The complementary DNAs for wildtype and tyrosine kinase-inactivated (K634A) forms of the PDGF β-receptor were expressed in porcine aortic endothelial cells. We examined the internalization and degradation of ligands and receptors after exposure of receptor expressing cells to PDGF-BB, which binds to the β-receptor with high affinity, and PDGF-AB, which binds with lower affinity. Cells expressing wildtype β-receptors were able to internalize and degrade the receptor, as well as the ligand, after exposure to PDGF-BB or -AB. Cells expressing the kinase-inactivated mutant receptor also internalized and degraded both receptor and ligand, but with lower efficiency compared with the wildtype receptor cells. The degradation of either form of receptor was inhibited by treatment of the cells with the lysosomotropic drug chloroquine. Exposure of wildtype and K634A receptor expressing cells to PDGF-AB resulted in a twofold slower rate of internalization of this ligand as compared with PDGF-BB, whereas the relative rate of degradation was similar for the two ligands. Our data indicate that tyrosine kinase activity promotes, but is not a prerequisite for, ligand-induced internalization and degradation of the ligand-receptor complex.

A major part of the mitogenic activity in human serum for mesenchymally derived cells, is contributed by PDGF (for reviews see references 20, 36). PDGF consists of disulphide-bonded dimers of homologous A and B polypeptide chains, which combine to create the three possible isoforms PDGF-AA, -AB, and -BB (1, 25, 26). Two PDGF receptor types, which interact with the three PDGF isoforms with different affinities, have been identified (18, 19). The PDGF β-receptor binds PDGF-BB with high affinity and PDGF-AB with lower affinity, whereas the PDGF α-receptor binds all three ligands with high affinity. cDNAs for both the α-receptor and the β-receptor have been isolated (7, 9, 15, 28, 43). The receptors belong to the family of growth factor receptors equipped with a ligand-stimulatable tyrosine kinase activity, and they are structurally very similar. Their extracellular parts contain five immunoglobulin-like domains and their tyrosine kinase domains are split into two segments by the insertion of ~100 amino acids. Binding of ligand has been shown to induce receptor dimerization, creating homo- as well as heterodimers of PDGF receptors, most likely through the bivalent interaction of one ligand with two receptor molecules (2, 17, 21, 38).

Binding of PDGF leads to a rapid internalization of both ligand and receptor (20, 36). Studies based on morphological examinations have indicated that at least the ligand is transferred to the lysosomes and degraded after internalization (30, 35). However, in other studies, no inhibition of PDGF receptor degradation was recorded after treatment of cells with lysosomotropic drugs (23, 27). Studies of the EGF receptor have shown that it is rapidly internalized and degraded in the lysosomes after ligand binding (5, 40); the effect of inactivation of the nucleotide-binding lysine residue in the EGF receptor has been extensively studied (13, 14, 22).

In the present study, we have examined the effects of inactivation of the tyrosine kinase of the PDGF β-receptor, on the internalization and degradation of ligand and receptor. Cells expressing the kinase negative PDGF β-receptor mutant, which was created by substituting the ATP-binding lysine residue in the kinase domain for an alanine residue, were found to mediate internalization and degradation of both receptor and ligands, albeit at a slower rate than wildtype receptor-expressing cells.

### Materials and Methods

#### Cells

The porcine aortic endothelial (PAE) cell line (29) was cultured in Ham's F12 medium (Gibco/Bethesda Research Laboratories, Gaithersburg, MD), supplemented with 10% FCS and 100 U of penicillin/ml. These cells lack endogenous expression of the PDGF α- and β-receptors, as assessed by Northern blot analysis, immunoprecipitation from metabolically labeled cells, as well as radio-receptor assay (41; and data not shown). Complement-
ory DNAs encoding the wildtype PDGF β-receptor (7) or a PDGF β-receptor in which the assumed nucleotide-binding lysine residue had been exchanged for an alanine residue (K634A) by site-directed mutagenesis, were cloned into the retroviral expression vector pZipNeo (6) and the constructions were introduced into PAE cells using electroporation, as described (26). Selection of transfected cells was initiated after 48 h by adding Gentamicin (G418 sulfate; Gibco/Bethesda Research Laboratories) at 0.5 mg/ml to the culture medium. G418-resistant colonies were analyzed for binding of 125I-PDGF.

