Stimulation of β_1 Integrins on Fibroblasts Induces PDGF Independent Tyrosine Phosphorylation of PDGF β-Receptors

Christian Sundberg and Kristofer Rubin

Department of Medical and Physiological Chemistry, Biomedical Center, S-751 23 Uppsala, Sweden

Abstract. We report that integrin-mediated signaling induces a rapid and transient tyrosine phosphorylation of platelet-derived growth factor (PDGF) β-receptors in human diploid foreskin AG 1518 fibroblasts. A transient tyrosine phosphorylation of PDGF β-receptors was evident one and two hours after cells had been plated on collagen type I and fibronectin, as well as on immobilized anti-integrin subunit IgG, but not on poly-L-lysine. In contrast EGF or PDGF α-receptors were not phosphorylated on tyrosine residues under these conditions. Tyrosine phosphorylation of PDGF β-receptors induced by plating on collagen type I was inhibited by cytochalasin D and herbimycin A, unaffected by cycloheximide and enhanced by orthovanadate. Furthermore, a transient phosphorylation of PDGF β-receptors occurred when AG 1518 fibroblasts were cultured in three-dimensional collagen lattices or exposed to external strain exerted through centrifugation. The latter

effect was evident already after two minutes. Clustering of cell surface β_1 integrins led to PDGF β-receptor phosphorylation both in suspended and firmly attached AG 1518 fibroblasts. Plating of cells on collagen type I, fibronectin, and anti-β_1-integrin IgG resulted in the formation of PDGF β-receptor aggregates as detected by immunofluorescence. Suramin or anti-PDGF-BB IgG had no effect on the plating-induced tyrosine phosphorylation of PDGF β-receptors. PDGF-B chain mRNA, or protein, were not detected in AG 1518 fibroblasts. Our data suggest that a ligand-independent PDGF β-receptor activation during cell adhesion and early phases of cell spreading is involved in integrin-mediated signaling in fibroblasts, and constitutes parts of a mechanism for cells to respond during the dynamic phases of externally applied tension as well as fibroblast-mediated tension during cell adhesion and collagen gel contraction.

C ELL adhesion to extracellular matrix (ECM)[1] regulates a plethora of cellular activities (6, 35, 68). Adhesion of cells to ECM components depends on a set of transmembrane receptors belonging to the integrin family. Integrins are heterodimers of non-covalently associated α and β subunits (2, 29, 32, 51), which link the ECM with the cytoskeleton, and act as signal transducing receptors (13, 16, 19, 35, 44, 55). In many types of cells, integrin stimulation by ligand occupation or clustering by antibodies elicit changes in the intracellular pH, cytoplasmic-free calcium concentration, phosphoinositide synthesis, protein tyrosine phosphorylation, and expression of certain genes. Proteins that are tyrosine phosphorylated in response to integrin-mediated adhesion include the focal adhesion kinase (p125^{FAK}) (9, 21, 25, 38, 53), paxillin (9), and the MAP kinases, ERK-1 and ERK-2 (72). Many of the responses elicited by integrin-mediated adhesion are also evoked by activation of the PDGF β-receptors (7, 11,

71). Thus, PDGF stimulation leads to tyrosine phosphorylation of p125^{FAK} and paxillin, as well as MAP kinases (11, 48) The role of PDGF β-receptors, if any, in integrin-induced signaling, is not known.

In addition to its well-known growth-promoting activities, PDGF stimulates several processes that depend on integrin activity. Examples are PDGF stimulated and $\alpha_2\beta_1$-mediated chemotaxis through collagen type I–coated membranes (61) and fibroblast-mediated contraction of three-dimensional collagen lattices (14, 24, 37, 42, 54). Conversely, PDGF β-receptors become functionally refractory in fibroblasts that have contracted a collagen gel (43, 64). PDGF specifically stimulates the synthesis of the collagen-binding integrin $\alpha_2\beta_1$ (1) and increases the apparent activity of β_1 integrins in fibroblasts (24). Thus, several experimental observations strongly suggest that PDGF β-receptors modulate, and are modulated by, cellular processes dependent on integrin activity.

Integrins are concentrated and co-localize with several cytoskeletal components at focal adhesion sites (8, 57, 67). Within focal adhesions, cytoskeletal components, as well as signaling molecules concentrate, and are believed to serve as regulatory complexes for integrin signaling. Stimulation of fibroblasts with PDGF induces a rapid and tran-

1. *Abbreviations used in this paper.* ECM, extracellular matrix; PAE, porcine aortic endothelium.

sient change of the cytoskeletal structure, involving the formation of membrane and circular ruffles (45). Stimulation with this growth factor also results in a redistribution, as well as a change in the phosphorylation pattern of the focal adhesion protein vinculin (30), a process dependent on changes in phosphoinositide turnover (18).

It has been suggested that integrins can function as mechanochemical transducers conveying strain from the extracellular matrix to the cytoskeleton leading to effects on the cell signaling machinery (reviewed in 33, 56). In a recent communication, Wang et al. (69) demonstrated that integrin-mediated adhesions will restrain external force applied to the structural components linked to the cell surface via integrins. Given the background that integrins transduce chemical signals that converge with responses elicited by the PDGF β-receptor, and that the latter in turn influence integrin activity, it is reasonable to propose an involvement of PDGF β-receptors in the mechanochemical transducing properties of integrins.

Here we report that integrins and integrin-mediated adhesion processes induce an early and transient phosphorylation and internalization of PDGF β-receptors in fibroblasts. These processes were independent of PDGF. The PDGF β-receptor autophosphorylation was stimulated by increases in mechanical tension or stress exerted on the cells. Thus our findings suggest that autophosphorylation of PDGF β-receptors constitutes parts of a mechanism for cells to respond during the dynamic phases of externally applied tension, as well as fibroblast-mediated tension during cell adhesion and collagen gel contraction. Furthermore, the data are compatible with that PDGF β-receptor activation during cell adhesion and early phases of cell spreading may be a component in integrin-induced signaling in fibroblasts.

Materials and Methods

Antibodies and Other Reagents

The characteristics of the PDGF β-receptor specific mouse mAb, PDGFR-B2, have been described elsewhere (28, 52). PDGFR-B2 was used at a concentration of 1 μg/ml (49, 65). Polyclonal rabbit anti–PDGF-BB IgG (34) was kindly donated by Dr. C.-H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden). The anti-phosphotyrosine mouse mAb (4G10) (15, 36), and polyclonal rabbit anti–human PDGF type B-receptor serum raised against a synthetic peptide corresponding to amino acids 1013–1025 (12) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). The anti-phosphotyrosine mouse mAb PY20 (20) was purchased from Transduction Laboratories (Lexington, KY). The anti-PDGF α-receptor mAb (anti-PDGF-Rα) (27) was purchased from Genzyme (Cambridge, MA). The anti–human EGF receptor mAb, clone 108, (3) was kindly donated by Dr. Andreas Batzer (New York University Medical Center, New York, NY). The mAb PGF 007, raised toward a synthetic peptide corresponding to amino acid residues 73–97 of the PDGF B-chain was purchased from Mochida Co. (Tokyo, Japan). The PGF 007 antibody specifically recognizes PDGF-AB and PDGF-BB (60). The anti–human fibroblast surface protein mAb (1B10) was purchased from Sigma Chemical Co. (St. Louis, MO). The anti-integrin α_1-subunit mAb TS2/7 (29) was kindly donated by Dr. Timothy Springer (Boston Blood Center, Boston, MA). The anti-integrin α_2-subunit mAb P1H5; anti-integrin α_3-subunit mAb P1B3; anti-integrin α_5-subunit mAb PID6; and the anti-integrin β_1-subunit mAb P4C10 (10, 63, 70) were all kindly donated by Dr. William Carter (Fred Hutchinson Cancer Research Center, Seattle, WA). Polyclonal rabbit anti-integrin β_1-subunit IgG was raised essentially as described (23, 50) with the modifications that purified rat hepatocytes were used as starting material and that collagen-binding proteins were eluted from collagen type I–Sepharose affinity columns by 10 mM EDTA. Cy-

tochalsin D, cyclohexamide, herbimycin A, and sodium orthovanadate were purchased from Sigma Chem. Co.

Rabbit anti–mouse IgG antibodies and goat anti–rabbit IgG antibodies were purchased from Sigma Chem. Co. and used at a concentration of 10 μg/ml. Affinity purified F(ab)$_2$ fragments of goat anti–mouse immunoglobulins (IgG, IgA, and IgM) was purchased from Cooper Biomedical (Malvern, PA). Biotinylated horse anti–mouse IgG, biotinylated goat anti–rabbit IgG, and Texas red avidin D were from Vector Laboratories (Burlingame, CA). Fluorescein-conjugated goat anti–mouse IgG, and goat anti–rabbit IgG were from Becton and Dickinson (Mountainview, CA) and Sigma Chem. Co., respectively. All antibodies were diluted in PBS (130 mM NaCl, 10 mM Na-phosphate, pH 7.4), and used in optimal concentrations determined after serial dilutions.

