The A-type receptor for platelet-derived growth factor mediates protein tyrosine phosphorylation, receptor transmodulation and a mitogenic response

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A clonal human glioma cell line, U-343 MGa 31L, which expresses the A-type but not the B-type receptor for platelet-derived growth factor (PDGF), was used in a functional study of the A-type receptor. PDGF-AA induced, in a dose- and time-dependent manner, phosphorylation on tyrosine residues of the receptor in metabolically labelled cells. The optimal dose was around 30 ng/ml; at 100 ng/ml, phosphorylation was maximal at 15 min and had almost returned to the control level after 60 min. The phosphorylation on tyrosine residues of the PDGF A-type receptor was stimulated by PDGF-AA, PDGF-AB and PDGF-BB; these isoforms also stimulated [³H]thymidine incorporation into U-343 MGa 31L cells. In addition, activation of the A-type PDGF receptor induced transmodulation of the epidermal growth factor receptor.

INTRODUCTION

Platelet-derived growth factor (PDGF) is a major mitogen in serum for connective tissue and glial cells (reviewed in Heldin *et al.*, 1985; Ross *et al.*, 1986). It is a disulphide-bonded dimer of two similar polypeptides, denoted A and B (Johnsson *et al.*, 1982). All three combinations of polypeptide chains (PDGF-AA, PDGF-AB and PDGF-BB) have been identified and purified from platelets or transformed cells (Doolittle *et al.*, 1983; Robbins *et al.*, 1983; Waterfield *et al.*, 1983; Stroobant & Waterfield, 1984; Heldin *et al.*, 1986; Hammacher *et al.*, 1988a,b). Large quantities of the various isoforms of PDGF are now available through expression in bacteria, yeast and mammalian cells (Östman *et al.*, 1988, 1989; Hoppe *et al.*, 1989). The PDGF isoforms differ in their functional effects on human fibroblasts; whereas PDGF-AA induces transmodulation of the receptor for epidermal growth factor (EGF) to the same extent as the other isoforms, it is a less potent mitogenic agent, and has no stimulatory effect on actin reorganization or chemotaxis (Nistér *et al.*, 1988). PDGF-AA is a potent mitogen, however, for Swiss 3T3 cells (Kazlauskas *et al.*, 1988) and O-2A progenitor cells of the rat optic nerve (Pringle *et al.*, 1989).

The action of PDGF is mediated by specific receptors on the target cells. The functional differences and binding characteristics of the isoforms have led to the identification of two classes of receptors (Hart *et al.*, 1988; Heldin *et al.*, 1988). The PDGF B-type receptor, which binds PDGF-BB with high affinity, and PDGF-AB with about 10 times lower affinity (Severinsson *et al.*, 1989), has been purified from pig uterus (Rönstrand *et al.*, 1987) and from 3T3 cells (Daniel *et al.*, 1985; Bishayee *et al.*, 1986). The PDGF B-type receptor is synthesized as a precursor of 160 kDa that matures to about 180 kDa (Claesson-Welsh *et al.*, 1987; Hart *et al.*, 1987; Keating

& Williams, 1987). Its amino acid sequence has been deduced from the nucleotide sequence of cDNA clones of murine (Yarden *et al.*, 1986) and human (Claesson-Welsh *et al.*, 1988; Escobedo *et al.*, 1988; Gronwald *et al.*, 1988) origin. The PDGF A-type receptor binds all three isoforms with high affinity (Heldin *et al.*, 1988); it is synthesized as a precursor of 140 kDa which is converted to a 170 kDa mature form (Claesson-Welsh *et al.*, 1989a). Recent cloning of the PDGF A-type receptor cDNA (Claesson-Welsh *et al.*, 1989b; Matsui *et al.*, 1989) revealed a close structural similarity to the B-type receptor; both receptors have extracellular parts which can be divided into five immunoglobulin-like domains, and the cytoplasmic parts possess split protein tyrosine kinase domains.

