfection of PAE Cells with the PDGF B-Type Receptor cDNA. PAE cells were initially analyzed with regard to their endogenous expression of the A- and B-type receptors as well as the A and B chains of PDGF. Northern blot analyses using ³²P-labeled probes corresponding to the extracellular domains of the PDGF receptors (18, 21) were negative (data not shown). The lack of endogenous expression of the PDGF B-type receptor was also confirmed on the protein level (see below).

The full-length PDGF B-type receptor cDNA was inserted into the murine leukemia virus-based expression vector pZip-Neo (26). Transfection of the DNA was performed using electroporation, and neomycin-resistant colonies were screened for PDGF B-type receptor expression by using an ¹²⁵I-labeled PDGF-BB binding assay (21). A mutated B-type receptor cDNA, in which the assumed nucleotide-binding lysine (Lys-634 according to ref. 21) had been changed to alanine by site-directed mutagenesis (K634A mutant; B. Ek and S. Wennergren, personal communication), was also inserted into pZipNeo and transfected into PAE cells. A closer examination of the mode of PDGF B-type receptor expression was performed on randomly chosen wild type or K634A mutant PAE cell clones. Representative results are shown in Fig. 1.

From metabolically labeled cells, expressing wild type or K634A mutant receptor, two components of 160,000 and 190,000 could be immunoprecipitated using a PDGF B-type receptor specific antiserum (Fig. 1, lanes a, e, i, and m). These components were not found in untransfected PAE cells

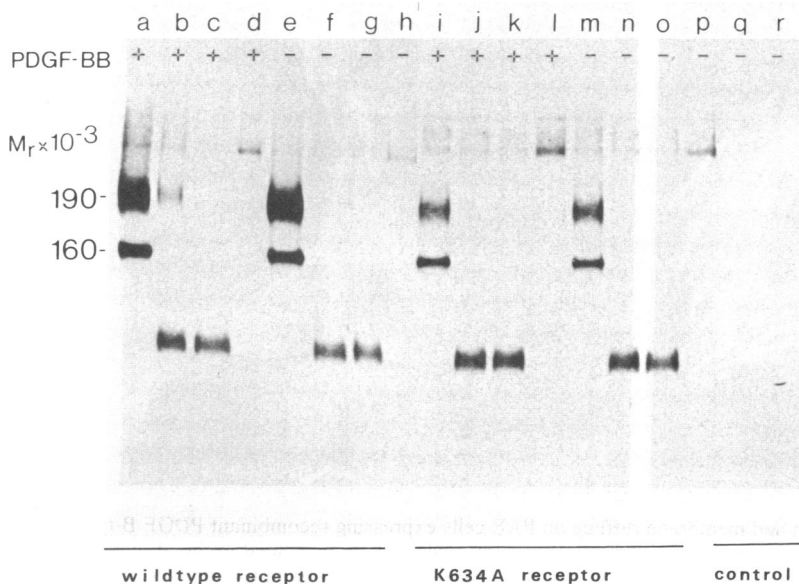


FIG. 1. Immunoprecipitation of PDGF B-type receptors from transfected PAE cells. Cells transfected with the wild-type receptor (lanes a–h), the K634A mutant receptor (lanes i–p), or untransfected PAE cells (lanes q–r) were metabolically labeled with [³⁵S]methionine and [³⁵S]cysteine for 3 hr and then treated with (+) or without (-) PDGF-BB at 100 ng/ml for 5 min. Immunoprecipitations were done by using a PDGF B-type receptor-specific antiserum, PDGFR-3 (lanes a, e, i, m, and q), a phosphotyrosine antiserum (lanes b, f, j, and n), phosphotyrosine antiserum preincubated with 25 μ g of phosphotyrosine per sample (lanes c, g, k, and o), or nonimmune serum (lanes d, h, l, p, and r). Molecular weights of the PDGF B-type receptor precursor (160,000) and mature (190,000) forms are indicated, as determined from marker proteins run in parallel (data not shown).