Recombinant human PDGF-AA and PDGF-BB (47a) was kindly donated by Dr. C.-H. Heldin. EGF was purchased from Sigma Chem. Co. Suramin, an agent which inhibits the binding of several growth factors to their receptors and is able to dissociate receptor-bound growth factors including PDGF (5, 31, 39), was kindly provided by Bayer (Leverkusen, Germany). Human plasma fibronectin was purified according to the method described by Miekka et al. (46). Calf skin collagen type I (Vitrogen 100) was obtained from Celltrix (Palo Alto, CA). DME, MCDB 104 medium used for serum-free cell culture and trypsin-EDTA were obtained from the National Veterinary Institute (Uppsala, Sweden). FBS was obtained from Sera-Lab limited (Sussex, UK).

Cells

Stock cultures of human diploid foreskin AG 1518 fibroblasts (Genetic Mutant Cell Repository, Camden, NJ), were grown in 175 cm^2 cell culture flasks (Costar Corp., Cambridge, MA) in DME supplemented with 10% FBS, 50 μg/ml streptomycinsulphate, 60 μg/ml penicillin G, and 2 mM L-glutamine (National Veterinary Institute). Cells were passaged once a week and fed fresh medium twice a week. All experiments were performed with cells in passages 15–20 (passage split 1:2). Cells grown to near confluence were detached by two rinses with PBS containing 10 mM EDTA, and subsequently treated for 3 min with trypsin-EDTA. Trypsin was neutralized with DME containing 10% FBS. Cells were then centrifuged and resuspended in MCDB 104 medium.

Preparation of Collagen Gels

For the manufacturing of three-dimensional collagen gels, tissue culture 24-well plates (Costar Corp.) were coated overnight with PBS containing 2% BSA (National Veterinary Institute) that had been heat inactivated at 55°C for 2 h, followed by washing the plates twice with PBS. Neutral collagen solutions were prepared by mixing two times concentrated MCDB 104 medium, 0.2 M Hepes, pH 8.0, and vitrogen 100, in the proportions 5:1:4 by volume. Fibroblasts suspended in two times MCDB 104 medium were mixed with the collagen solution to bring the final cell concentration to 800,000 cells/ml, and the final collagen concentration to 1.1 mg/ml. The collagen/cell solution was incubated at 37°C for 5 min, whereafter 400 μl of the mixture was added to each well. The collagen/cell mixture was allowed to polymerize for 60 min at 37°C. After polymerization, 1 ml of MCDB 104 was added and gels that were to be flotated (relaxed) were dislodged from the walls of the plates with a spatula and allowed to float freely in the well. Gels that were not dislodged are referred to as stressed.

Cell Staining

Cells were seeded onto coverslips in 24-well plates (Costar Corp.) and cultivated for the designated time periods. Cells were fixed in fresh 2% paraformaldehyde 4°C for 10 min. After rinsing in TBS (0.15 M NaCl and 0.01 M Tris, pH 7.4) cells were incubated for 15 min in TBS with 0.1% Tween, rinsed in TBS containing 0.1% BSA, and 0.1% Tween, thereafter remaining free aldehyde groups were blocked by incubating in 0.1 M glycine containing 0.5% BSA, pH 7.4, for 1 h. Coverslips were incubated with the primary mouse monoclonal antibody for 45 min, rinsed, and incubated with biotinylated horse anti–mouse IgG diluted for 30 min, rinsed, and incubated with a mixture of Texas red avidin D and Bodipy FL Phallacidin (Molecular Probes Inc.), rinsed, and subsequently mounted in glycerin/PBS (9:1) containing 0.1% phenylenediamine (Aldrich, Steinheim, Germany).

Detection of β_1-integrin Clustering

AG 1518 fibroblasts were grown on glass coverslips in the presence of 10% FBS for 2 d, washed, and then serum depleted in MCDB 104 for 24 h.

Cells were either incubated with polyclonal anti-β_1 integrin IgG (250 μg/ml) only or with polyclonal anti-β_1 integrin IgG (250 μg/ml) followed by goat anti–rabbit IgG (20 μg/ml) prior to fixation in 2% paraformaldehyde 4°C for 10 min and rinsed in TBS. Cells were incubated for 15 min in TBS with 0.1% Tween, rinsed in TBS containing 0.1% BSA and 0.1% Tween, and free aldehyde groups were blocked by incubating in 0.1 M glycine with 0.5% BSA, pH 7.4, for 1 h. IgG was visualized by incubation with goat anti–rabbit IgG FITC (Sigma Chem. Co.) or rabbit anti–goat IgG FITC (Sigma Chem. Co.) for 30 min, rinsed in TBS, and mounted as described above.

Experimental Conditions

Plating Experiments. 60 mm dishes were coated at 37°C overnight with 30 μg/ml collagen type I, 20 μg/ml of plasma fibronectin or 4 mg/ml of poly-L-lysine diluted in PBS. To coat dishes with different IgG:s or IgM, 100 μg/ml of affinity-purified F(ab)$_2$ fragments of goat anti–mouse immunoglobulins (IgG, IgA, and IgM) were incubated in PBS on 60 mm dishes for 8 h at 4°C. All dishes were washed in PBS and remaining free plastic protein-binding sites blocked for 2 h in PBS containing 2% BSA that had been heat inactivated, and again washed twice in PBS. Dishes were then incubated overnight with monoclonal anti–integrin subunit antibodies diluted in PBS, and subsequently washed twice in PBS. AG 1518 fibroblasts were trypsinized for 2 min at 37°C, and then washed and resuspended in MCDB 104 medium. 800,000 cells were plated in the 60 mm cell culture dishes unless otherwise stated and incubated at 37°C for the designated time periods.

Integrin Cross-linking Experiments. 400,000 AG 1518 fibroblasts were seeded in 60 mm dishes and grown to near confluence in DME supplemented with 10% FBS. Before the initiation of experiments cells were serum starved in MCDB 104 for 24 h, washed twice in MCDB 104 and incubated with polyclonal anti-β_1 integrin IgG (250 μg/ml) for 30 min. Cells were washed once in MCDB 104 and incubated with secondary goat anti–rabbit antibody (20 μg/ml) for different time periods to cluster cell surface β_1 integrins.

Experiments on Cells in Suspension Were Performed as Follows. 500 μl of protein A–Sepharose beads (Pharmacia, Piscataway, NJ) were incubated in 4 ml of a solution containing 250 μg/ml polyclonal anti-β_1 integrin IgG for 2 h at 4°C and then washed three times in PBS. 50 μl of the beads were incubated with 800,000 cells in 600 μl MCDB 104 in suspension at 37°C for the designated time periods. Alternatively, 800,000 cells were incubated in 600 μl MCDB 104 containing 250 μg/ml polyclonal anti-β_1 integrin antibodies in suspension at 37°C.

Solubilization and Immunoprecipitation

Cell monolayers were removed from the culture dishes by scraping with a rubber policeman and solubilized for 30 min in 400 μl solubilization buffer (0.5% Triton X-100, 0.5% sodiumdeoxycholate, 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 30 mM disodium pyrophosphate, 2 mM EDTA, 0.4 mM Na-vanadate, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 1 mM PFA block, and 250 KIE/ml aprotinin). Collagen gels were pooled and volumes were equilibrated to 1.5 ml with MCDB 104. Thereafter 500 μl of four times concentrated solubilization buffer was added. Samples were then homogenized (15 strokes) with a 2 ml Dounce homogenizer at 4°C and incubated for 30 min.

Lysates were centrifuged at 16,000 g for 30 min. Supernatants were precleared by incubating with normal rabbit IgG and protein A– or G–Sepharose (Pharmacia) for 1.5 h. Supernatants were then immunoprecipitated with polyclonal anti-PDGF β-receptor, or monoclonal anti-PDGF α-receptor IgG, anti-phosphotyrosine IgG or anti-EGF receptor IgG for 2 h, and subsequently precipitated with protein A– or G–Sepharose. The Sepharose pellets were washed three times in 0.5 M NaCl and three times in 0.15 M NaCl solubilization buffer and subjected to SDS-PAGE.

Immunoblotting

Samples for SDS-PAGE were mixed with reducing sample buffer (0.2 M Tris-HCL, pH 8.8, 18% glycerol, 4% SDS, 0.01% bromophenol blue, 10% mercaptoethanol) and were subjected to a 7.5% acrylamide gel electrophoresis (40) using minigel aggregates (Bio-Rad Labs, Boston, MA). Rainbow-colored protein molecular weight markers (Amersham) were used. Gels were transferred to Immobilin polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA).

