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PDGF Stimulates Transient Phosphorylation of 180,000 Dalton Protein

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Cell-free extracts of platelet-derived growth factor (PDGF) treated, density-arrested, quiescent BALB/c-3T3 cells are capable of phosphorylating a 180,000 dalton protein (PP180). The phosphorylation of PP180 was observed in SDS polyacrylamide gel electrophoresis profiles of Nonidet P-40 solubilized cell preparations that had been incubated with [γ-^{32}P]ATP. When quiescent BALB/c-3T3 cell cultures were incubated at 37°C with PDGF, phosphorylation of PP180 in cell extracts could be detected after a 3-min exposure of the intact cells to PDGF, which was maximal after 10–15 minutes and had diminished by 30–60 min. PDGF stimulation of PP180 phosphorylation also was observed in extracts of cells that had been incubated with PDGF at 4°C; however, in contrast to PDGF exposure at 37°C, the ability of cell extracts to phosphorylate PP180 did not decrease even after 4 hr of cell exposure to PDGF at 4°C. When cells exposed to PDGF at 4°C were transferred to 37°C for 30 min, the ability of cell extracts to phosphorylate PP180 decreased to a nonstimulated level. After cells stimulated by PDGF showed a diminished ability to phosphorylate PP180, immediate restimulation with PDGF did not induce the ability to phosphorylate PP180. Incubation for 11 hr at 37° C was required before readdition of PDGF allowed observable phosphorylation of PP180 in cell extracts, but maximum PDGF stimulation of the phosphorylation of PP180 was found after the cells were incubated for 24 hr in culture conditions.

The amount of the stimulation of PP180 phosphorylation was dependent on the concentration of PDGF. The stimulation of DNA synthesis by PDGF was correlated to the phosphorylation of PP180. This phosphorylation activity was not observed in extracts of cells that had been treated with epidermal growth factor (EGF), somatomedin C, insulin, plasma, or fibroblast growth factor (FGF). This novel experimental approach allows the investigation of a PDGF-stimulated phosphorylation activity in relation to the cell cycle and growth regulation.

Key words: platelet-derived growth factor, phosphorylation, membrane protein, cell cycle

The abbreviations used are: PDGF, platelet-derived growth factor; PPP, platelet-poor plasma; DMEM, Dulbecco's Modified Eagle's Medium; PBS, phosphate-buffered saline; PMSF, phenylmethyl-sulfonyl-fluoride; Hepes, N-2-hydroxethylpiperazine-N'-2-ethane sulfonic aid; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; EGF, epidermal growth factor; FGF, fibroblast growth factor; RSB, reticulocyte saline buffer; Ins, Insulin; NP40, Nonident-P40.

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Platelet-derived growth factor (PDGF) is a potent mitogen stored in the α-granules of platelets and is released into serum during the clotting of blood [1–4]. Nontransformed fibroblastic cells require PDGF or other agents with apparently similar function to initiate cellular proliferation, whereas transformed cells have an abrogated PDGF requirement for growth [5].

Quiescent cells exposed to PDGF rapidly develop cellular growth signals that are more stable than the association of PDGF molecules with the cells [6,7]. These stable growth initiation signals induced by PDGF can be transferred by cell fusion of PDGF treated cells to nontreated quiescent cells [8]. Induction of the growth initiation phase by PDGF also has been shown to be dependent upon RNA synthesis [8]. Several unique mRNAs [9,10] and proteins [11] are induced in quiescent cells in response to PDGF treatment. One PDGF-induced protein, pI, is synthesized and appears in the nucleus within 40 min [12]. These inducible cellular components may be the necessary signals for initiation of cellular proliferation. These data lead to the conclusion that initiation of the cell cycle by PDGF requires expression of specific genes.

Specific receptors for PDGF have been demonstrated on fibroblasts and smooth muscle cells [13–16]. However, the mechanism whereby PDGF binds specific receptors and then induces the expression of specific genes is unknown. It has been suggested that changes in ion flux, nutrient uptake, cyclic AMP concentrations, cellular pH, or Ca^{++} effects may be involved in the transfer of mitogenic signals from the receptor to the nucleus [17].

Recently, PDGF was shown to stimulate the phosphorylation of a 180,000–185,000 dalton protein in membrane and solubilized membrane preparations isolated from fibroblasts [18–20]. This PDGF-stimulated phosphorylation of a membrane protein shares many similarities with the putative EGF-stimulated EGF receptor-kinase activity (for review of EGF receptor–kinase activity, [21]). The PDGF-induced phosphorylation of the 180–185 kDa protein has been shown to be in part a tyrosine phosphorylation, dependent on Mg^{++} or Mn^{++}, independent of cyclic nucleotides, and suggested to be the result of a PDGF receptor-kinase activity [18,20]. At present it cannot be concluded whether this PDGF-induced phosphorylation is on the PDGF receptor or if the stimulated kinase activity is the PDGF receptor or is associated with the PDGF receptor. Data reported by Ek and Heldin [20] suggest that this kinase activity is a domain on the PDGF receptor protein. Recently, Pike et al [22] showed that PDGF-induced down regulation of PDGF receptors correlated with a decrease in the putative PDGF receptor-kinase activity. The exact relation of the putative PDGF-stimulated kinase activity to the PDGF receptor has not been delineated; however, a possible involvement of a PDGF receptor-kinase in growth control is attractive. The role of the PDGF receptor-kinase complex in growth regulation has been difficult to determine. One major reason for this difficulty is that the PDGF-stimulated activity has been investigated mainly in isolated plasma membranes. Using this methodology, little has been learned about the regulation of the putative PDGF-induced kinase activity.