Antibodies

The rabbit polyclonal antibody PDGFR-3 was generated using a synthetic peptide corresponding to the murine PDGF β-receptor amino acids 981-994; this antiserum reacts in a specific manner with the human PDGF β-receptor (8). The mouse monoclonal antibody PDGFR-B2 was established using purified porcine PDGF receptor preparations as antigen. The characteristics of this antibody have been described (34). Rhodamine-labeled swine anti-rabbit Ig was from Dako (Copenhagen, Denmark), rhodamine-labeled goat anti-mouse Ig from Sigma Chemical Co. (St. Louis, MO), fluorescein-labeled goat anti-mouse Ig from Becton Dickinson. The secondary antibodies were checked and found not to cross-react with Ig from the other species (not shown).

125I-PDGF-Binding Experiments

Recombinant PDGF-BB was purified from a Saccharomyces cerevisiae expression system (31). PDGF-AB was purified by immobilized metal ion affinity chromatography from PDGF isolated from human platelets (16). Alternatively, CHO cells expressing recombinant PDGF-AB were used as a source for purification (32). PDGF-BB was labeled with 125I using the Bolton and Hunter procedure (4) to specific activities of 50,000-70,000 cpm/ng, and PDGF-AB was labeled using the chloramine T method (24) to a specific activity of 150,000 cpm/ng. The iodinated ligands were added to cells in MEM supplemented with 20 mM Hepes, pH 7.4, and 0.1% BSA (henceforth demoted MEM-BSA). After incubation, the cell monolayers were washed five times using MEM-BSA. In some experiments, the cells were thereafter lysed directly in 20 mM Hepes, pH 7.5, 1% Triton X-100, 10% glycerol, for 30 min at room temperature, to estimate the total amount of cell-associated 125I-PDGF. To estimate cell surface-bound but not internalized ligand, the cells were treated with 0.2 M acetic acid, 0.5 M NaCl, pH 2.7, for 5 min on ice, followed by a wash in the same buffer. Both washes were combined and analyzed for radioactivity. This treatment removed 90-95% of the total cell-associated 125I-PDGF from cells incubated with the ligand at 4°C. After treatment with the acidic buffer, cells were lysed in lysis buffer as described above, and analyzed for intracellular radioactivity. In certain experiments, milder acidic conditions were used to remove cell surface-bound, but not internalized, ligand: cells were treated with 20 mM sodium acetate, pH 3.7, 150 mM NaCl and 0.2% BSA for 8 min on ice, and then washed with MEM-BSA. After this mild acidic treatment the cells were able to bind, internalize, and degrade the ligand with the same efficiency. The extent of degradation of iodinated ligand was determined by TCA precipitation (10% TCA for 2 h at 4°C, followed by centrifugation). TCA nonprecipitable radioactivity was taken as an estimate of degraded 125I-PDGF.

For Scatchard analyses, the cells were incubated in MEM-BSA containing 1-2 ng/ml of iodinated PDGF-BB or -AB and 0-400 ng/ml of corresponding unlabeled ligand, for 2 h at 2°C, in CO2 atmosphere. The monolayers were washed 5 times in MEM-BSA and lysed in lysis buffer as described above. The data were analyzed using the RIBINDING computer program for the linear subtraction method (44).

Results

Binding of PDGF-AB and -BB to Porcine Aortic Endothelial Cells Expressing Wildtype and K634A PDGF β-Receptors

PAE cells expressing wildtype PDGF β-receptor or β-receptor mutates at the ATP-binding lysine residue (K634A) were generated by transfecting PAE cells with the appropriate cDNAs cloned into the expression vector pZipNeo (6). The tyrosine kinase-inactive PDGF B-receptor was previously shown to be unable to mediate ligand-induced receptor autophosphorylation, actin reorganization (41), and mitogenesis (12). Scatchard analyses were performed to estimate the number of PDGF-BB and PDGF-AB binding sites and apparent affinities for wildtype receptor expressing cells and for two independently isolated cell lines expressing K634A receptors. As seen in Fig. 1, PDGF-BB bound with high and approximately similar apparent affinities to wildtype, K634A-2, and K634A-6 β-receptor-expressing cells (Fig. 1, A, C, and E). In each case, PDGF-BB bound with 4-10-fold lower apparent affinity (Fig. 1, B, D, and F). These recordings are consistent with earlier estimations for the wildtype receptor expressed in CHO cells (39).