In order to characterize further the A-type receptor, we have utilized a human clonal glioma cell line, U-343 MGa 31L, that has only type A receptors (Claesson-Welsh *et al.*, 1989a). Data presented in this paper establish that activation of the PDGF A-type receptor induces EGF receptor transmodulation and protein tyrosine phosphorylation, and provide an initial characterization of the phosphorylation of the receptor. In addition, in contrast to the situation prevailing in human fibroblasts (Nistér *et al.*, 1988), we show that PDGF-AA is an approximately equally potent mitogen as PDGF-BB for U-343 MGa 31L cells.

EXPERIMENTAL

Purification of PDGF

Recombinant PDGF-AA from the yeast strain *Saccharomyces cerevisiae* was purified as described (Östman *et al.*, 1989). PDGF-AB and PDGF-BB were purified from human platelet PDGF (Heldin *et al.*, 1987) using immobilized metal-ion-affinity chromatography (Hammacher *et al.*, 1988b).

Culturing and metabolic labelling of cells

U-343 MGa 31L clonal human glioma cells were cultured in Eagle's minimal essential medium (MEM) containing 10% fetal calf serum (Flow Laboratories), 100 units of penicillin/ml and 50 µg of streptomycin/ml (Sigma). For ^{32}P -labelling, cells forming a confluent monolayer in 50 mm-diam. Petri dishes (Falcon) were incubated in phosphate-free medium (Dulbecco's MEM/Ham's F12 medium, 1:1, v/v) supplemented with 10 µg of transferrin/ml, 30 nM- Na_2SeO_3 (kindly donated by Dr. E. J. J. van Zoelen, Nijmegen, The Netherlands), and 0.04 mg of human serum albumin/ml (Kabi Vitrum) for 4.5 h. The cells were washed once in the same medium and further incubated for 2 h in the phosphate-free medium/0.04 mg of human serum albumin/ml/15 mM-Hepes, pH 7.4, supplemented with $[\text{P}^{32}]\text{P}_i$ (Amersham; specific radioactivity 10 mCi/ml) at a concentration of 1 mCi/ml. For ^{35}S -labelling, cells forming a confluent monolayer in 50 mm Petri dishes were labelled in cysteine- and methionine-free MCDB 105 medium (McKeehan *et al.*, 1977) (1 mM- Ca^{2+}), supplemented with $[\text{S}^{35}]\text{cysteine}$ and $[\text{S}^{35}]\text{methionine}$ (Amersham; specific activities > 800 Ci/mmol and 600 Ci/mmol respectively). The ^{35}S -labelled amino acids were added at concentrations of 50–100 µCi/ml each.

Antibodies

The rabbit antiserum PDGFR-1 was raised against a highly purified pig PDGF B-type receptor preparation (Rönstrand *et al.*, 1987) and was found to recognize both the PDGF A- and B-type receptors (Claesson-Welsh *et al.*, 1989a). The rabbit antiserum PDGFR-3 was raised against a synthetic peptide corresponding to amino acids 981–994 in the deduced murine PDGF B-type receptor sequence (Yarden *et al.*, 1986); it specifically recognizes B-type receptors (Claesson-Welsh *et al.*, 1989a). The phosphotyrosine antiserum was raised in rabbits against phosphotyrosine (Ek & Heldin, 1984) and was used with or without blocking with 40 mM-phenyl phosphate (Sigma).

Lysis of cells and immunoprecipitation

Cells were lysed in 0.15 M-NaCl/20 mM-Tris (pH 7.5)/1% Triton X-100 (Merck)/0.1% SDS (BDH)/1% deoxycholate (Merck)/10 mM-EDTA/50 µM-sodium orthovanadate (Sigma)/1 mM-phenylmethanesulphonyl fluoride (Sigma)/1% Trasylol (Bayer, Leverkusen, Germany). The lysates were centrifuged at 10000 g for 30 min at 4°C in an Eppendorf table-top centrifuge and the supernatants were precleared by incubation with Protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology Inc.) for 30 min at 4°C before addition of antiserum. Typically, 10 µl of immune serum was added to each sample and incubated for 2–12 h at 4°C, followed by 50 µl (bead volume) of Protein A-Sepharose CL-4B for 30 min. The beads were washed four times in 0.5 M-NaCl/20 mM-Tris (pH 7.5)/0.1% Triton X-100/0.1% Tween 80 (Merck)/5 mg of bovine serum albumin (Boehringer Mannheim)/ml, and once in 20 mM-Hepes (pH 7.4) or water. Immune complexes were eluted by heating the beads for 3 min at 96°C or for 20 min at 65°C in sample buffer containing 0.4% SDS/0.2 M-Tris (pH 8.8)/0.5 M-sucrose/0.01% Bromophenol Blue (Merck)/20 mM-dithiothreitol.