To characterize further the cellular behavior of the PDGF-stimulated kinase activity, we have examined protein phosphorylation with [γ-^{32}P]ATP in solubilized cell preparations of PDGF-treated, density-arrested BALB/c-3T3 cells. In solubilized cell extracts of PDGF-treated cells, the stimulation of a phosphorylation of a protein was observed with a molecular weight of approximately 180,000 daltons (PP180).

The stimulation of the phosphorylation in cell-free extracts was dependent on the concentration and length of time intact cells were incubated with the PDGF. Stimulation of the phosphorylation was observed in extracts of cells pretreated with PDGF at 37°C or at 4°C. In extracts of cells pretreated with PDGF at 37°C, the phosphorylation of PP180 was transient. However, if cells were incubated with PDGF at 4°C, the ability of cell-free extracts to phosphorylate PP180 did not diminish with increasing time of PDGF exposure.

The experimental conditions developed in this study allow investigation of the kinetics of the PDGF-stimulated phosphorylation. Stimulation of the phosphorylation of PP180 was specific for extracts of PDGF-treated cells and was not observed in extracts of cells pretreated with EGF, insulin, somatomedin C, or plasma. The phosphorylation was not observed after incubation of cells with medium containing calcium phosphate or fibroblast growth factor, both of which have been shown, like PDGF, to induce cell proliferation of BALB/c-3T3 cells. The PDGF-stimulated phosphorylation of PP180, as observed in the experimental approach employed here, provides a system whereby the amount of PDGF-stimulated phosphoprotein can be correlated with cell cycle regulation.

MATERIALS AND METHODS

Cell Cultures

BALB/c-3T3 (A31) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (FLOW) with 10% calf serum (Colorado Serum Co.) in a humidified 5% CO_2 atmosphere. Stock cells were grown in 100 mm culture dishes and passaged to 35 mm dishes. Cultures in 35 mm dishes were refed with media containing 10% calf serum after three days and reached confluent densities two to three days later. Cultures were used after they became quiescent in spent growth medium.

Cellular Preparations

In all experiments density-arrested cell cultures in 35 mm dishes were exposed to various growth factors at the desired concentration in 1 ml of DMEM for the indicated times. These incubations were performed at either 37°C or 4°C as indicated in the text. After appropriate incubations, the cell cultures were placed on ice and washed two to three times with cold PBS. Cells were scraped from the dishes and pelleted by low-speed centrifugation. The cell pellet was solubilized in reticulocyte saline buffer (RSB) (0.01M NaCl/0.01M Tris-HCl/1.5 mM $MgCl_2$, pH 7.4) containing 1% Nonidet P-40, 10 mM NaF, and 0.5 mM PMSF by keeping the cell suspension on ice for 10 min with occasional agitation. The nuclei were removed from these preparations by low-speed centrifugation.

Phosphorylation Reactions

The phosphorylation reactions were performed on ice and the reaction was initiated by the addition of 10 μl of a solution containing 20 mM Hepes, pH 7.4, 20 mM $MnCl_2$, 0.1% BSA with 10 μCi [γ-^{32}P]ATP (6000 Ci/mmol) to 100 μl of solubilized cell preparation. Phosphorylation reactions were allowed to incubate 10 min. The reactions were terminated by addition of an equal volume of 2% SDS and 2% β-mercaptoethanol, and this mixture was placed in boiling water for 3 min.

SDS Polyacrylamide Gel Electrophoresis

Standard Laemmli SDS polyacrylamide gel electrophoresis was used to identify phosphorylated proteins [23]. Gels were stained, dried, and processed for autoradiography. Equal amounts of acid insoluble ^{32}P counts from each reaction were applied to gels. Molecular weights were estimated from known standards. Where indicated in the text, the amount of ^{32}P incorporated into PP180 was detected by scanning the autoradiogram with a Kontes fiber optic scanner.

Growth Factors and Other Reagents

PDGF activity was purified by previously described procedures. Boiled platelet extracts were passed through CM-Sephadex as described by Antoniades et al [24]. The PDGF activity was then chromatographed with Blue Sepharose by the method of Heldin et al [25]. The activity was dialyzed against 1M acetic acid, concentrated by lyophilization, and chromatographed on Biogel P-150 in 1M acetic acid [24]. The PDGF activity was concentrated and used as highly purified PDGF (10 ng/unit) for experiments except when HPLC chromatographically pure PDGF was used to verify specificity of the phosphorylation reactions. EGF was a gift from Dr. Ed O'Keefe, and somatomedin C was obtained from Dr. J.J. VanWyk; FGF was obtained from Core chemicals. Plasma was made as previously described [6]. Acrylamide and most other reagents were purchased from Sigma, [γ-^{32}P]ATP (6000 Ci/mmol) was obtained from New England Nuclear, and ^{3}H-thymidine (6 Ci/mmol) was obtained from ICN.