Membranes were blocked overnight at 4°C in TBS with 5% BSA and 0.2% Tween-20 and then were incubated for 2 h with the anti-phosphoty-

rosine mAb PY20 or P4G10 diluted in blocking solution. Membranes were washed in TBS with 0.1% BSA and 0.2% Tween followed by 1.5-h incubations with horseradish peroxidase–linked sheep anti–mouse IgG (Amersham) in blocking solution. Bands were developed using the enhanced chemiluminescence substrate kit (Amersham) for 1–3 min with medical x-ray film (Fuji). Films were analyzed by laser scanning densitometry on an Ultroscan XL (LKB-Wallac, Bromma, Sweden).

Centrifugation Experiments

Plastic dishes were coated with collagen type I (30 μg/ml) or fibronectin (20 μg/ml) as described above. AG 1518 fibroblasts were plated at a density of 800,000 cells in 60 mm dishes, and allowed to adhere for 4 h in MCDB 104. Dishes were then incubated on ice until used in centrifugation experiments. Immediately before centrifugation, dishes were incubated at 37°C for 5 min, washed once with MCDB 104, and then completely filled with MCDB 104 containing 10 mM Hepes and 100 μM sodium orthovanadate, pH 7.4, at 37°C. Dishes were covered with parafilm and airbubbles removed with a syringe. The cell plates were immediately centrifuged for the designated time periods. The dishes were placed in the centrifuge so that the centrifugal force was directed upward and perpendicular to the plane of the interphase between the cell and the substrate. The Omnifuge 2.0 RS centrifuge (Heraeus Sepatech, Hanau, Germany) used enables precise regulation of temperature, acceleration, deceleration, and final G-force value. Cell plates were centrifuged upside-down at 25°C, varying the final G-force values. Parallel control plates that were not subjected to centrifugation were incubated in MCDB 104, 10 mM Hepes, and 100 μM sodium orthovanadate at 25°C.

Results

Plating of Cells on ECM Substrates Induces Tyrosine Phosphorylation of PDGF β-Receptors

Plating of human AG 1518 fibroblasts on dishes coated with collagen type I or fibronectin in serum-free MCDB 104 medium resulted in a rapid tyrosine phosphorylation of PDGF β-receptors (Fig. 1, *A* and *B*). Maximal PDGF β-receptor tyrosine phosphorylation was detected 1 and 2 h, respectively, after plating. The tyrosine phosphorylation response elicited by plating cells on collagen type I and fibronectin was transient, 6 h after plating a clear decrease in PDGF β-phosphorylation was apparent on both substrates. In cells plated on poly-L-lysine only a limited phosphorylation of PDGF β-receptors was detected 2 h after plating (Fig. 1 *C*). The limited phosphorylation of PDGF β-receptors in cells plated on poly-L-lysine was completely abrogated by the presence of 10 μg/ml cycloheximide (Fig. 1 *D*). In contrast the PDGF β-receptor phosphorylation induced by plating cells on fibronectin or collagen type I was not affected by cycloheximide (Fig. 1 *D*) thus the PDGF β-receptor phosphorylation response after plating cells on poly-L-lysine was due to synthesis of a cell attachment protein. Furthermore, the plating-induced tyrosine phosphorylation of PDGF β-receptors was not altered by the presence of 10% FBS, or when cells were plated at a four times lower density, but was enhanced in the presence of orthovanadate and inhibited in the presence of herbimycin A (data not shown). As expected no tyrosine phosphorylation of PDGF β-receptors was detected in AG 1518 fibroblasts that had been plated in the presence of 10% FBS, grown to confluence and serum depleted for 24 h. PDGF-BB stimulation of such cells resulted, however, in a rapid and effective tyrosine phosphorylation of PDGF β-receptors (Fig. 1, *A* and *D*).

The plating-induced phosphorylation of PDGF β-receptors was sensitive to disruption of the actin cytoskeletal in-
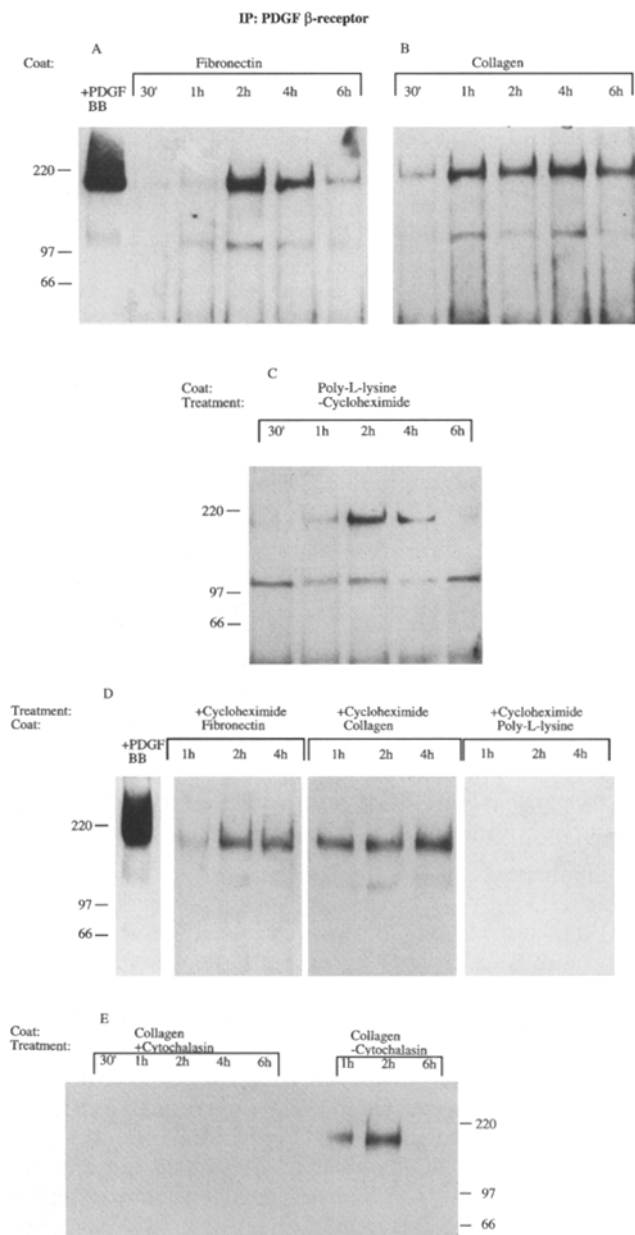
Figure 1. Plating-induced phosphorylation of PDGF β-receptors in AG 1518 fibroblasts. Cells were plated and cultured in MCDB 104 medium for the indicated time periods under serum-free conditions. (A) Cells plated on fibronectin; (B) cells plated on collagen type I; (C) cells plated on poly-L-lysine; (D) cells plated on poly-L-lysine and cultured in the presence of cycloheximide (10 μg/ml); and (E) cells plated on collagen type I and cultured in the presence or absence of (5 μg/ml) cytochalasin D. Phosphorylation of PDGF β-receptors achieved after stimulation of cells with 20 ng/ml of PDGF-BB for 15 min is shown in A and D. PDGF β-receptors were immunoprecipitated with specific polyclonal anti-PDGF β-receptor IgG, and tyrosine phosphorylation was detected by immunoblotting after separation of precipitated proteins by SDS-PAGE. The relative migration of pre-stained molecular weight standard proteins are indicated. 220, Myosin; 97, phosphorylase B; and 66, bovine serum albumin. These marker proteins migrate as proteins with M_r:s 220,000, 97,000, and 66,000 respectively. IP, Immunoprecipitation.

tegrity as evidenced by experiments in which cells were plated on ECM molecules in the presence of 5 μg/ml cytochalasin D. Cells plated in the presence of cytochalasin D adhered but did not spread on collagen type I and fibronectin. Addition of this drug to the medium completely abolished the plating-induced tyrosine phosphorylation of PDGF β-receptors (Fig. 1 E).

The degree of PDGF β-receptor tyrosine phosphorylation, measured as optical density of exposed films by laser densitometric scanning, was directly proportional to the PDGF-BB concentration used to stimulate cells for the film exposure times, 1–3 min, that were used in the present studies. The relative values obtained for plating-induced tyrosine phosphorylation of PDGF β-receptors in AG 1518 fibroblasts amounted to 19% ($n = 6$) for cells plated on collagen type I, and 27% ($n = 3$) for cells plated on fibronectin, compared to the signal resulting from stimulation of the cells with 20 ng/ml PDGF-BB for 15 min. Cells stimulated with 20 ng/ml PDGF-BB for 15 min were included in all of the following experiments, and the magnitude of the PDGF β-receptor tyrosine phosphorylation response is referred to as the maximal response in the following text.