Polyacrylamide-gel electrophoresis

Reduced samples were electrophoresed in 5–12% gradient SDS/polyacrylamide slab gels as described (Blobel & Dobberstein, 1975). Chemicals were from Bio-Rad. Gels were prepared for fluorography by soaking in Amplify (Amersham), and were dried and exposed to Hyperfilm MP (Amersham).

Incorporation of $[\text{H}^3]\text{thymidine}$

U-343 MGa 31L cells were seeded sparsely on to 35 mm Petri dishes in Eagle's MEM with 10% newborn calf serum (Gibco) and antibiotics as above. At 4 days after subcultivation, the medium was changed to serum-free MCDB 104 with a reduced Ca^{2+} concentration (0.5 mM) (Betsholtz & Westermark, 1984), supplemented with 0.04 mg of human serum albumin/ml. After 48 h, the medium was changed and $[\text{H}^3]\text{thymidine}$ (0.02 µCi/ml) was added, together with the PDGF isoforms at various concentrations. Another 48 h after the additions, the cells were harvested by trichloroacetic acid precipitation.

Fig. 1. Immunoprecipitation of ^{32}P -labelled PDGF A-type receptor

Confluent U-343 MGa 31L cells were incubated for 4.5 h in phosphate-free medium containing human serum albumin, washed once in the same medium and labelled with 1 mCi of $[\text{P}^{32}]\text{P}_i$ /ml for 2.5 h at 37°C. The cells were then incubated without (–) or with (+) 100 ng of PDGF-AA/ml for an additional 10 min before lysis and immunoprecipitation. The immunoprecipitates were analysed by SDS/gel electrophoresis and autoradiography as described in the Experimental section. Samples were precipitated consecutively with non-immune serum (NRS) and PDGFR-3, divided into three equal parts and incubated with PDGFR-1 or phosphotyrosine antiserum (P-Tyr) incubated with (+) or without (–) 40 mM-phenyl phosphate. The relative migration positions of M_r standards (myosin, 200000; *Escherichia coli* β -galactosidase, 116250; rabbit muscle phosphorylase b, 97000; bovine serum albumin, 66200) run in parallel are indicated in this Figure and in Figs. 2, 3 and 4, as well as the migration position of the mature form of the PDGF A-type receptor (arrow).

Fig. 2. Time-dependent phosphorylation of PDGF A-type receptor by PDGF-AA

Confluent U-343 MGa 31L cells were labelled with [35 S]cysteine and [35 S]methionine for a total of 4 h at 37 °C before lysis and immunoprecipitation. At the end of the labelling time, the cells were incubated with 100 ng of PDGF-AA/ml for 5 min (lanes b), 15 min (lanes c), 30 min (lanes d) or 60 min (lanes e). Incubation for 5 min with diluent was used as control (lanes a). The lysates were divided into two equal parts and immunoprecipitated with phosphotyrosine antiserum (*P-Tyr*) blocked with 40 mM-phenyl phosphate (+) or not (–), followed by normal rabbit serum (NRS) and PDGFR-1 as controls for residual antigen. The immune complexes were analysed by SDS/gel electrophoresis and fluorography as described in the Experimental section. Samples immunoprecipitated with phosphotyrosine antiserum and PDGFR-1 were exposed for 5 and 0.5 days respectively.

Incorporated radioactivity was measured by liquid scintillation counting.