RESULTS

Rapid PDGF-Stimulated Phosphorylation in Quiescent BALB/c-3T3 Cells

Density-arrested BALB/c-3T3 cells were treated with PDGF to determine if unique phosphorylations stimulated by PDGF could be observed. Quiescent cells in 35 mm culture dishes were transferred to fresh medium containing 500 ng of highly purified PDGF and incubated at 37°C for 3, 10, and 30 min. Additional cultures were incubated under identical conditions in medium alone or medium supplemented with 5% plasma for comparative nonmitogenic controls. At appropriate times, cell cultures were placed on ice and washed twice with cold PBS; cells were collected and NP40-soluble preparations were made. After the phosphorylation reactions (outlined under Materials and Methods), equal amounts of acid insoluble ^{32}P from each condition were applied to standard Laemmli acrylamide gels. Figure 1 shows the SDS polyacrylamide electrophoresis profiles of the phosphorylated proteins from these reactions.

One phosphorylated protein band, not present in extracts of cells exposed to medium alone or plasma-supplemented medium, was observed in the extracts of PDGF-treated cells (Fig. 1, arrow). Clearly, PDGF induced the cells to bring about a unique protein phosphorylation that could be detected in this assay system after quiescent cells were exposed to PDGF for 3 min. This phosphoprotein also was observed in extracts of cells exposed to PDGF for 10 and 30 min; however, incubation of the cell culture in PDGF at 37°C for 60 min or longer revealed no detectable amounts of this unique phosphoprotein (data not shown). These data suggested that PDGF exposure to the quiescent cells rapidly induced the ability to phosphorylate a high molecular weight protein, and the PDGF-stimulated phosphorylation activity

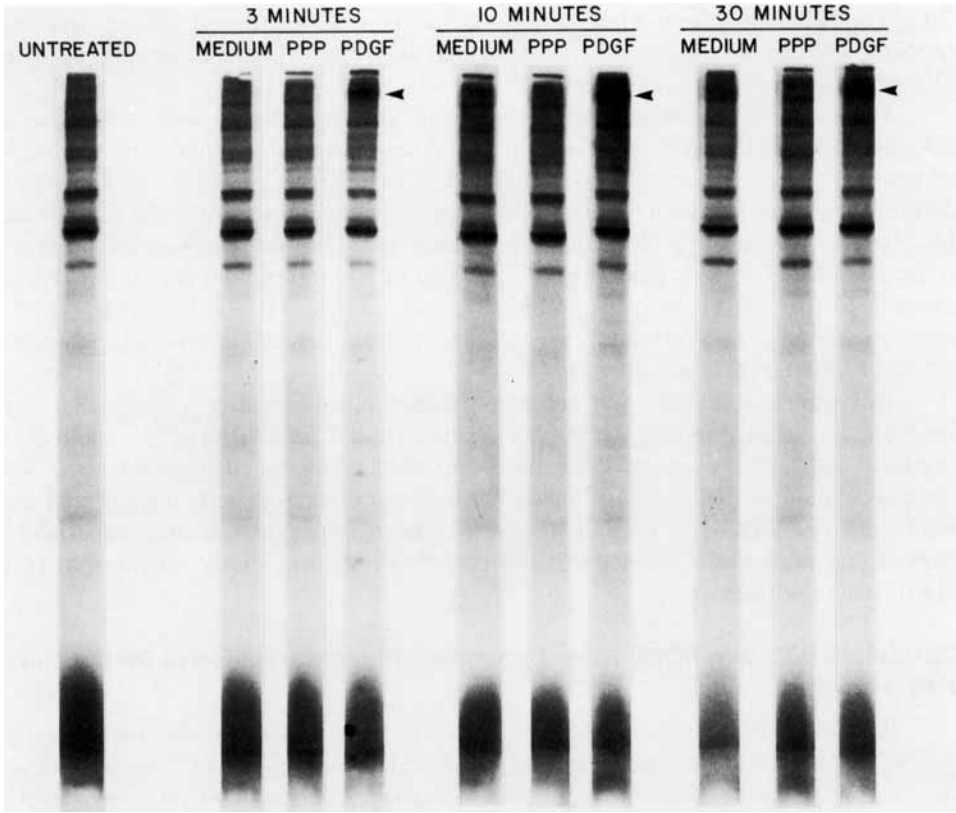

Fig. 1. PDGF stimulated a unique phosphorylation in density-arrested BALB/c-3T3 cells. Cultures of density arrested BALB/c-3T3 cells in 35 mm culture dishes were transferred to 1 ml of fresh medium containing 5% plasma, 500 ng highly purified PDGF, or no additions. After 3, 10, or 30 min incubations at 37°C, cultures were placed on ice. The cells from these cultures and one untreated culture were washed twice with cold PBS, scraped from the plates, and centrifuged to collect cells. The resultant cell pellets were solubilized in 100 μl RSB with 1% NP40, 10 mM NaF, and 0.5 mM PMSF. After cells were solubilized, nonsoluble material was removed by centrifugation. The phosphorylation reactions were performed on ice as outlined in Materials and Methods. The phosphorylated soluble cell preparation was made to 1% SDS and 1% β-mercaptoethanol and heated at 100°C for 3 min. Equal amounts of acid insoluble counts of ^{32}P were applied to a 12.5% polyacrylamide gel (standard Laemmli). The gel was fixed in 15% TCA, stained, and an autoradiogram of the ^{32}P-phosphoproteins was made. The arrow points out a phosphorylation present only in the PDGF treated cultures.

was transient. Plasma, unlike PDGF, does not stimulate cellular proliferation of quiescent BALB/c-3T3 cells and also did not stimulate the observed phosphorylation.