Human AG 1518 fibroblasts express EGF receptors as well as PDGF α-receptors. Tyrosine phosphorylation of EGF receptors and PDGF α-receptors was detected after stimulation for 15 min with 100 ng/ml of EGF and 50 ng/ml of PDGF-AA, respectively (Fig. 2, A and B). Densitometric scanning revealed a maximum tyrosine phosphorylation response of 80% for EGF receptors and 20% for PDGF α-receptors of the maximal tyrosine phosphorylation response of PDGF β-receptors. In contrast, plating of the fibroblasts on collagen type I (Fig. 2, A and B) or fibronectin did not induce any detectable tyrosine phosphorylation of EGF receptors or PDGF α-receptors.

Addition of polyclonal anti-PDGF-BB IgG (Fig. 2 C), known to inhibit auto- or paracrine stimulation of PDGF receptors in v-sis transfected cells (34), or 100 μM suramin (data not shown) had no effect on the phosphorylation of PDGF β-receptors in response to plating on collagen type I or fibronectin. The concentrations of anti-PDGF-BB IgG used, 40 μg/ml, and suramin, 100 μg/ml were able to inhibit the tyrosine phosphorylation of PDGF β-receptors induced by 50 ng/ml PDGF-BB (Fig. 2 D and data not shown). No expression of PDGF-AB/BB was detected in the AG 1518 fibroblasts, either as protein using the anti-PDGF-AB/BB antibody PGF 007 in immunofluorescence staining, or as mRNA using Northern blotting (data not shown). Taken together, these findings suggest that the plating-induced phosphorylation of PDGF β-receptors is not dependent on para- or autocrine stimulatory loops involving PDGF-BB.

Plating of AG 1518 Fibroblasts on ECM Substrates Induces Clustering of PDGF β-Receptors

Stimulation of AG 1518 fibroblasts with PDGF-BB, but not with PDGF-AA, results in the formation of intracellular granular accumulations, or clusters, of PDGF β-receptors (62, 65). These receptor clusters can be detected by immunofluorescence using PDGFR-B2 IgG (Fig. 3, A–L). Plating of AG 1518 fibroblasts on collagen type I or on fi-
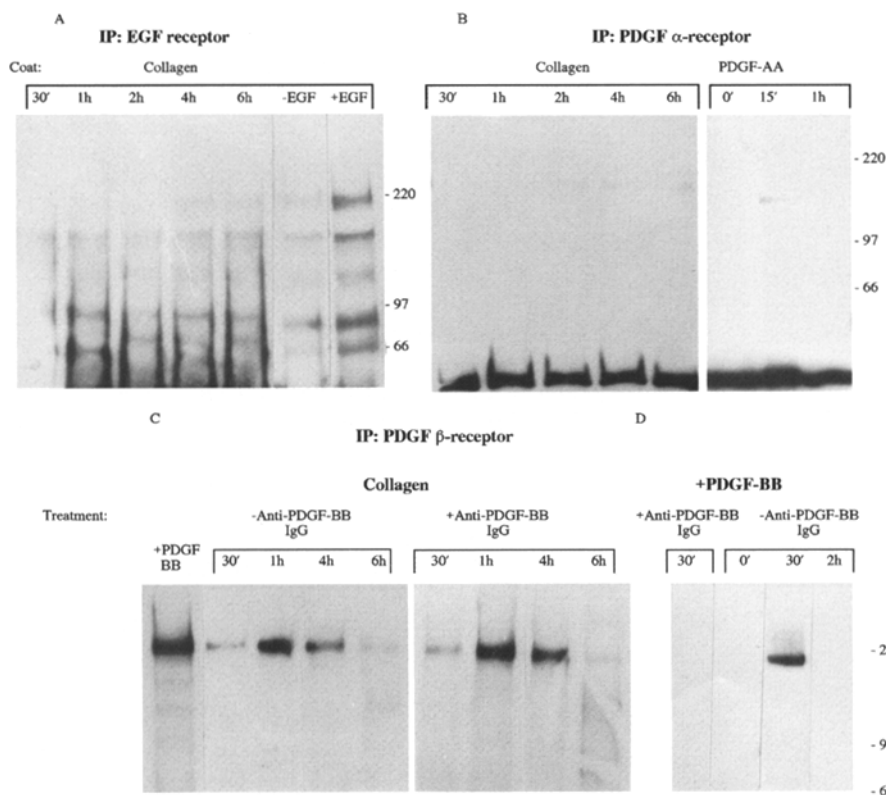
Figure 2. Effects of plating on EGF and PDGF α-receptor phosphorylation (*A* and *B*) and effects of anti-PDGF-BB on plating-induced tyrosine phosphorylation of PDGF β-receptors (*C* and *D*) in AG 1518 fibroblasts. AG 1518 fibroblasts were plated on collagen type I and cultured for the indicated time periods in MCDB 104 medium. Cells were cultured in *C*, the absence or presence of 40 μg/ml of polyclonal anti-PDGF-BB IgG; or (*D*) cells were stimulated with 50 ng/ml of PDGF-BB in the presence or absence of 40 μg/ml of polyclonal anti-PDGF-BB IgG. Extracts were immunoprecipitated with: (*A*) monoclonal anti-EGF receptor IgG; (*B*) monoclonal anti-PDGF α-receptor IgG; and (*C* and *D*) polyclonal anti-PDGF β-receptor IgG. Phosphorylation of PDGF β-receptors achieved after stimulation of cells with 20 ng/ml of PDGF-BB for 15 min is shown in *D*. Immunoprecipitations and detection of tyrosine phosphorylated proteins by immunoblotting was performed as described in Materials and Methods. The relative migration of pre-stained molecular weight standard proteins are indicated, 220, myosin; 97, phosphorylase B; and 66, bovine serum albumin. These marker proteins migrate as proteins with M_r:s 220,000, 97,000, and 66,000 respectively, in SDS-PAGE. *IP*, Immunoprecipitation.

bronectin led to the formation of PDGF β-receptor vesicles suggesting that the plating-induced phosphorylation resulted in assembly and internalization of the PDGF β-receptors. The receptor clusters were evident two hours after plating of cells on collagen type I (Fig. 3, *E* and *F*) or on fibronectin (Fig. 3, *G* and *H*). Cells plated on poly-L-lysine did not exhibit clusters of PDGF β-receptors 2 h after plating (Fig. 3, *K* and *L*).

Plating of AG 1518 Fibroblasts on Anti-integrin Subunit IgG Induces Phosphorylation and Clustering of PDGF β-Receptors

The involvement of β_1 integrins in the plating-induced phosphorylation of PDGF β-receptors was investigated. In a first series of experiments AG 1518 fibroblasts were seeded on dishes to which monoclonal anti-integrin α subunit or anti-integrin β_1-subunit IgG had been immobilized (see Materials and Methods). Monoclonal antibodies used were TS2/7 (anti-α_1), P1H5 and P1E6 (anti-α_2), P1B5 (anti-α_3), P1D6 (anti-α_5), and P4C10 (anti-β_1). AG 1518 fibroblasts attached and spread readily to surfaces coated with all of these antibodies, anti-α_5 and anti-β_1 being the most effective in supporting cell spreading. A rapid tyrosine phosphorylation of PDGF β-receptors was evident after plating of cells on all of the anti-integrin antibodies (Fig. 4). Phosphorylation of PDGF β-receptors occurred most rapidly in cells plated on monoclonal anti-α_5 and

anti-β_1 integrin IgG with a maximal level achieved 30 min after plating and thereafter decreasing during a 2-h incubation period. AG 1518 fibroblasts, plated on monoclonal anti-fibroblast surface marker IgM (1B10) were able to adhere and spread but did not display any phosphorylation of the PDGF β-receptors during the time period investigated (Fig. 4). Cells did not adhere to dishes coated solely with the anti–mouse F(ab)₂ fragments used to immobilize the anti-integrin subunit antibodies.

Plating of cells on polyclonal anti-β_1 integrin IgG resulted in rapid formation of PDGF β-receptor clusters visible 2 h after plating (Fig. 3, *I* and *J*), these clusters gradually decreased during a 6-h incubation period (data not shown), as assessed by immunofluorescence using PDGFR-B2 IgG. When omitting the primary antibody in the immunofluorescence staining procedure no patches were detected indicating that the secondary biotinylated horse anti-mouse IgG does not cross-react with rabbit IgG (data not shown). No PDGF β-receptor clusters were detected in cells plated on poly-L-lysine (Fig. 3, *K* and *L*).