The estimated number of binding sites for PDGF-AB as well as PDGF-BB was slightly lower on K634A-2 and K634A-6 (35,000-40,000 mol wt) receptor expressing cells as compared with wildtype receptor-expressing cells (45,000-55,000 mol wt). For each cell type, the apparent number of binding sites was similar for PDGF-AB and PDGF-BB. However, the lower affinity recorded for PDGF-BB makes the estimation of binding sites for this ligand more difficult to evaluate.
Internalization and Degradation of PDGF-BB and PDGF-AB

To examine the rate of endocytosis of bound ligand, wildtype, K634A-2, and K634A-6 receptor-expressing cells were incubated in the presence of 1 ng/ml of $^{125}$I-PDGF-BB or $^{125}$I-PDGF-AB at 37°C for various time periods. The cell cultures were washed with an acidic buffer to release surface bound ligand and the cells were then solubilized (Fig. 2 A). Since internalized ligand is protected from the acid and released only after solubilization of the cells, this procedure makes it possible to distinguish between cell surface and internalized ligand. Under the conditions used, ~10% of the cell surface receptors were occupied and this number did not change significantly with time. Also, the number of available binding sites remained constant, since the concentration of ligand was too low to appreciably downregulate the surface receptors. Therefore, the increase in ratio between internalized and surface bound ligand should reflect the relative rate of internalization under steady-state conditions (42). The internalization rate of $^{125}$I-PDGF-BB as well as of $^{125}$I-PDGF-AB was similar in K634A-2 and K634A-6 cells (not shown) and slower than in wildtype cells. In all types of cells, the internalization of $^{125}$I-PDGF-BB occurred faster than that of $^{125}$I-PDGF-AB. Similar differences in rates of internalization were seen when cells were preincubated in the presence of labeled ligands for 1 h at 4°C to load the receptors with ligand, before incubation at 37°C (data not shown).

The relative rates of ligand degradation were measured by incubating wildtype, K634A-2, and K634A-6 receptor-expressing cells with 2 ng/ml of $^{125}$I-PDGF-BB or $^{125}$I-PDGF-AB for 5 min at 37°C, followed by 90 min at 17°C, to allow internalization to the endosomal, but not lysosomal compartment; cells were then washed to remove unbound ligand, and further incubated for 90 min at 17°C. Degradation was initiated by increasing the incubation temperature to 37°C (Fig. 2 B). The kinetics of ligand degradation was expressed as the ratio between TCA-nonprecipitable radioactivity in the medium and the intracellular ligand. The rate of degradation was similar for the K634A-2 and K634A-6 receptor-expressing cells (not shown) and slower than for wildtype receptor cells, irrespective of whether
PDGF-BB or PDGF-AB was used. The relative number of cell surface receptors on wildtype or K634A-6 receptor cells remaining after 2 h incubation at 37°C with various concentrations of PDGF-BB or PDGF-AB, was estimated through binding of 125I-PDGF-BB after a mild acidic wash and transfer to 4°C. On wildtype and K634A-6 receptor cells exposed to saturating concentrations of PDGF-BB, 30-40% of the binding sites remained, whereas PDGF-AB-treated cells had 70-80% of the sites available on untreated cells (Fig. 3). The binding sites remaining after PDGF-AB treatment could be downregulated by exposure of the cells of PDGF-BB (not shown).

Internalization and Degradation of Wildtype and K634A PDGF B-Receptors Examined by Immunofluorescence Staining

Internalization and degradation of PDGF B-receptors was examined through fluorescence microscopy using the B-receptor-specific monoclonal antibody PDGFR-B2 (34). This antibody reacts with the external domain of the B-receptor. It mediates a weak and diffuse cell surface staining when used at higher concentrations. When the antibody is used at very low concentrations on ligand-stimulated cells, patchy staining is observed (Tingström, A., manuscript in preparation), but only after detergent treatment of the cells. Consequently, with the antibody concentrations used in the experiments described below (100-150 ng/ml), only clustered receptors, presumably concentrated in endosomes/lysosomes, were stained.