EGF receptor transmodulation

U-343 MGa 31L cells were seeded on to 12-well Linbro plates and allowed to grow for 3–4 days in Eagle's MEM with 10% newborn calf serum. At sub-confluence, cells were incubated for 1 h at 37 °C in 0.5 ml of serum-free Eagle's MEM with antibiotics as above/0.5 mg of human serum albumin/ml/20 mM-Hepes (pH 7.4), and diluent, EGF (Collaborative Research) or PDGF at different concentrations. The medium was then changed and 2 ng of [125 I]-EGF/ml, labelled according to the chloramine-T method (Hunter & Greenwood, 1962) to a specific radioactivity of 80000 c.p.m./ng, was added for an incubation period of 1 h at room temperature. The cells were washed four times with Eagle's MEM containing antibiotics as above/0.5 mg of human serum albumin/ml/20 mM-Hepes, pH 7.4, in 1% Triton X-100, 20 mM-Hepes (pH 7.4), 10% (v/v) glycerol, 0.1 mg of human serum albumin/ml. Membrane-bound radioactivity was counted in a gamma counter.

RESULTS

Analysis of the PDGF A-type receptor in [32 P] P_i -labelled cells

The PDGF A-type receptor was examined for ligand-inducible protein tyrosine kinase activity. U-343 MGa

31L cells were metabolically labelled with [32 P] P_i and stimulated with 100 ng of PDGF-AA/ml for 10 min, before lysis and immunoprecipitation. Anti-phosphotyrosine serum, but not non-immune serum, immunoprecipitated a component of 170 kDa in PDGF-AA-stimulated cells (Fig. 1). This component was not precipitated from control cells stimulated with diluent, nor when the phosphotyrosine antiserum was blocked with 40 mM-phenyl phosphate. PDGFR-1, but not the B-type specific antiserum PDGFR-3, immunoprecipitated two components from the PDGF-AA-stimulated cells as well as from the control cells. The component which has a molecular mass similar to the 170 kDa compound, precipitated by the phosphotyrosine antiserum, is most probably the PDGF A-type receptor. A component of about 190 kDa was also precipitated by PDGFR-1; the relationship between this component and the A-type PDGF receptor remains to be determined.

The fact that a 32 P-labelled 170 kDa component was also precipitated by PDGFR-1 from unstimulated cells may indicate that the A-type receptor is also phosphorylated on serine and/or threonine residues in a ligand-independent fashion; the kinases involved in such phosphorylations are at present unknown.

Activation of the PDGF A-type receptor thus induces tyrosine phosphorylation. Together with recent data showing that the A-type receptor cDNA contains a sequence coding for a tyrosine kinase domain (Claesson-

Fig. 3. Dose-dependent phosphorylation of PDGF A-type receptor by PDGF-AA

Confluent U-343 MGa 31L cells were labelled with [35 S]cysteine and [35 S]methionine for 3 h at 37 °C and then incubated with 0, 3, 10, 30, 100 or 300 ng of PDGF-AA/ml for 5 min (lanes a, b, c, d, e and f respectively) before lysis and immunoprecipitation. Cells were lysed and treated as described in the legend to Fig. 2 (precipitations with normal rabbit serum are not shown). Samples immunoprecipitated with phosphotyrosine antiserum and PDGFR-1 were exposed for 5 days and 1 day respectively.

Welsh *et al.*, 1989b; Matsui *et al.*, 1989), this indicates that the receptor has an intrinsic kinase activity.