Molecular Weight Determination and Specificity of the PDGF-Induced Phosphorylation

In order to analyze the specificity of the stimulation of the phosphorylation of the phosphoprotein shown in Figure 1, density-arrested cultures of BALB/c-3T3 cells were transferred to fresh medium with one of the following additions: 5% plasma;

PDGF (1.25 μg, 0.60 μg, 0.30 μg); EGF (25 ng); 10^{-5}M insulin; somatomedin C (20 ng) or 10% calf serum. After 15 min incubation at 37°C, treated cultures and one untreated culture were processed as described for Figure 1 and electrophoresed in 10% or 12.5% polyacrylamide gels with SDS.

The resulting autoradiograms demonstrate that extracts of cells treated with PDGF were capable of phosphorylating, in a dose-dependent manner, a protein with an apparent molecular weight of 180,000 daltons (Fig. 2, see arrow). The other culture conditions, including additions of EGF and somatomedin C, did not induce this phosphorylation of PP180. Longer incubation times in these medium supplements or the addition of greater quantities of EGF (up to 100 ng) did not reveal detectable amounts of the 180kDa phosphoprotein that was termed PP180. FGF and Ca^{++}, which are BALB/c3T3 cell mitogens, did not stimulate detectable levels of phosphorylation of PP180 (data not shown).

To ensure that PDGF, and not a preparation contaminant was responsible for the induction of the observed phosphorylation activity, pure PDGF was applied to quiescent cells and the ability of solubilized extracts of these cells to bring about the phosphorylation of PP180 was compared to a preparation from cells treated with the highly purified PDGF as used in Figures 1 and 2. Results from this experiment showed that pure PDGF induced PP180 phosphorylation as did the highly purified PDGF (data not shown).

Correlation Between PDGF Dose-Dependent Phosphorylation of PP180 and DNA Synthesis

Treatment of quiescent BALB/c-3T3 cells with PDGF, in the presence of plasma, results in a dose-dependent entry of these cells into S phase. To determine if there was a quantitative relationship between PDGF induction of PP180 phosphorylation and PDGF stimulation of DNA synthesis, the following experiment was done. Duplicate sets of cultures were transferred into fresh medium containing varying amounts of PDGF, 5% plasma, or medium alone. After incubation at 4°C for 1 hr, half of the cultures were processed for the phosphorylation reaction, as outlined in the legend to Figure 1, and were electrophoresed in a 10% SDS-polyacrylamide gel. An autoradiogram of the gel was made and scanned with a densitometer to estimate the amount of ^{32}P incorporated into PP180. To the remaining cultures, plasma (10%) and 5 μCi of ^{3}H-thymidine were added, and cultures were returned to 37°C and incubated for 24 hr. The percentage of labelled nuclei was determined as outlined in the legend to Figure 3, and results of this experiment are shown in Figure 3. The increase in the incorporation of ^{32}P into PP180 and the stimulation of DNA synthesis were related to the amount of PDGF. The insert in Figure 3 illustrates the close correlation of the increased PP180 phosphorylation with the stimulation of DNA synthesis in the quiescent cell cultures treated with PDGF.

PDGF Stimulation of PP180 In Cell Cultures Exposed to PDGF at 4°C

The data presented thus far indicate that in extracts of cells exposed to PDGF at 37°C there is a rapid stimulation of PP180 phosphorylation, followed by a time-dependent decrease in the ability to detect this phosphoprotein. To determine if the induction of the ability to phosphorylate PP180 or the diminution of this PDGF-stimulated phosphorylation activity was temperature dependent, the following exper-