To follow internalization and degradation of receptors, the wildtype, K634A-2, and K634-6 receptor cells were exposed to saturating concentrations of either PDGF-BB or PDGF-AB for 30 min at 37°C, and processed for immunostaining. Both PDGF-BB (100 ng/ml) (Fig. 4 a) and PDGF-AB (400 ng/ml) (Fig. 4 c) induced the appearance of brightly stained vesicles in the cell periphery and perinuclear region in cells expressing wildtype receptors. Although a similar pattern of staining was displayed, the brightness of staining and number of fluorescent structures were somewhat lower in cells expressing K634A-6 receptors, irrespective of ligand used (Fig. 4, e and g). The fluorescent staining disappeared from cells with wildtype receptors when the incubation period in the presence of ligand was extended to 2 h (Fig. 4, b and d), whereas some staining remained in cells with K634A-6 receptors after exposure to ligand for the same time (Fig. 4, f and h). Complete disappearance of K634A-6 receptors was attained by 4 h of incubation in the presence of ligand (data not shown). The K634A-2 cell line displayed a pattern and kinetics of disappearance of staining indistinguishable from that for the K634A-6 cells (not shown).

Chloroquine-induced Block in Degradation of Wildtype B-Receptors Visualized by Immunofluorescence Staining

Wildtype B-receptor cells were incubated in the presence of 100 ng/ml of PDGF-BB for 5 min at 37°C and then for 3 h at room temperature. Chloroquine was added to the medium 15 min before the end of the incubation period. Unbound ligand was then washed away, and the cells were further incubated for 2 h at 37°C, in the continued presence of 100 μM chloroquine. At this point, the cells were fixed and treated for immunofluorescence using the PDGFR-B2 antibody, to stain intracellularly located B-receptors.

At room temperature, PDGF-BB induced internalization and accumulation of wildtype B-receptors within endosome-like structures (Fig. 5 a). The following incubation at 37°C led to disappearance of the staining, due to degradation of the molecules (Fig. 5 b). Under the same conditions, but in the presence of chloroquine, a considerable accumulation of staining was seen (Fig. 5 c). The chloroquine-induced block of receptor degradation could also be seen when cells were stained with the antibody PDGFR-3 (8), which recognizes the intracellular domain of the B-receptor. In fact, the staining mediated by PDGFR-3 colocalized with that seen with PDGFR-B2, which recognizes the extracellular domain of the B-receptor (Fig. 5, d and e). A similar block in degradation was seen also when cells were incubated with either PDGF-BB and PDGF-AB at 37°C, without prior preincubation at room temperature, or when preincubation was performed at 4°C. The degradation of B-receptors in K634A-2 and K634A-6 cells were inhibited to the same extent as in wildtype cells by chloroquine after ligand exposure (not shown). Furthermore, inhibition of degradation of wildtype, K634A-2 and K634A-6 receptors was also seen after treatment of the cells with monensin, as well as other amines (not shown).