Time- and dose-dependent phosphorylation of the PDGF A-type receptor induced by PDGF-AA

In order to determine the time dependence of the ligand-induced phosphorylation of the PDGF A-type receptor, cells were metabolically labelled in serum-, cysteine- and methionine-free MCDB 105 medium supplemented with [35 S]cysteine and [35 S]methionine, and then incubated with 100 ng of PDGF-AA/ml for different time periods at 37 °C (Fig. 2). The phosphotyrosine antiserum precipitated a component of slightly higher molecular mass than the 170 kDa PDGF A-type receptor from PDGF-AA-stimulated cells, but not from control cells (Fig. 2, lanes a–e; compare precipitations with anti-phosphotyrosine serum and PDGFR-1); the phosphorylation was noticeable after 5 min and reached a maximum at 15 min (lane c). The component was not precipitated when the phosphotyrosine antiserum was blocked with 40 mM-phenyl phosphate. The immunoprecipitated molecule is most probably the mature form of the PDGF A-type receptor phosphorylated on tyrosine and migrating differently in SDS/gel electrophoresis due to the change in net charge. A similar change in mobility in SDS/gel electrophoresis has been noticed for the EGF receptor (Gates & King, 1982) and for the B-type PDGF receptor (L. Rönstrand, unpublished work). After 60 min of incubation with PDGF-AA (lane e) the amount of phosphorylated receptor decreased. As a control, normal rabbit serum followed by PDGFR-1 was added to

Fig. 4. Phosphorylation of PDGF A-type receptor by PDGF-AA, PDGF-AB and PDGF-BB

Confluent U-343 MGa 31L cells were labelled with [35 S]cysteine and [35 S]methionine for 3 h at 37 °C and then incubated with diluent (lanes a) or 100 ng/ml of PDGF-AA (lanes b), PDGF-AB (lanes c) or PDGF-BB (lanes d) for 5 min. Cells were lysed and treated as described in the legend to Fig. 2 (precipitations with normal rabbit serum are not shown).

supernatants from samples immunoprecipitated with phosphotyrosine antiserum without block. Concomitant with the increase in the amount of 170 kDa mature receptor precipitated by the anti-phosphotyrosine serum, a decreasing amount of this component remained to be precipitated with PDGFR-1. As expected, the amount of 140 kDa precursor form precipitated with PDGFR-1 was constant.

In order to determine the dose-dependence of the phosphorylation, cells metabolically labelled with [35 S]cysteine and [35 S]methionine were stimulated with different concentrations of PDGF-AA for 5 min at 37 °C (Fig. 3). Tyrosine phosphorylation was seen already at 3 ng/ml (lane b) and reached a plateau at 30 ng of PDGF-AA/ml (lane d). As a control, PDGFR-1 was added to supernatants from samples immunoprecipitated with phosphotyrosine antiserum without block (Fig. 3).

Phosphorylation of PDGF A-type receptor by different isoforms of PDGF

Cells were metabolically labelled with [35 S]cysteine and [35 S]methionine and stimulated with 100 ng of PDGF-AA, PDGF-AB or PDGF-BB/ml for 5 min (Fig. 4; lanes b, c and d respectively). All three isoforms of PDGF were found to induce tyrosine phosphorylation of the type A receptor to a similar degree, as determined by immunoprecipitation with the phosphotyrosine antiserum.

Fig. 5. Effects of PDGF-AA, PDGF-AB and PDGF-BB on $[^3\text{H}]$ thymidine incorporation into U-343 MGa 31L cells

Sparse and serum-starved U-343 MGa 31L cells were incubated for 2 days with $[^3\text{H}]$ thymidine and different concentrations of PDGF-AA (○), PDGF-AB (●) or PDGF-BB (□). The cells were harvested and incorporated radioactivity was determined as described in the Experimental section.

Fig. 6. Effects of PDGF-AA and PDGF-BB on binding of ^{125}I -EGF to U-343 MGa 31L cells

Subconfluent cells were preincubated for 1 h at 37 °C with different concentrations of PDGF-AA (○), PDGF-BB (□) or EGF (▲), followed by a 1 h incubation at room temperature with ^{125}I -EGF. Cell-bound radioactivity (expressed as mean value of duplicate samples; the difference between duplicates was less than 10 %) was determined as described in the Experimental section.