(Fig. 1, lane q). Pulse chase analyses indicated that the M_r 160,000 band represented the B-type receptor precursor, which was converted to the M_r 190,000 mature form with the same kinetics in cells expressing the wild type and the K634A mutant receptor (data not shown). A similar mode of biosynthesis has been described for the endogenous B-type receptor in human foreskin fibroblasts (29). That the PAE cells transfected with the wild-type receptor cDNA expressed a molecule carrying a stimutable protein-tyrosine

kinase domain was shown by immunoprecipitation using a phosphotyrosine antiserum, of a M_r 190,000 component after exposure of [35 S]methionine- and [35 S]cysteine-labeled cells to PDGF-BB at 100 ng/ml for 5 min (Fig. 1, lane b). The M_r 190,000 component, which most likely represents the autophosphorylated B-type receptor, was not seen in the absence of PDGF-BB (Fig. 1, lane f). Cells expressing the K634A mutant receptor were unable to mediate ligand-induced receptor autophosphorylation (Fig. 1, lanes j and n). The lack

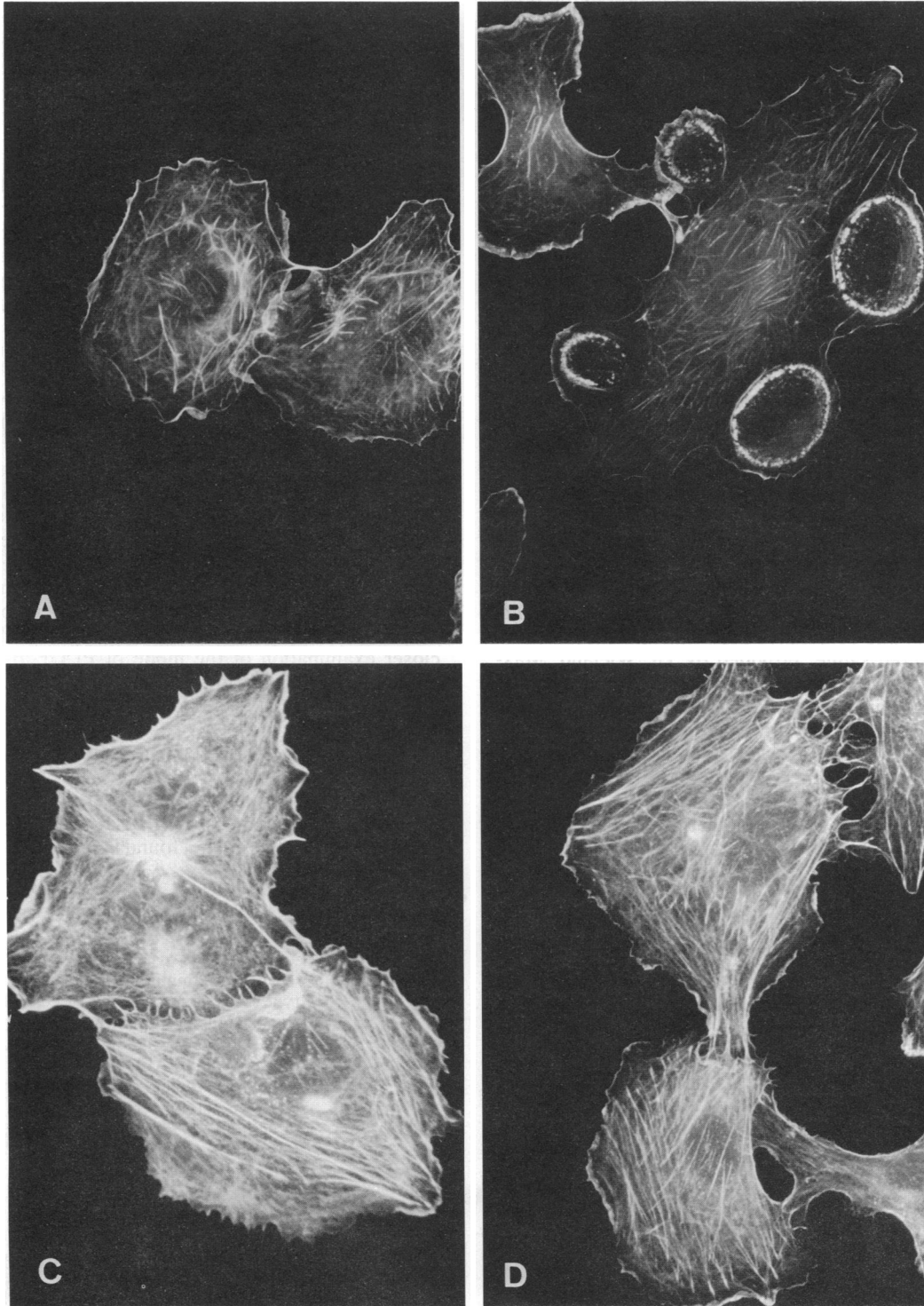


FIG. 2. Effect of PDGF-BB on actin reorganization and membrane ruffling on PAE cells expressing recombinant PDGF B-type receptors. PAE cells expressing the wild-type receptor (A and B) or the K634A mutant receptor (C and D) were incubated in the absence (A and C) or presence (B and D) of 20 ng of PDGF-BB per ml. Actin filaments were visualized by staining with rhodamine-conjugated phalloidin, as described.