Turnover of PDGF B-Receptors in Metabolically Labeled Cells

Ligand-induced internalization and degradation of PDGF B-receptors was further followed by immunoprecipitation from metabolically labeled wildtype, K634A-2, and K634A-6
Figure 5. PDGF β-receptor immunofluorescence staining of cells expressing wildtype receptors treated with chloroquine after exposure to PDGF-BB. The cells were incubated in the presence of 100 ng/ml of PDGF-BB for 5 min at 37°C, and further incubated for 3 h at 17°C (a). Chloroquine (100 µM) was added 15 min before the end of the 3 h incubation, thereafter the cells were transferred to 37°C for a continued 2-h incubation in the presence (c, d, and e) or absence (b) of 100 µM chloroquine. The cells were fixed and processed for immunofluorescence staining using PDGFR-B2 (a–d) or PDGFR-3 (e). Arrows show colocalization of staining with PDGFR-B2 and PDGFR-3. Bar, 10 µm.
Turnover of β-receptors in metabolically labeled cells with wildtype and K634A receptors. Cells with wildtype (lanes a-j) and K634A-6 (lanes k-t) receptors were cultured in the presence of [35S]methionine and [35S]cysteine for 3 h and then in MEM-BSA for 30 min at 37°C. Samples were either removed for processing, (p), (lanes a and k) or transferred to fresh medium, containing an excess of unlabeled methionine and cysteine ("chase" medium) without ligand (lanes b-d and l-n) or supplemented with 100 ng/ml of PDGF-BB (lanes e-g and o-q) or 400 ng/ml of PDGF-AB (lanes h-j and r-t). Chase periods in the absence or presence of ligands were 30 min (c1; lanes b, e, h, l, o, and r), 120 min (c2; lanes c, f, i, m, p, and s), and 240 min (c3; lanes d, g, j, n, q, and t). Cell samples were processed for immunoprecipitation using PDGFR-3. Immunoprecipitated material were analyzed by SDS-gel electrophoresis and fluorography. The relative migration rates of the mature (190 kD) and precursor (160 kD) forms of the β receptor are indicated.

Cells. The cells were cultivated in the presence of [35S]methionine and [35S]cysteine for 3 h, whereafter the medium was changed to MEM-BSA; after an additional 30 min, ligands were added. Four times higher concentrations of PDGF-AB (400 ng/ml) than PDGF-BB (100 ng/ml) were used to compensate for the differences in receptor binding affinities. PDGF β-receptors were immunoprecipitated from solubilized cells using the β-receptor specific peptide antiserum PDGFR-3. From all three cell lines, this antiserum precipitated two components of 190 and 160 kD, corresponding to the mature and the precursor forms of the receptors, respectively (8). The intensity of the immunoprecipitated 190-kD band was substantially reduced when wildtype (Fig. 6, lanes e, f, and g) receptor expressing cells were exposed to PDGF-BB. As seen from densitometric scanning of the fluorogram (Fig. 7), the amount of immunoprecipitated 190-kD band from wildtype receptor cells was reduced to close to zero after 1 h exposure to PDGF-BB. Exposure of wildtype receptor cells to PDGF-AB also cause a disappearance of the mature receptor band, albeit with a slower rate (Fig. 6, lanes h, i, and j). The intensity of the β-receptor band after ligand stimulation is expressed as percent of that for the β-receptor band from untreated cells. Thus, although the effect of PDGF-AB was less pronounced, it did increase the turnover rate of the β-receptor in a ligand-specific way.

The rate of degradation of the K634A receptor was slower than that of the wildtype receptor; 4 h exposure to PDGF-BB was needed to cause complete disappearance of the 190-kD band (Fig. 6, lanes o, p, and q show that K634A-6 cells). An even slower rate of disappearance of the 190-kD component was seen when PDGF-AB was added to K634A receptor expressing cells (Fig. 6, lanes r and s, and t show the K634A-6 cells). The rate of ligand-induced receptor turnover was very similar in the two cell lines expressing the mutated β-receptor; the plot shown in Fig. 7 is therefore averaged from several experiments using K634A-2 or K634A-6 cells.

The simultaneous expression of the PDGF α-receptor changes the efficiency of PDGF-AB induced downregulation of the PDGF β-receptor. Fig. 8 shows immunoprecipitation from metabolically labeled, ligand-stimulated human fore-
skin fibroblasts. In this cell line, which expresses both types of PDGF receptors, a complete downregulation of the β-receptor band was seen after 1 h exposure of the cells to 50 ng/ml of PDGF-BB or 100 ng/ml of PDGF-AB.

Discussion

PAE cells, which lack endogeneous expression of PDGF receptors, were used for expression of the wildtype and tyrosine kinase-inactivated form of the PDGF β-receptor. The characteristics of the wildtype β-receptor expressed in PAE cells were very similar to those described for the endogeneous β-receptor in human foreskin fibroblasts, as well as the β-receptor expressed in CHO cells after transfection (39; and data not shown). The turnover rates of the wildtype and mutated β-receptors in PAE cells, in the absence of ligand, were similar (4 h; Fig. 6). The small but significant differences in the efficiency of ligand-mediated internalization and degradation between the wildtype receptor expressing cells and two independent isolates of cells expressing the mutated receptor, can therefore not be ascribed to differences in cellular constitutive turnover rates.