Induction of $[^3\text{H}]$ thymidine incorporation by different isoforms of PDGF

The mitogenic activities of PDGF-AA, PDGF-AB and PDGF-BB were tested with U-343 MGa 31L cells grown under serum-free conditions. A low amount of human serum albumin added to the culture medium increased the reproducibility of the assay (results not shown). Addition of either one of the isoforms significantly increased the incorporation of $[^3\text{H}]$ thymidine above background in a dose-dependent manner. In contrast with the situation prevailing for human foreskin fibroblasts (Nistér *et al.*, 1988), the mitogenic signal induced by PDGF-AA was at least equally potent to those of PDGF-AB and PDGF-BB (Fig. 5).

Transmodulation of EGF receptors by PDGF-AA and PDGF-BB

Preincubation of U-343 MGa 31L cells with either PDGF-AA or PDGF-BB decreased the binding of ^{125}I -EGF in a dose-dependent manner (Fig. 6). The isoforms were of similar potency in the EGF receptor transmodulation assay. As expected, preincubation of the cells with EGF led to an efficient down-regulation of EGF receptors (Fig. 6).

DISCUSSION

The amino acid sequence of the PDGF A-type receptor, as deduced from the nucleotide sequence of cDNA clones (Claesson-Welsh *et al.*, 1989b; Matsui *et al.*, 1989), together with data presented in this report, indicate that the PDGF A-type receptor is a protein tyrosine kinase. The receptor kinase becomes activated after ligand binding, which leads to autophosphorylation of the receptor. A clonal human glioma cell line, U-343 MGa 31L, was chosen for these studies, since it was previously found to express only PDGF A-type receptors and no B-type receptors (Claesson-Welsh *et al.*, 1989a). Thus the possibility that the observed phosphorylation involved B-type receptors was ruled out.

With human fibroblasts, which have 3–4 times more B-type than A-type PDGF receptors, PDGF-AB and PDGF-BB were found to be potent mitogens, whereas PDGF-AA induced only a limited mitogenic stimulation (Östman *et al.*, 1989). In contrast, in U-343 MGa 31L cells, PDGF-AA was slightly more potent than PDGF-AB and PDGF-BB (Fig. 5). This is consistent with the finding that the K_d values for binding to the A-type receptor are 2–3-fold lower for PDGF-AA and PDGF-AB than for PDGF-BB (Claesson-Welsh *et al.*, 1989b).

PDGF induces EGF receptor transmodulation in human fibroblasts (Heldin *et al.*, 1982; Bowen-Pope *et al.*, 1983; Nistér *et al.*, 1988). The data presented here establish that this effect can be mediated by the A-type receptor. Whether the B-type receptor also mediates transmodulation of EGF receptors is not yet known.

The A-type and B-type PDGF receptors are structurally related molecules (Claesson-Welsh *et al.*, 1989a,b; Matsui *et al.*, 1989). Whereas both receptors can transduce a mitogenic signal, only the B-type receptor appears to be able to mediate chemotaxis or the formation of circular membrane ruffles in human foreskin fibroblasts (Nistér *et al.*, 1988). It is possible that the functional differences between the two PDGF receptor types are due to differences in the substrate specificities of the two kinases; substrates involved in the chemotactic and ruffling responses may be recognized only by the B-type

receptor. The kinase domains of the A-type and B-type receptors show a high degree of amino acid sequence similarity (about 80 %), whereas the insert sequences are less similar (about 36 %). It is possible that the insert sequence has a role in determining the substrate specificities of the kinases. Comparison of the substrate specificities of the wild-type B-type receptor with a mutant lacking the insert sequence have provided evidence in support of this possibility (Escobedo & Williams, 1988; L. Severinsson, B. Ek, K. Mellström, L. Claesson-Welsh & C.-H. Heldin, unpublished work).

An important aspect of future research on the PDGF receptors will thus be to identify and characterize substrates for the two receptor kinases. A few substrates have been described for the B-type receptor (Ek & Heldin, 1984). Careful inspection of the anti-phosphotyrosine immunoprecipitates (Figs. 1–4), however, revealed no reproducible substrate bands after activation of the A-type receptor in U-343 MGa 31L cells; more sensitive methods will apparently be needed to identify substrates for the A-type receptor in intact cells.

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