To further assess the effects of β_1 integrin stimulation on phosphorylation of PDGF β-receptors in AG 1518 fibroblasts, antibody cross-linking experiments were conducted. Fibroblasts were grown to near confluence in DME with 10% FBS, serum-depleted in MCDB 104 medium for 24 h, and stimulated with 250 μg/ml polyclonal anti-β_1 integrin IgG for 30 min. After removal of unbound anti-β_1 IgG by washing with MCDB 104, cultures were incubated with
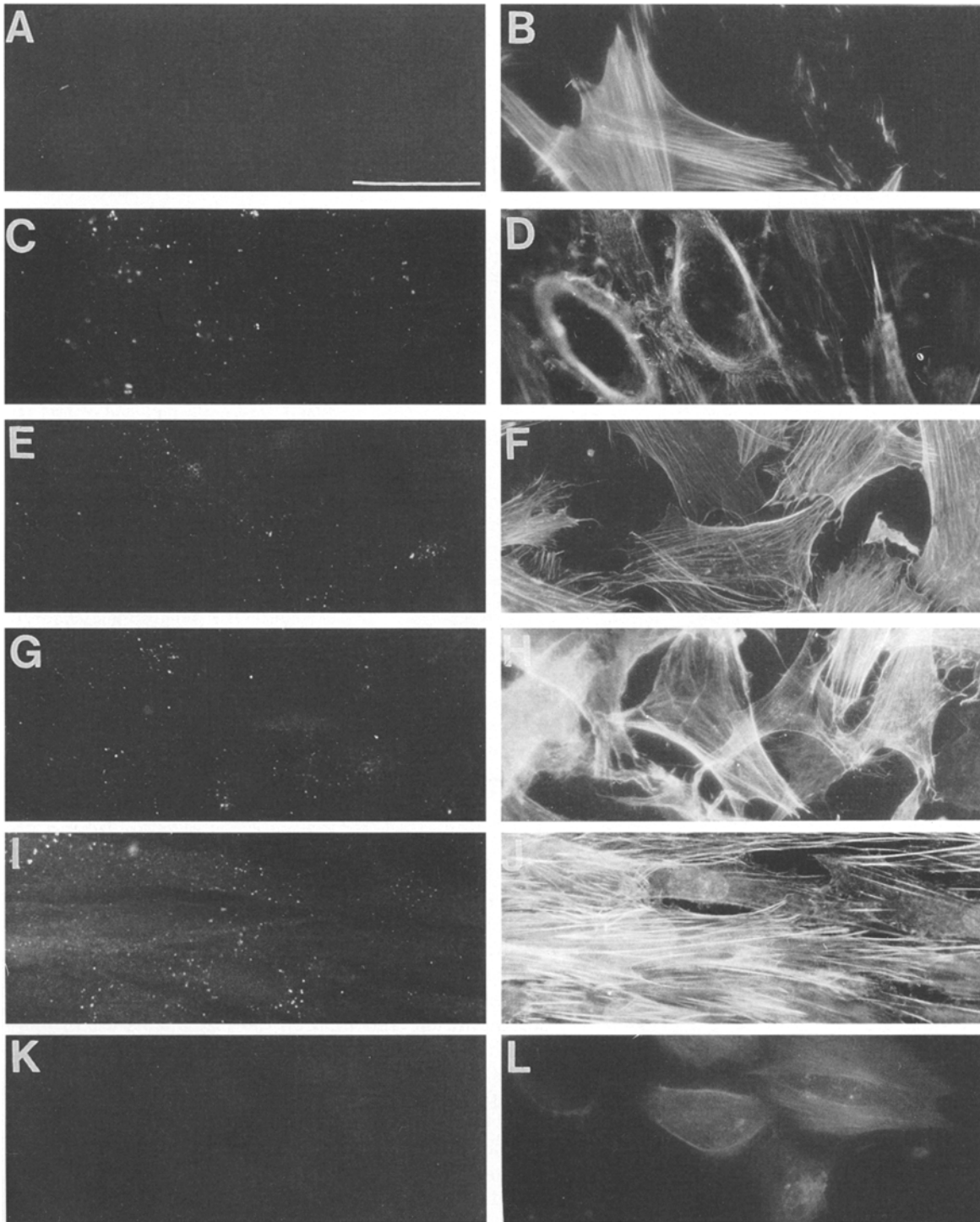
Figure 3. Plating-induced formation of PDGF β-receptor granules in AG 1518 fibroblasts. (*A, C, E, G, I,* and *K*), immunofluorescence stainings of PDGF β-receptors using PDGFR-B2 IgG; (*B, D, F, H, J,* and *L*), detection of F-actin by Bodipy FL-Phallacidin. Both reagents were used in the double fluorescence staining procedure described in Materials and Methods. Double-stained areas are represented side by side in the figure. In *A–D* cells had been cultured on glass coverslips in DME containing 10% FBS and subsequently serum depleted for a 24-h time period in MCDB 104 medium. In *E–L* cells had been cultured on glass coverslips coated with the indicated substances for 2 h in MCDB 104 medium. (*A* and *B*) Nonstimulated AG 1518 fibroblasts; (*C* and *D*) AG 1518 fibroblasts stimulated for 15 min with 50 ng/ml of PDGF-BB. Note the formation of PDGF β-receptor granules in *C*, and circular actin ruffles in the same cells (*D*); (*E* and *F*) AG 1518 fibroblasts plated on collagen type I–coated coverslips; (*G* and *H*) AG 1518 fibroblasts plated on human plasma fibronectin-coated coverslips; (*I* and *J*) AG 1518 fibroblasts plated on polyclonal anti-β$_1$ integrin IgG-coated coverslips; (*K* and *L*) AG 1518 fibroblasts plated on poly-L-lysine-coated coverslips. Note the presence of PDGF β-receptor granules in (*E, G,* and *I*), but absence of circular actin ruffles in the corresponding cells (*F, H,* and *J*). No PDGF β-receptor granules or circular actin ruffles were detected in cells plated on poly-L-lysine (*K* and *L*). Bar, 40 μm.
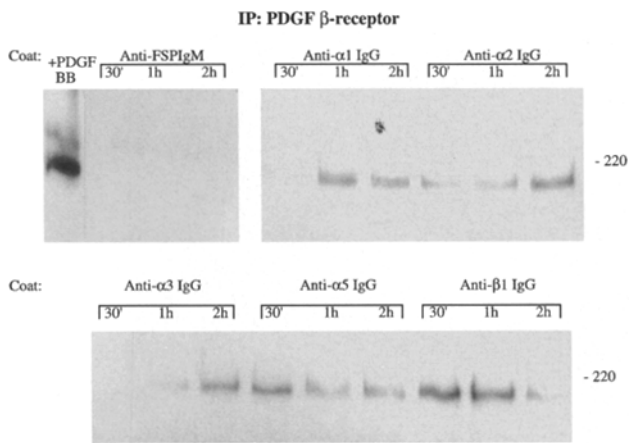
IP: PDGF β-receptor

Figure 4. Tyrosine phosphorylation of PDGF β-receptors induced by plating AG 1518 fibroblasts on anti-integrin subunit IgG. Cells were plated and cultured in MCDB 104 medium for the indicated time periods. Monoclonal anti-integrin subunit IgG, or anti-fibroblast surface marker IgM were immobilized on plastic culture dishes pre-coated with goat anti–mouse immunoglobulin F(ab)₂ fragments as described in Material and Methods. Antibodies used were: TS2/7 (anti-α1); P1E6 (anti-α2); P1B3 (anti-α3); P1D6 (anti-α5); P4C10 (anti-β1); and 1B10 (anti–human fibroblast surface protein; FSP IgM). PDGF β-receptors were immunoprecipitated with specific polyclonal anti-PDGF β-receptor IgG, and tyrosine phosphorylation was detected by immunoblotting after separation of precipitated proteins by SDS-PAGE. The position of pre-stained myosin, migrating as a 220,000-M_r protein in SDS-PAGE is indicated. Phosphorylation of PDGF β-receptors achieved after stimulation of cells with 20 ng/ml of PDGF-BB for 15 min is shown.

goat anti–rabbit IgG for different time periods. This treatment resulted in a weak tyrosine phosphorylation of PDGF β-receptors after a 30-min incubation period and an increased phosphorylation at 120 min (Fig. 5 *D*) amounting to 28% of the maximal response that was achieved after PDGF-BB stimulation. Cells incubated with 250 μg/ml of polyclonal anti-β₁ integrin IgG only, exhibited a weak tyrosine phosphorylation throughout the 2-h time course, averaging 3.5% of the maximal PDGF-BB response (Fig. 5 *C*). Cells incubated with 250 μg/ml of

polyclonal anti-β₁ integrin IgG and then secondary antibodies exhibited extensive integrin receptor patching as determined by immunofluorescence (Fig. 6 *B*). However, when cells were incubated with primary antibody alone, only low amounts of patches were discerned (Fig. 6 *A*). No integrin patches were observed when incubating with non-immune rabbit IgG (250 μg/ml) or secondary antibodies alone (data not shown).