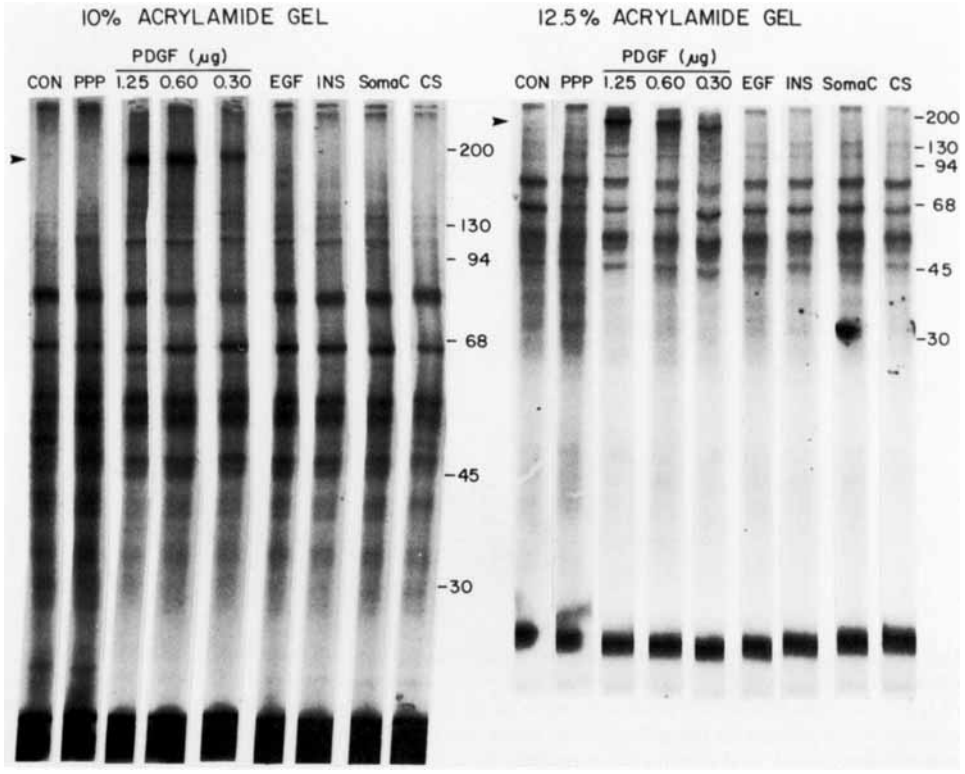

Fig. 2. Molecular weight determination and specificity of PDGF-stimulated phosphorylation. Density arrested cultures of BALB/c-3T3 cells in 35 mm culture dishes were transferred to 1 ml of fresh medium containing 5% plasma (PPP), highly purified PDGF (1.25 μg, 0.60 μg, 0.30 μg), 25 ng EGF, 10^{-5}M Ins, 20 ng Somatomedin C, or 10% calf serum. One culture (CON) was untreated. After 15 min at 37° C solubilized cell preparations were made and phosphorylated with [γ-^{32}P]ATP as described in Figure 1. Standard Laemmli electrophoretic gels (10% and 12.5% acrylamide) were performed. The arrow indicates the unique PDGF-stimulated phosphoprotein. Molecular weights of coelectrophoresed protein standards are indicated.

iment was done. Cells were exposed to various concentrations of PDGF at 4°C for 90 min; cells then were washed, and soluble cell preparations made and reacted with [γ-^{32}P]ATP as described for Figures 1–3. Figure 4 illustrates the SDS-polyacrylamide gel electrophoretic profile of solubilized phosphoproteins. The apparent stimulation of PP180 phosphorylation in extracts of cells treated with the PDGF for 90 min at 4°C was as much or more than that determined for the same PDGF concentrations exposed to cells for 15 min at 37°C (see Figs. 1 and 2).

The kinetics of PP180 phosphorylation when cells were pretreated with the PDGF at 4°C differed from those observed for cells exposed to PDGF at 37°C. As can be seen in Figure 5, extracts of cells treated with PDGF at 37°C showed a maximum phosphorylation when incubated with PDGF for 15 min, and the phospho-

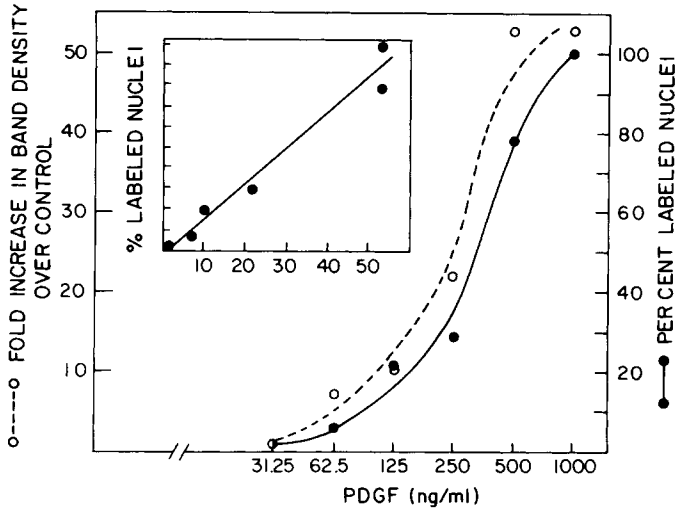

Fig. 3. Correlation of PP180 phosphorylation with the stimulation of DNA synthesis. Duplicate cultures of density-arrested BALB/c-3T3 cells were transferred to fresh medium containing the indicated amounts of highly purified PDGF, 5% plasma or media alone, and were incubated at 4°C for 1 hr. One set of cultures was processed for phosphorylation. To the other set, plasma and 5 μCi of ^{3}H-thymidine (6 Ci/mmole) were added, and cultures were returned to a 37°C incubator. After 24 hr, cells were rinsed twice with PBS, fixed with methanol, and processed for autoradiography. The percent labeled nuclei was calculated from duplicate counts of at least 200 cells. Inset: The correlation coefficient was .98 inclusive of the two highest PDGF concentrations tested and .93 when these values were excluded.

rylation was not observed in extracts of cells exposed to PDGF for 60 min. In contrast to these results, extracts of cells exposed to PDGF at 4°C did not show diminished phosphorylation after 4 hr PDGF pretreatment. These results indicated that the ability of PDGF to stimulate the ability to phosphorylate PP180 was not temperature dependent; however, the diminution of detectable PP180 phosphorylation was temperature dependent.

The temperature dependence of the decrease in phosphorylation activity was substantiated further by transferring cells treated with PDGF for 4 hr at 4°C (as in Fig. 5) to 37°C. In these experiments, cells treated with PDGF at 4°C were washed twice with medium then transferred either to plasma or to PDGF-supplemented medium and then incubated at 37°C for the times indicated in Figure 6. The electrophoretic profiles revealed a time and temperature dependent decrease in the phosphorylation of PP180. The ability to phosphorylate PP180 was not observed in the soluble preparations after the cells had been incubated for 60 min at 37°C.