of response to PDGF-BB in the K634A mutant receptor-expressing cells was not due to defects in PDGF-BB binding because Scatchard analyses showed a similar number of high-affinity (K_d 0.3–0.5 nM) PDGF-BB binding sites ($3\text{--}5 \times 10^4$ binding sites per cell) on the K634A mutant as on the wild-type receptor expressing PAE cells (A. Sorkin, L.C.-W., C.-H.H., and B.W., unpublished data).

Effect of PDGF-BB on Actin Reorganization and Membrane Ruffling. The effect of PDGF-BB (10 ng/ml) on actin reorganization and membrane ruffling in transfected PAE cells is shown in Fig. 2. In cells expressing the wild-type PDGF B-type receptor, PDGF-BB elicited a response, similar to that induced in human fibroblasts (22, 23). Thus, a large proportion ($\approx 60\%$) exhibited circular arrangements of filamentous actin, as visualized by staining with rhodamine-conjugated phalloidin. These structures corresponded to membrane ruffles that were visible in the phase-contrast microscope (data not shown). PAE cells expressing the K634A receptor, devoid of protein-tyrosine kinase activity, were completely unresponsive to PDGF-BB (Fig. 2), as were untransfected PAE cells and cells transfected only with vector (pZipNeo) DNA and isolated by G418 selection (data not shown). We, therefore, conclude that PDGF-induced actin reorganization can be mediated by the PDGF B-type receptor and that the receptor protein-tyrosine kinase is essential for the effect.

Chemotaxis. We next analyzed the effect of PDGF on chemotaxis of PAE cells expressing the recombinant PDGF B-type receptor. Fig. 3 shows that PDGF-BB induced a strong, dose-dependent chemotactic response in cells expressing the wild-type receptor. The dose-dependency of the chemotactic response was similar to that recorded in human foreskin fibroblasts with an initial response occurring at 1 ng/ml and a maximal response at 10–50 ng/ml. No chemotactic response was elicited in control cells transfected with vector DNA only or in cells expressing the mutant receptor lacking protein-tyrosine kinase activity.

DISCUSSION

The results presented in this communication provide formal evidence that the PDGF B-type receptor mediates a chemo-

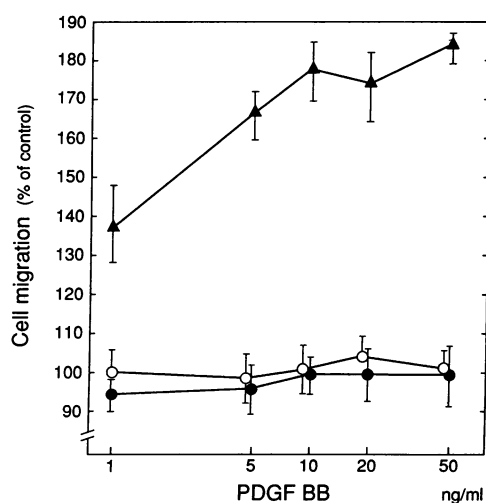


FIG. 3. Chemotactic response of PAE cells to PDGF-BB. A significant ($P < 0.01$; Student's t test) chemotactic response to PDGF-BB at 1 ng/ml was achieved with PAE cells expressing the wild-type receptor (▲). PDGF-BB at 10–50 ng/ml gave a maximal response ($P < 0.001$). PDGF-BB induced no chemotaxis in PAE cells expressing the K634A mutant receptor (○) or in PAE cells transfected with vector DNA (●). Data points represent the mean \pm SEM of three experiments.

tactic response. PAE cells that have no endogenous PDGF receptor expression were unresponsive to the chemotactic activity of PDGF-BB. When expressing a moderate level of a human recombinant PDGF B-type receptor, however, a strong chemotactic response to PDGF-BB was recorded. The magnitude of this response equaled that of human fibroblasts, tested in the same assay system (22–24).