Suspended AG 1518 fibroblasts incubated in MCDB 104 containing 250 μg/ml of polyclonal anti-β₁ integrin IgG exhibited no tyrosine phosphorylation of PDGF β-receptors (Fig. 5 *A*). However, when cells were incubated in suspension with polyclonal anti-β₁ integrin IgG antibodies immobilized on protein A–Sepharose, tyrosine phosphorylation of PDGF β-receptors was evident at 30 min, thereafter the signal decreased over the 2-h observation period (Fig. 5 *B*).

PDGF β-Receptors are Phosphorylated in AG 1518 Fibroblasts during the Process of Collagen Gel Contraction

AG 1518 fibroblasts readily contract three-dimensional collagen gels in a process that is stimulated by, but not dependent on, PDGF or serum (64), and that is mediated by β₁ integrins (24). A prominent phosphorylation of PDGF β-receptors in AG 1518 fibroblasts during the process of contracting free-floating gels (relaxed condition) was evident 4 h after that gels had been flotated (20% of maximal response), corresponding to 5 h after seeding of the collagen/cell mixture (Fig. 7 *B*). Fibroblasts cultured in attached collagen gels (stressed conditions) also exhibited a strong phosphorylation of PDGF β-receptors, but the response followed a different time course with a peak in the phosphorylation at 1.5 and 5 h after initiation of cultures with relative levels of phosphorylation corresponding to 13 and 11% of the maximal response, respectively, (Fig. 7 *A*). In lysates of AG 1518 fibroblasts cultured both under stressed and relaxed conditions a ∼110,000-M_r protein that was phosphorylated on tyrosine co-precipitated with the PDGF β-receptor. The identity of this latter protein is not known but the degree of tyrosine phosphorylation of this protein closely followed that of PDGF β-receptors.
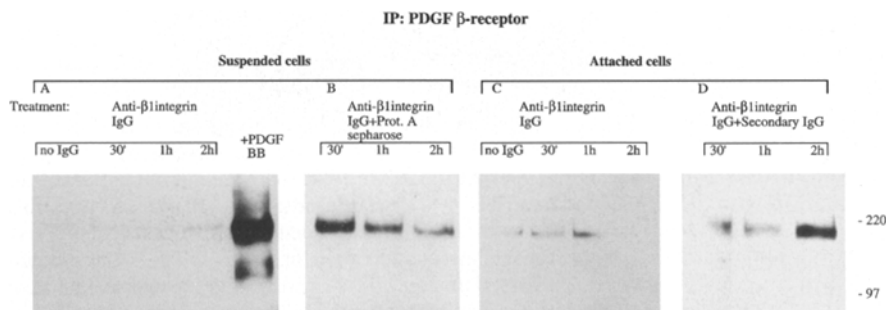

IP: PDGF β-receptor

Figure 5. Tyrosine phosphorylation of PDGF β-receptors in AG 1518 fibroblasts induced by clustering cell surface β₁ integrins by antibodies. (*A* and *B*), AG 1518 fibroblasts suspended in 600 μl MCDB 104 medium; (*C* and *D*) AG 1518 fibroblasts grown to confluence in DMEM with 10% FBS and subsequently serum-depleted for 24 h in MCDB 104 medium. (*A* and *C*) cells incubated with 250 μg/ml polyclonal anti-β₁ integrin IgG for the indicated time periods; (*B*) cells incubated with 150 μg polyclonal anti-β₁ integrin IgG coupled to 50 μl protein A–Sepharose; (*D*) cells incubated with 250 μg/ml polyclonal anti-β₁ integrin IgG for 30 min and subsequently with 10 μg/ml goat anti–rabbit IgG for the indicated time periods. PDGF β-receptors were immunoprecipitated with specific polyclonal anti-PDGF β-receptor IgG, and tyrosine phosphorylation was detected by immunoblotting after separation of precipitated proteins by SDS-PAGE. The positions of pre-stained myosin and phosphorylase B, migrating as 220,000- and 97,000-M_r proteins are indicated. Phosphorylation of PDGF β-receptors achieved after stimulation of cells with 20 ng/ml of PDGF-BB for 15 min is shown in *A*.
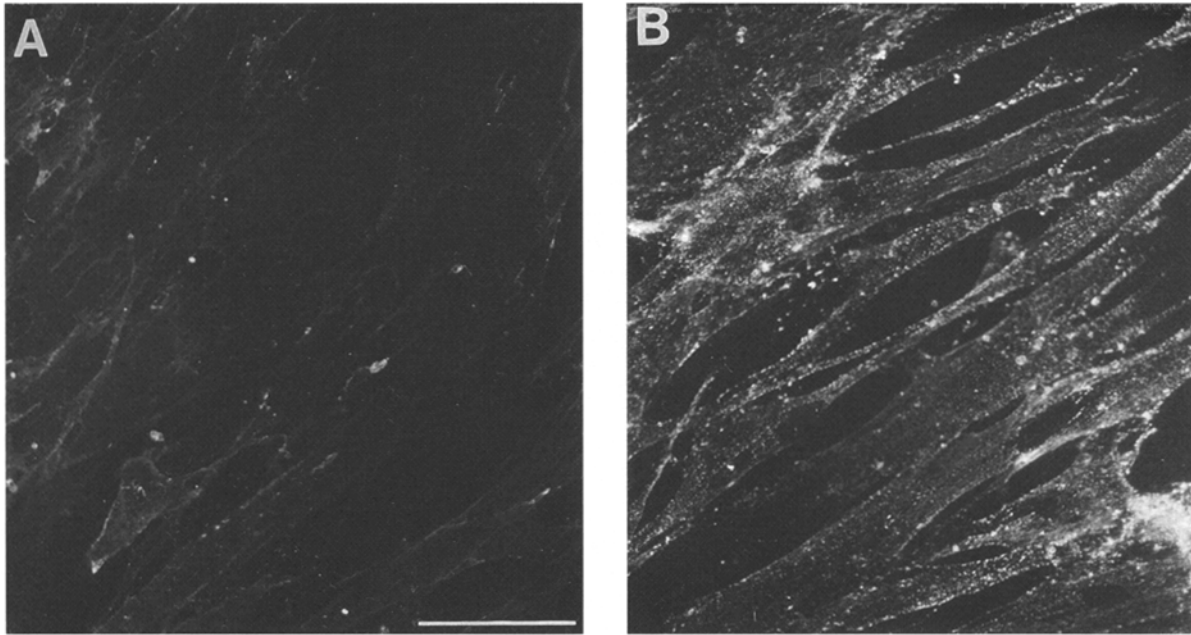
Figure 6. Appearance of clustered cell-surface β_1 integrins on AG 1518 fibroblasts. AG 1518 fibroblasts grown to confluence in DME with 10% FBS and subsequently serum-depleted for 24 h in MCDB 104 medium. (*A*) Fibroblasts incubated with 250 μg/ml polyclonal anti-β_1 integrin IgG for 30 min prior to fixation and staining with goat anti–rabbit FITC; (*B*) fibroblasts incubated with 250 μg/ml polyclonal anti-β_1 integrin IgG for 30 min, washed, and further incubated with 10 μg/ml of goat anti–rabbit IgG prior to fixation and staining with goat anti–rabbit FITC. Bar, 40 μm.

PDGF β-Receptor Phosphorylation is Induced in AG 1518 Fibroblasts in Response to the Application of External Tension on the Cells

To assess the effect of the PDGF β-receptor phosphorylation by external strain on cell–substrate adhesions AG 1518 fibroblasts attached to collagen type I and fibronectin were subjected to a centrifugal force that was directed perpendicular to the plane of the cell–substrate interphase using the technology detailed in Materials and Methods. The centrifuge used, enabled precise control of acceleration, de-acceleration, temperature, and final G-force value. Cultures were centrifuged with constant acceleration and deceleration rates but with final G-force values ranging from 2–64 g. Tyrosine phosphorylation of PDGF β-receptors was evident in cells plated on collagen type I that had been exposed to centrifugal force for 2 min with a peak in phosphorylation after 5 min (Fig. 8 *A* and 9) and a subsequent decrease in tyrosine phosphorylation after 10 min. Tyrosine phosphorylation of PDGF β-receptors increased with increasing final G-force values up to 32 g. Fibroblasts remained attached during centrifugation even at 64 g for 10 min, and, in addition, responded to stimulation with 20 ng/ml PDGF-BB by tyrosine phosphorylation of PDGF β-receptors when stimulated immediately after being exposed to centrifugal forces (Fig. 8 *D*). It can therefore be concluded that the decrease in tyrosine phosphorylation, noticeable after centrifugation for 10 min, was not due to detachment of cells, nor to downregulation of PDGF β-receptors from the cell surface. Cells attached to fibronectin exhibited a similar response of tyrosine phosphorylation of PDGF β-receptors when exposed to centrifugal force, however, the peak in tyrosine phosphorylation

was observed already after 2 min (Figs. 8 *D* and 9). Cells incubated in medium containing 100 μM sodium orthovanadate but not subjected to centrifugation exhibited a slight increase in tyrosine phosphorylation (3% of maximal response) of PDGF β-receptors above background levels (0.5% of maximal response) after a 5-min incubation period compared to uncentrifuged cells incubated in the absence of orthovanadate (Fig. 8 *C*).
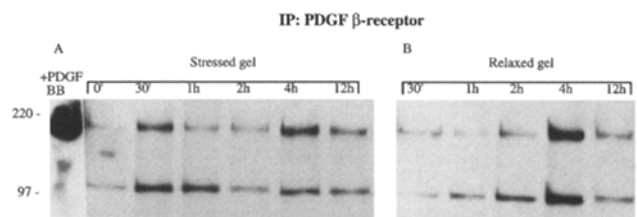