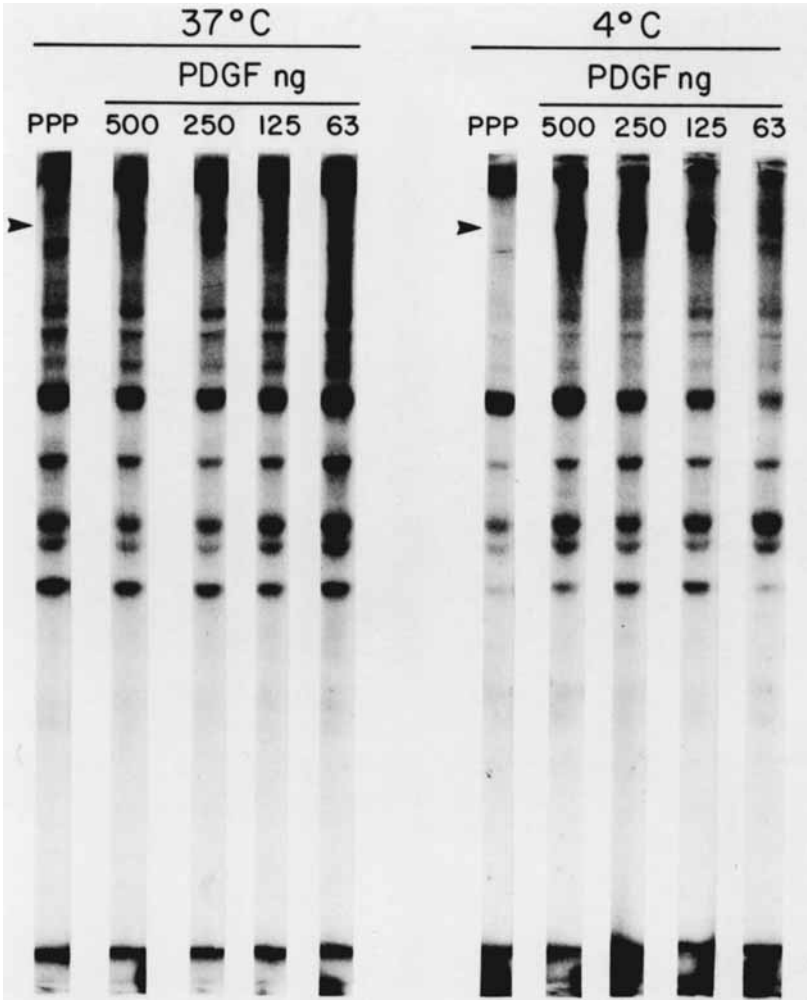

Fig. 4. PDGF-stimulated phosphorylation of PP180 was temperature independent. Density-arrested BALB/c-3T3 cells were transferred to fresh medium containing plasma or varying amounts of highly purified PDGF. Duplicate cultures were prepared: one set was incubated for 15 min at 37°C; another set was precooled to 4°C and exposed to highly purified PDGF for 90 min at 4°C. Cultures were washed at approximate times and processed as in Figure 1. The arrow indicates PP180.

Recovery of PP180 Phosphorylation Activity

In order to determine the time required in cultured conditions for PDGF-treated cells to regain the ability to phosphorylate PP180 after its decrease, density-arrested cells were treated as described for experiments reported in Figures 5 and 6; that is, the cells were treated with PDGF at 4°C, washed, and then incubated at 37°C in plasma (10%) supplemented medium. Stimulation of the PP180 phosphorylation was obtained as shown in Figure 6; after 30–60 min incubation at 37°C, there was no detectable phosphorylation of the PP180, as seen in Figure 6. Cultures were incubated

Fig. 5. Transient nature of the increased PP180 phosphorylation is temperature dependent. Quiescent cells were treated with 200 ng/ml highly purified PDGF in 1 ml fresh medium for varying times at 37°C or 4°C. At the indicated times cells were processed, phosphorylated, run on 10% polyacrylamide gels. One lane (PPP) indicates cells treated with plasma. The arrow indicates the PP180.

at 37°C for increasing periods of times and then duplicate cultures were placed on ice. One culture dish received PDGF for 60 min, whereas the other received no additions. After this reexposure to PDGF, treated and nontreated cultures were processed and reacted with [γ-^{32}P]ATP, as previously described, and then separated on SDS polyacrylamide gels. Figure 7 illustrates that after 2 and 4 hr of incubation in the plasma supplemented medium at 37°C, the cells did not repond to PDGF by inducing the phosphorylation of PP180. However, after 11 hr of incubation at 37°C,