The motility response of the PAE cells expressing the recombinant B-type receptor included a rapid reorganization of actin and the formation of circular ruffles. A similar response is also elicited in fibroblasts that are natural target cells for PDGF (22–24). Such ruffles are only induced by PDGF-AB and PDGF-BB and are not induced by PDGF-AA (23); the effect is therefore likely to be mediated by the B-type receptor. Because phalloidin binds preferentially to filamentous actin (34), we can conclude that the circular ruffles are formed as a result of a focal polymerization of actin. Detailed electron microscopy studies have, indeed, shown that PDGF-induced ruffles contain a network of actin filaments (35).

Cell locomotion seems to result from a coordinated polymerization/depolymerization of actin along the long axis of a polarized cell (36). Thus, in pioneering studies, Abercrombie *et al.* observed that ruffles occur particularly at the front end of moving fibroblasts (37), and it is generally believed that ruffles are a sign of active actin polymerization required for the advancement of the cell margin over the substratum. Chemoattractants are known to induce changes in cell shape, including ruffle formation (1). We consider it likely that the chemotactic response and the formation of cell-surface ruffles as mediated by the PDGF B-type receptor have a common denominator in the sense that both responses are generated by *de novo* actin polymerization; although a global response (visible as dorsal ruffles) is induced when PDGF is added diffusely, a concentration gradient of the ligand may stimulate actin polymerization preferentially where the concentration is the highest and thereby change cell polarity and induce a directional motility response. This model adheres to that proposed for other chemoattractants (1).

Previously identified receptors for chemoattractants in eukaryotic cells include a cell-surface cAMP-binding protein in *Dictyostelium* (38) and a receptor for fMet-Leu-Phe in neutrophils (39). The cAMP receptor in *Dictyostelium* has been molecularly cloned and found to contain seven putative membrane-spanning domains, a typical feature of receptors that interact with G proteins. There is also circumstantial evidence that the fibronectin receptor mediates a chemotactic response in 3T3 cells (40). Thus, the chemotactic response to fibronectin can be blocked by the Arg-Gly-Asp (RGD) group (40) that blocks the binding of fibronectin to its receptor (41). The fibronectin receptor, a member of the integrin family (41, 42), is made up as a noncovalently linked dimer of α and β subunits and is believed to link the cytoskeleton and fibronectin, thereby enabling a dynamic interaction between the motility apparatus and the extracellular matrix. Thus, receptors for chemoattractants appear to form a rather heterogeneous family of molecules with principal differences in their structural and functional properties.

The present study proves a role for a protein-tyrosine kinase-dependent event in the transduction of a chemotactic signal: a PDGF B-type receptor with its protein-tyrosine kinase inactivated by a point mutation in the putative nucleotide-binding site, was completely nonfunctional in transducing the chemotactic signal. As cells expressing the K634A mutant receptor showed no signs of actin rearrangement upon PDGF-BB addition, we conclude that PDGF-dependent actin polymerization is brought about by a mechanism that involves tyrosine-specific phosphorylation(s).

Several lines of evidence suggest that phosphatidylinositol derivatives mediate a chemotactic response in neutrophils (for reviews, see refs. 1–3). The consensus idea is that the

activated receptor couples to a G protein, whereby phospholipase C is activated. Hydrolysis of phosphatidylinositol 4,5-bisphosphate generates two putative second messengers—namely, diacylglycerol, which activates protein kinase C (43), and inositol 1,4,5-trisphosphate, which releases calcium from intracellular stores (44). A similar mechanism may explain the chemotactic response mediated by the ligand-activated PDGF B-type receptor. Thus, PDGF induces phosphatidylinositol 4,5-bisphosphate turnover in responsive cells (45, 46), and this event requires an intact protein-tyrosine kinase activity of the B-type receptor (47). However, our findings that actin reorganization is not induced by an alternative activator of protein kinase C, or calcium ionophore, or combination of both (22) argue against the possibility that breakdown products of phosphatidylinositol 4,5-bisphosphate mediate the effect of PDGF on both chemotaxis and reorganization of actin.

The present finding that the expression of a recombinant PDGF B-type receptor confers a motility response to PDGF-BB on endothelial cells makes possible further detailed analyses of signal-transduction mechanisms in the stimulation of actin rearrangement and chemotaxis. Thus, structure-function relationships can be delineated by the use of receptor mutants. One example is the K634A mutant receptor, which has already allowed us to functionally link the motility response to the protein-tyrosine kinase activity of the PDGF B-type receptor.

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