Figure 7. Induction of tyrosine phosphorylation of PDGF β-receptors in AG 1518 fibroblasts cultured in three-dimensional collagen gels. (*A*) AG 1518 fibroblasts cultured and maintained under "stressed" (see text) conditions; stressed; and (*B*) AG 1518 fibroblasts cultured and maintained under "relaxed" (see text) conditions. Incubations were performed in MCDB 104 medium for the indicated time periods referring to the times after gelation of the lattices. PDGF β-receptors were immunoprecipitated with specific polyclonal anti-PDGF β-receptor IgG, and tyrosine phosphorylation was detected by immunoblotting after separation of precipitated proteins by SDS-PAGE. The positions of pre-stained myosin and phosphorylase B, migrating as 220,000- and 97,000-M_r proteins are indicated. Phosphorylation of PDGF β-receptors achieved after stimulation of cells with 20 ng/ml of PDGF-BB for 15 min is shown in *A*.
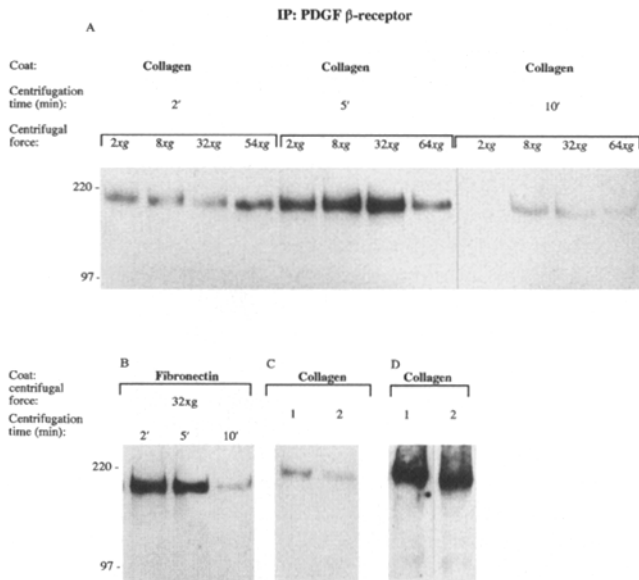
Figure 8. Induction of PDGF β-receptor tyrosine phosphorylation in AG 1518 fibroblasts exposed to centrifugal forces. Cells were plated on the respective substrates and cultured in MCDB 104 medium prior to exposure to centrifugal forces as detailed in Material and Methods. Final G-force values (*g*) and time periods are indicated in *A* and *B*. (*A*) Fibroblasts plated on collagen type I; (*B*) fibroblasts plated on human plasma fibronectin. (*C*) Fibroblasts plated on collagen type I and cultured in MCDB 104, and then incubated (without being subjected to centrifugation) for 5 min in the presence (lane *1*), or absence (lane *2*) of 100 μM orthovanadate; (*D*) fibroblasts plated on collagen type I and cultured in MCDB medium, centrifuged for 10 min at 2 *g* (lane *1*) and 64 *g* (lane *2*) and thereafter stimulated with 50 ng/ml of PDGF-BB for 15 min. PDGF β-receptors were immunoprecipitated with specific polyclonal anti-PDGF β-receptor IgG, and tyrosine phosphorylation was detected by immunoblotting after separation of precipitated proteins by SDS-PAGE. The positions of prestained myosin and phosphorylase B, migrating as 220,000- and 97,000-M_r proteins are indicated in *A* and *B*.

Figure 9. Time dependence of the induction of PDGF β-receptor tyrosine phosphorylation by centrifugal forces. Laser densitometric scanning was performed on the exposed films from the experiment shown in Fig. 8, *A* and *B*. Phosphorylation signals are expressed as percentages of the signal recorded for confluent fibroblasts that had been stimulated with 20 ng/ml of PDGF-BB for 15 min. (□) Cells plated on collagen type I, 2 *g*; (○) cells plated on collagen type I, 8 *g*; (△) cells plated on collagen type I, 32 *g*; (◇) cells plated on collagen type I, 64 *g*; (▲) cells plated on human plasma fibronectin, 32 *g*.

Discussion

We have demonstrated that PDGF β-receptors in AG 1518 diploid human fibroblasts become phosphorylated on tyrosine residues in response to changes in cell shape, or in response to external strain exerted on the cells. This tyrosine phosphorylation occurred in the absence of exogenously added PDGF. The response was apparent during attachment, and early phases of spreading on fibronectin or collagen type I, but not when fibroblasts were plated on poly-L-lysine. Furthermore, tyrosine phosphorylation of PDGF β-receptors occurred during collagen gel contraction and when cells were exposed to a centrifugal force. As assessed from laser densitrometric scanning, the magnitudes of the tyrosine phosphorylation responses in all these cases amounted to between 20 and 30% of the response achieved by stimulation with 20 ng/ml of PDGF-BB for 15 min. Furthermore, we demonstrated that PDGF β-receptors become clustered in response to plating of AG 1518 fibroblasts on fibronectin and collagen type I. These receptor clusters were morphologically indistinguishable from the intracellular granular accumulations seen after
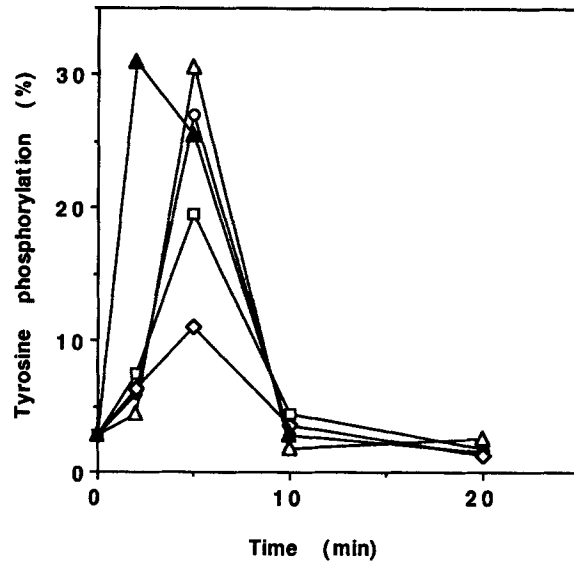
stimulation of these cells with PDGF-BB (62, 65), indicating that plating-induced tyrosine phosphorylation leads to an internalization of PDGF β-receptors. Tyrosine phosphorylation of PDGF β-receptors occurred when cells were plated on immobilized anti-$β_1$ integrin IgG, but not when they were plated on IgG directed to a fibroblast (nonintegrin) cell surface marker. It can therefore be concluded that the plating-induced response is a result of $β_1$ integrin-mediated adhesion reactions. We also investigated if the effects on PDGF β-receptors could be attributed to a specific $β_1$-integrin. Experiments in which AG 1518 fibroblasts were plated on immobilized monoclonal anti-integrin subunit IgG revealed that PDGF β-receptor tyrosine phosphorylation was elicited in response to engagement of the integrins $α_1β_1$, $α_2β_1$, $α_3β_1$, and $α_5β_1$. These results demonstrate that the various integrin α subunits do not confer specificity to the plating-induced tyrosine phosphorylation of PDGF β-receptors. Taken together, our findings demonstrate the existence of a cross-talk between $β_1$ integrin-mediated adhesion reactions and tyrosine phosphorylation of PDGF β-receptors. Furthermore, the findings raise the interesting possibility that part of the signal transduction events that are evoked by $β_1$ integrin engagement (13, 55) in fact may be dependent on activation of PDGF β-receptors.

The present data suggest that $β_1$ integrin-mediated tyrosine phosphorylation of PDGF β-receptors does not involve auto- or paracrine stimulation by PDGF-AB/BB. This notion is based on several observations. The kinetics of the reactions, displaying maximal responses at early

time points; minutes in the case of exposure to centrifugal forces; and within one and two hours in the cases of plating and collagen gel contraction, makes it unlikely that the observed effect depends on de novo synthesis of PDGF B-chains. Moreover, PDGF B-chain mRNA was not detected in AG 1518 fibroblasts by Northern blotting (data not shown) and no expression of PDGF-AB/BB protein using the specific monoclonal anti-PDGF-AB/BB antibody PGF 007 in immunofluorescence studies could be detected in AG 1518 fibroblasts. More importantly, the plating-induced tyrosine phosphorylation of PDGF β-receptors was insensitive to suramin, as well as to specific anti-PDGF-BB IgG, both being able to inhibit the activation of PDGF β-receptors in response to exogenous PDGF-BB, added in concentrations of 50 ng/ml or less. Taken together, our data exclude that the β_1 integrin-mediated PDGF β-receptor tyrosine phosphorylation is dependent on stimulation of PDGF B-chain synthesis or on release of stored PDGF-AB/BB.