The discrete impairment in the rate of ligand-induced downregulation noted for the mutated receptor indicates that there is no absolute requirement for kinase activity in endocytosis of the PDGF β-receptor complex. A slower turnover rate was also seen for both the wildtype and K634A receptor after exposure to PDGF-AB, as compared with PDGF-BB. In human foreskin fibroblasts, which express both types of PDGF receptors, PDGF-AB efficiently induced down regulation of the β-receptor (Fig. 8). The ligand-dependent delay in PAE cells appeared to be in the rate of internalization and not in the rate of degradation (Fig. 2, A and B) at least when the ligand itself was examined. It is possible that the delay seen when using PDGF-AB is a consequence of a suboptimal conformational state of the receptor molecule induced by this ligand, which binds to the β-receptor with lower affinity, as compared with PDGF-BB. It has recently been shown that PDGF β-receptors dimerize as a consequence of ligand binding, before and possibly a prerequisite for activation of the tyrosine kinase domain (2, 21). PDGF-AB has been shown to induce formation of heterodimers of α- and β-receptor (17, 38), in which the A-chain would be bound to the α-receptor, and the B-chain to the β-receptor. If no α-receptors are available to bind the A-chain, as is the case in PAE cells, β-receptors that have bound PDGF-AB might remain in a monomeric configuration. Such ligand-receptor complexes may be internalized less efficiently compared to dimeric receptor complexes, with or without activated tyrosine kinase domains.

Our data on the block in degradation induced by treatment of cells with chloroquine, indicate that the two ligands, as well as the wildtype and K634A receptors follow the lysosomal pathway for degradation, eliminating the need to postulate a novel pathway for degradation of the PDGF β-receptor (23, 27). Receptors which accumulated after treatment of cells with chloroquine could be detected by immunofluorescence staining using two different antibodies, directed against the extra- or the intracellular domains, respectively (Fig. 5). However, in agreement with data reported by others (23, 27), we were unable to see a chloroquine-mediated block in degradation of β-receptors, using immunoprecipitation from metabolically labeled cells as an assay, irrespective of which antibody was used (not shown). The reason for the discrepancy between the results of the two assays is not clear. However, it is possible that accumulated receptors might have become less soluble, e.g., through association with the detergent insoluble cell fraction, which in our procedure was removed by centrifugation from the lysate, before immunoprecipitation.

Studies addressing the question of the role of tyrosine kinase activity in endocytosis have been performed on other growth factor receptors with tyrosine kinase domains. In the case of the EGF receptor, inactivation of the kinase resulted in a receptor that failed to undergo downregulation in response to ligand, such that the mutant receptor in the presence of EGF retained the half-life of the unoccupied wild-type receptor. There are conflicting interpretations regarding the mechanisms involved, however. Whereas Honegger et al. (22) and Felder et al. (13) reported that their EGF receptor kinase mutant expressed in NIH 3T3 cells was internalized, but escaped degradation and recycled back to the cell surface; Glenney et al. (14) failed to record any internalization of their mutant, expressed in B82 or CHO cells.
a kinase negative CSF-1 receptor mutant is expressed in NIH 3T3 cells revealed that the ligand-induced downregulation was abolished, but the mechanisms involved were not analyzed (11). On the other hand, downregulation and receptor degradation after ligand binding was recorded for the tyrosine kinase-inactivated insulin receptor (37).

Several mechanisms could account for the discrete impairment in efficiency in downregulation and receptor degradation which we report for the PDGF β-receptor tyrosine kinase mutant expressed in PAE cells. It is possible that the rate of endocytosis can be modulated by receptor mediated tyrosine phosphorylation of key molecules in the endocytic machinery. Alternatively, the observed slower endocytic rate of the tyrosine kinase-inactivated receptor may be due to an inefficient sorting to the lysosomal route after internalization. A fraction of the internalized receptors might recycle back to the cell surface and thereby escape degradation. Future studies will be aimed at examining whether the kinase-inactivated PDGF β-receptor undergoes recycling.

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