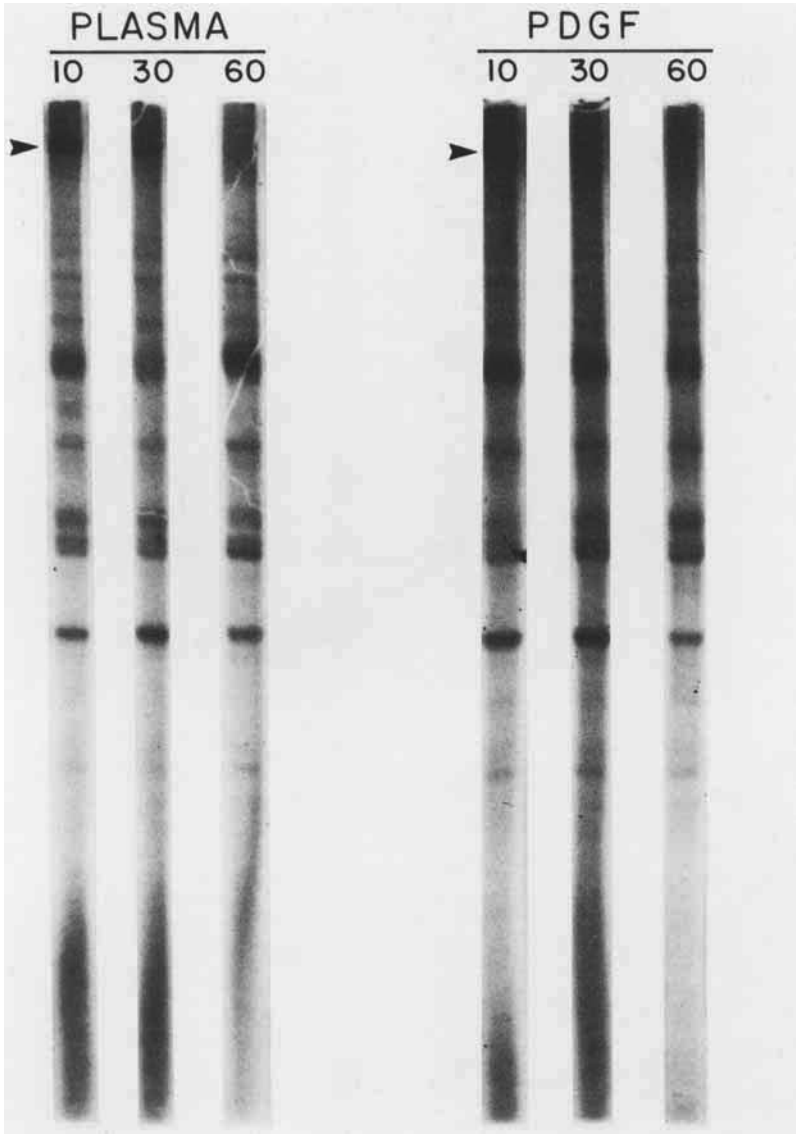

Fig. 6. Loss of PP180 phosphorylation is temperature dependent. Density arrested cells were treated with highly purified PDGF at 4°C as in Figure 6. The cells were washed and transferred to medium (37°C) containing 5% plasma or 200 ng/ml highly purified PDGF. At the indicated times the cells were harvested. The arrow indicates the PP180.

some phosphorylation of PP180 could be detected, and high levels of phosphorylation of PP180 could be obtained after 24 hr incubation in these conditions.

DISCUSSION

The PDGF-stimulated cellular events leading to altered gene expression and initiation of proliferation are mediated by PDGF receptors; however, the mechanism

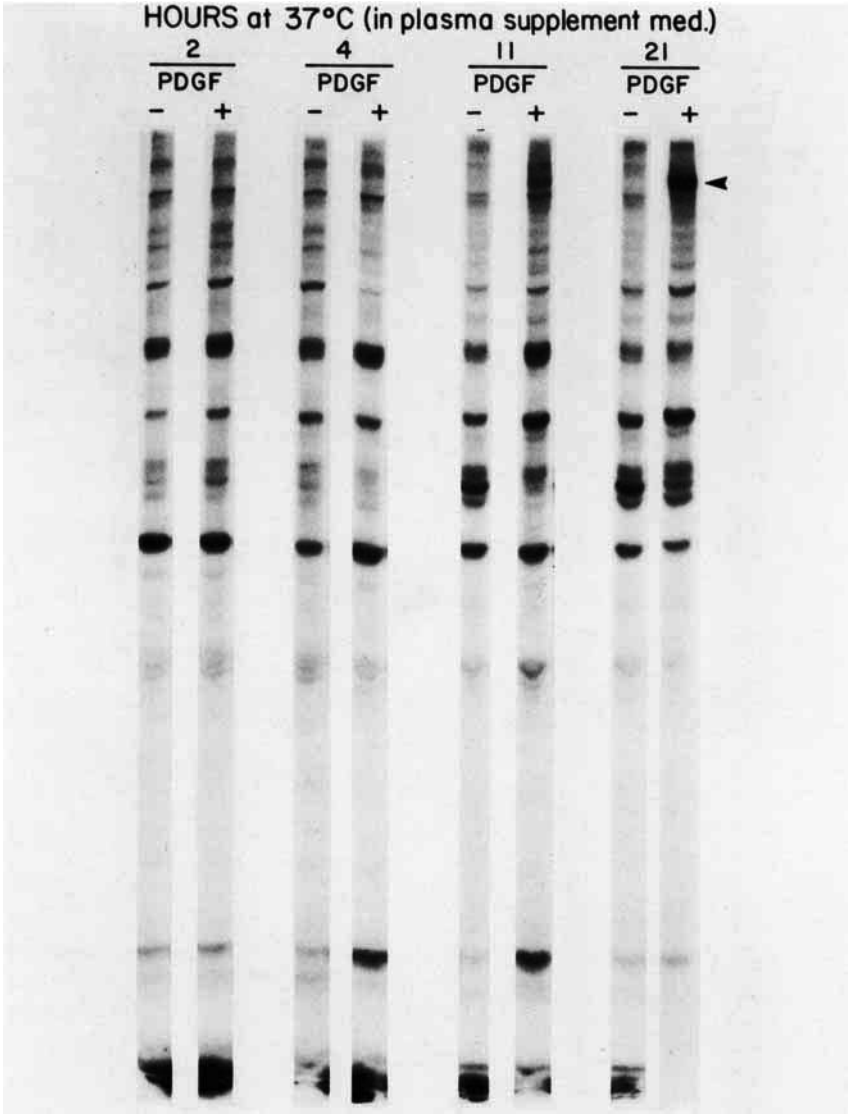

Fig. 7. Restimulation of phosphorylation of PP180 by PDGF. Cells were treated with highly purified PDGF at 4°C for 2 hr then transferred to 37°C in medium containing 10% plasma. At the indicated times, duplicate cultures were placed at 4°C. One culture received highly purified PDGF for 60 min at which times cells were harvested and phosphorylation of PP180 determined. The (-) indicates cell cultures left untreated at 4°C, the (+) indicates cultures retreated with PDGF. The arrow points to PP180.

whereby PDGF interacts with specific receptors and stimulates initiation of proliferation is not known.