Cell surface EGF receptors on AG 1518 fibroblasts were not phosphorylated in response to plating on fibronectin or collagen type I. Similarly, no tyrosine phosphorylation of PDGF α-receptors was detected in response to plating of the fibroblasts on these ECM glycoproteins. Since human diploid foreskin fibroblasts express low levels of PDGF α-receptors compared to PDGF β-receptors (58), it can, however, not be excluded that a possible plating-induced tyrosine phosphorylation of PDGF α-receptors remained undetected by the present assay system. Nevertheless, the fact that EGF receptors were not phosphorylated suggests a degree of specificity regarding the observed tyrosine phosphorylation of PDGF β-receptors, among tyrosine kinase receptors, elicited by plating on ECM components.

A transient tyrosine phosphorylation of PDGF β-receptors was elicited after clustering of integrin β_1 subunits at the cell surface. Binding of rabbit polyclonal anti-β_1 integrin IgG to fibroblasts that had been cultured for several days and possessed well-developed focal adhesions, did not induce a tyrosine phosphorylation response. In order for PDGF β-receptor tyrosine phosphorylation to occur, bound anti-β_1 integrin IgG had to be clustered by anti-rabbit IgG. Similarly, mere binding of anti-β_1 integrin IgG to the surface of suspended AG 1518 fibroblasts did not lead to tyrosine phosphorylation of PDGF β-receptors. Such a response could, however, be elicited in suspended cells, by means of presenting the anti-β_1 integrin IgG immobilized to protein A–Sepharose, in a process that involves clustering of integrins. Thus, induction of β_1 integrin clusters at the cell surface provides the necessary signal for induction of PDGF β-receptor tyrosine phosphorylation, whereas integrins in already established focal adhesions are unable to induce such a response. These data suggest that PDGF β-receptor tyrosine phosphorylation may be an important element during the dynamic phase of β_1 integrin-mediated adhesion reactions.

The above concept was also supported by the transient tyrosine phosphorylation of PDGF β-receptors initiated during contraction of AG 1518 fibroblast-populated collagen matrices. Cell-mediated collagen gel contraction is an obviously dynamic process, dependent on an intact actin cytoskeleton, and resulting from compaction of the col-

lagen fiber network by elongated fibroblasts (4, 22, 66) through the process of "cell traction" (26). During this process, integrins form stable contacts with the collagen fibers; these contacts should be operative for a certain time period after which they are to be dismantled allowing for new contacts to be formed. Such a model is supported by the recent finding that the β_1 integrin-activating antibody TS2/16 paradoxically inhibits contraction of smooth muscle cell–populated collagen matrices (42). In anchored AG 1518 fibroblast-populated collagen gels, in which contraction is mechanically impeded ("stressed" conditions; 43), tyrosine phosphorylation of PDGF β-receptors peaked at 1 and 4 h. In floating collagen matrices ("relaxed" conditions) tyrosine phosphorylation peaked at 4 h. This difference in timing of the response may be related to differences in the mechanical tension during contraction. Floating collagen gels remain mechanically relaxed during contraction in contrast to anchored lattices where a high mechanical stress is generated (47). Our results indicate that tyrosine phosphorylation of PDGF β-receptors in collagen gels coincides with the buildup of mechanical stress and subsequent collagen gel contraction. Thus our findings support the view that tyrosine phosphorylation of PDGF β-receptors is elicited during dynamic phases of β_1 integrin mediated adhesion processes. It has been reported that PDGF β-receptors become functionally refractory in fibroblasts that are cultured in contracting relaxed collagen gels (43, 64). The present results add the possibility that a persistent remodelling of integrin-mediated contacts during contraction, leading to the observed PDGF-BB–independent phsphorylation of PDGF β-receptors, makes the latter nonavailable for stimulation by added ligand.

A correlation between alterations in mechanical tension and PDGF β-receptor tyrosine phosphorylation in AG 1518 fibroblasts was further supported by experiments in which cells were exposed to centrifugal forces. Under the experimental conditions used focal adhesions already formed with the underlying substratum were challenged by forces induced through centrifugation. A rapid and transient PDGF β-receptor tyrosine phosphorylation was elicited in response to a centrifugal force directed upwards from, and perpendicular to, the plane of the culture dishes. This phosphorylation response was dependent on the magnitude of the centrifugal force applied to the cells up to a force of 32 g, at 64 g the phosphorylation response decreased in magnitude. The latter effect was not due to that cells detached from the substrate. Furthermore, immediately after centrifugation fibroblasts responded to stimulation by PDGF-BB with a rapid and prominent tyrosine phosphorylation of PDGF β-receptors. However, it can not be ruled out that the observed decrease in tyrosine phosphorylation after centrifugation at 64 g is due to mechanical damage inflicted on the cells. The time needed to reach the final G-force value in the centrifuge varied from 30 s to over 2 min when cells were centrifuged at 64 g. The transient character of the tyrosine phosphorylation response, with maximal phosphorylation achieved after 5 min, thereafter declining to background levels regardless of final G-force value, indicate that when cells have achieved a constant tension, adaption occurs. Mechanoreceptors often register dynamic rather than static mechanical forces, and adaption occurs when a displacement stim-

ulus is constant (41) as is the case in our experimental system when cells have reached the final G-force value. Adaption enables mechanoreceptors to remain highly sensitive to changes in environment. These results further support that the phosphorylation response of PDGF β-receptors is elicited in response to changes in tension exerted on the cells, and that this effect can be induced in already established focal adhesions.

The signal transduction pathway involved in plating-induced tyrosine phosphorylation of PDGF β-receptors is not known. One possibility is that the tyrosine phosphorylation of PDGF β-receptors is carried out by tyrosine kinases, e.g., p125^{FAK}, that become activated as part of the integrin-signaling pathway (13, 55). Alternatively, integrin engagement could induce changes in the spatial distribution of PDGF β-receptors in a manner that leads to their dimerization and activation. In experiments aimed to discriminate between these possibilities we took advantage of porcine aortic endothelial (PAE) cells transfected with PDGF β-receptor cDNA constructs (17). Plating-induced PDGF β-receptor tyrosine phosphorylation was observed when PAE cells, transfected with the wildtype receptor, were plated on fibronectin (manuscript in preparation). In PAE cells expressing kinase-inactivated (K634A) receptors (62) no plating-induced tyrosine phosphorylation of PDGF β-receptors was observed. From these experiments it is possible to conclude that plating-induced PDGF β-receptor tyrosine phosphorylation is dependent on the intrinsic tyrosine kinase activity of the receptor. Thus it seems less likely that integrin-mediated activation of tyrosine kinases such as p125^{FAK} phosphorylate PDGF β-receptors.

The cross-talk between PDGF β-receptors and β_1 integrins has several implications. An obvious possibility is that this phosphorylation response, at least in part, is responsible for integrin-mediated signaling events. Thus, PDGF β-receptor activation could constitute an upstream signaling event in the phosphorylation of p125^{FAK}, paxillin, and the MAP kinases. Furthermore, the integrin-mediated phosphorylation of PDGF β-receptors could be part of a positive feed-back loop-strengthening cell–ECM contact. In favor of such a hypothesis are the findings that the phosphorylation response was observed during cell spreading, collagen gel contraction and when cells were exposed to external strain. Evidence that receptor tyrosine kinases stimulates integrin-mediated adhesion in reactions not likely to be dependent on stimulation of de novo biosynthesis of integrins include stem cell factor–enhanced β_1 integrin-mediated adhesion of mast cells to fibronectin (59), PDGF-BB stimulated contraction of β_1 integrin-mediated fibroblast-populated collagen gel contraction (1, 24) and smooth muscle cell migration through collagen-coated membranes (61).

In summary, the present study provides evidence for a coupling between β_1 integrin-mediated adhesion reactions and ligand-independent activation of PDGF β-receptors in AG 1518 fibroblasts. This activation is elicited during the dynamic phases of adhesion and spreading, collagen gel contraction and when external force is applied to the cells. Furthermore, the signal leading to activation of PDGF β-receptors is dependent on clustering of β_1 integrins at the cell surface. The results demonstrate a novel, potential regulatory mechanism for cell behavior based on a cross-talk between adhesion and tyrosine kinase receptors.

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