One biochemical reaction suggested to be involved in the mechanism responsible for stimulation of required mitogenic events is the phosphorylation of specific proteins. It has been proposed that the PDGF receptor may have an associated kinase

activity. When PDGF binds to its receptor in isolated plasma membranes preparations, a kinase activity is stimulated resulting in phosphorylation of the receptor and other cellular substrates [18,20,26,27]. In this regard, the PDGF receptor-kinase activity is similar to the activity of the putative EGF receptor-kinase [28], the insulin receptor-kinase [29], and the somatomedin C receptor-kinase [30]. The majority of PDGF-receptor-kinase activity investigations have employed isolated plasma membranes from fibroblasts. Generally, studies of the EGF receptor-kinase activity have included isolated plasma membranes from A431, liver, and fibroblastic cells.

Using a solubilized cell assay system, this study has shown that extracts of density-arrested BALB/c-3T3 cells, pretreated in culture with PDGF, induced a dose-dependent phosphorylation of a 180kDa protein. The molecular weight of the PDGF-stimulated phosphoprotein, the temperature-dependent disappearance of the activity, and two other preliminary observations suggest that the PP180 may be the phosphorylated PDGF receptor. These two preliminary results are that the PP180 band in gels similar to the one shown in Figure 2 is stable to 1M KOH at 55°C and that PP180 fractionates with plasma membranes. Further investigations are required to confirm the identity of PP180 as the PDGF receptor.

The phosphorylation of PP180 was specific for extracts of PDGF-treated cells, as it was not observed in extracts of cells pretreated with other purified growth factors. The ability of extracts of PDGF-treated cells to phosphorylate PP180 correlated with PDGF stimulation of cellular proliferation. The data presented in this paper does not prove that phosphorylation of PP180 brings about stimulation of DNA synthesis, but it does suggest that PP180 phosphorylation is in the chain of events leading to PDGF stimulation of growth. If PP180 were the PDGF receptor, it could be expected that the solubilized cell assay should have allowed the detection of the EGF, insulin, and somatomedin C-receptor-kinase activities. However, there are several reasons why these activities may not have been detected: 1) the number of receptors for each hormone on density-arrested BALB/c-3T3 cells differs (50,000 EGF receptors/cell [31], 34,000 somatomedin C receptors/cell, [32], and 160,000 PDGF receptors/cell [33]); 2) quiescent, density-arrested BALB/c-3T3 cells may be more synchronized with respect to a PDGF stimulation of a transient phosphorylation; and 3) the PP180 may not be the PDGF receptor, but rather a phosphoprotein induced by PDGF required for growth initiation. The BALB/c-3T3 cell line used in these experiments has been demonstrated to respond poorly to growth stimulation by EGF [34] and may be why an EGF-stimulated phosphoprotein was not detected in our assays.

When density-arrested fibroblasts were pre-exposed to PDGF at 37°C, there was a rapid stimulation of the phosphorylation of PP180 followed by a time and temperature decrease in the ability to detect the observed phosphorylation. Extracts of cells exposed to the PDGF at 4°C also displayed the increase in phosphorylation of PP180 but not the time-dependent decrease in the PDGF stimulated phosphorylation. However, when cells pretreated with PDGF at 4°C were shifted to 37°C prior to solubilization, there was a rapid decrease in the ability to detect the stimulated phosphorylation of PP180. Once the cells had lost the PDGF-stimulated phosphorylation activity, it was not restimulated by PDGF until after 11–24 hr incubation at 37°C.

The high concentrations of the PDGF activity used in these experiments were required for two reasons. First, when growth stimulations are performed on surfaces larger than those of microtiter wells, greater concentrations of PDGF are required.

Secondly, because the stimulation of PP180 was transient, it was important to stimulate as many cells as possible, as rapidly as possible, thereby increasing our ability to detect PP180.

The data presented here tentatively suggest that the phosphorylation that was identified may be similar or identical to the reported phosphorylation occurring on the PDGF receptor. The stimulation of PP180 observed only in extracts of PDGF-treated cells may not be required for the initiation of cellular proliferation. Ca^{++} and FGF have been shown to be mitogenic, [34] yet extracts of these cells did not induce phosphorylation of PP180. This suggests that PP180 may be required for other PDGF responses or associated specifically with the mechanism whereby PDGF functions to stimulate proliferation.

Because the identification of a tyrosine kinase activity associated with the src gene product, pp60 [35,36], tyrosine kinase activities also have been identified with the EGF [29], somatomedin C [31], insulin [30], and PDGF [20] receptor complexes. The majority of studies concerning receptor-associated kinase activities thus far have been performed using purified membrane receptor preparations and immunoprecipitates. Results from these studies have provided necessary information concerning the physical and chemical requirements for the receptor-kinase activities. However, the significance of a kinase activity associated with a hormone-receptor complex is not known. The solubilized cell assay developed in this study may provide a suitable technique for comparing the regulation of receptor and kinase activities associated with the PDGF receptor to the initiation and control of cellular proliferation.

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