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ARTICLES

Rapid Induction of Competence Formation Is PDGF-Isoform Specific

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Abstract Platelet-derived growth factor (PDGF) stimulates the expression of a number of genes associated with entry of quiescent Balb/c-3T3 fibroblasts into the cell cycle. We determined that two of these genes, c-*myc* and c-*fos*, are induced equivalently in medium supplemented with platelet-poor plasma (PPP) and either PDGF-BB or PDGF-AA. The rate at which fibroblasts entered S phase was also similar in PDGF-BB– and AA–treated cells as was the expression of the late G_1 gene, thymidine kinase (TK). However, PDGF-AA must be present for a period of 16 h to stimulate the proliferation of 90% of the cells, whereas PDGF-BB was required for only 4 h. Exposure of cells to PDGF-AA for 4 h, a time during which maximum expression of c-*fos* and c-*myc* occurred, only induced 20% of the cells in a quiescent population to enter the cell cycle. Therefore, PDGF-AA–mediated expression of the immediate early genes c-*fos* and c-*myc* may be necessary but is not sufficient to rapidly stimulate density-arrested Balb/c-3T3 fibroblasts into the competent state. Thus, these data suggest that PDGF-AA and PDGF-BB initiate traverse of the cell cycle by distinct mechanisms.

Key words: PDGF-AA and BB, cell cycle, c-*myc*, fibroblasts, receptors

The coordinate interaction of specific growth factors stimulates quiescent cells arrested in G_0 to enter and traverse G_1. PDGF acts as a "competence" factor, enhancing the sensitivity of Balb/c-3T3 fibroblasts to growth factors contained in platelet-poor plasma that are required for progression through G_1 [1–3]. Competence has been shown to require only a transient exposure of cells to PDGF and to be stable for 12–18 h [4]. A number of genes, including c-*fos*, c-*myc*, JE, and KC are temporally expressed in fibroblasts following serum stimulation [5–8]. The expression of c-*fos* and c-*myc* has been demonstrated to be required for serum stimulation of growth as blocking the expression of these genes with antisense mRNA inhibits the ability of cells to proliferate [9–11]. It has been proposed that c-*myc* expression either alone or in concert with other cellular events renders cells competent [12–14].

PDGF is composed of two distinct polypeptide chains, A and B, that dimerize to form three isoforms: PDGF-AA, PDGF-BB, and PDGF-AB [15–17]. The PDGF receptor is composed of two subunits, α and β [18–20]. The receptor subunits are also capable of forming dimers, thereby establishing three receptor classes, αα, ββ, and αβ [21,22]. The PDGF α receptor binds all three isoforms of PDGF with high affinity while the β receptor binds PDGF-BB with high affinity and PDGF-AB with low affinity but does not bind PDGF-AA [23,24]. A 1:4 ratio of PDGF α to β receptors exists in Balb/c-3T3 fibroblasts [25]. Early studies used PDGF from human platelets which mainly consists of the PDGF-AB isoform. Since PDGF-AB binds and activates both PDGF α and β receptors, the specific effect of PDGF α receptor activation, in terms of growth stimulation and gene expression, was not addressed. Using PDGF-AA we examined whether activation of PDGF α receptors alone was sufficient to induce competence and a growth response in Balb/c-3T3 fibroblasts and, in addition, determined whether early gene expression correlated with the ability of PDGF-AA to induce competence and the proliferation of fibroblasts.

MATERIALS AND METHODS

Cell Culture

Stock cultures of Balb/c-3T3 mouse fibroblasts (clone A31) were grown in Dulbecco-Vogt

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modified Eagle's medium supplemented with 10% calf serum, 4 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin in humidified 5% CO_2/95% air. For all experimental procedures, cells were grown to confluency in serum containing medium and used 2–3 days after cessation of growth.

Mitogenic Assays

Density-arrested Balb/c-3T3 fibroblasts in 35 mm dishes were treated with Dulbecco's modified Eagle's medium (DMEM) containing 10% PPP and either 50 ng/ml PDGF-AA or 25 ng/ml PDGF-BB for the times indicated. Cells were then rinsed and incubated in medium containing 10% PPP [26] and [^{3}H]thymidine (5 μCi/ml) for an additional 30 h at which time the cells were fixed and processed for autoradiography as described previously [27]. For the rate of entry into S phase experiments density-arrested Balb/c-3T3 fibroblasts in 35 mm dishes were treated with DMEM containing 10% PPP and [^{3}H]thymidine (5 μCi/ml) and either 50 ng/ml PDGF-AA or 25 ng/ml BB for the appropriate times. Cells were then fixed and processed for radiography. For all experiments percent labeled nuclei was determined as compared to the total number of nuclei present.

Northern Analysis of c-*myc*, c-*fos*, and TK Expression

Density-arrested Balb/c-3T3 fibroblasts in 100 mm dishes were exposed to medium containing 10% PPP and either 50 ng/ml PDGF-AA or BB for the appropriate times. Cells were then harvested for poly(A)RNA by a modification of the method described by Schwab et al. [28]. Cells were lysed and subsequently sheared by passage through a 22 gauge needle; Proteinase K was added and the mixture incubated at 37°C for 1 h. Pre-washed oligo-dT was added and the samples were mixed overnight. The oligo-dT mixture was poured into columns and washed 3 times with high salt buffer before RNA was eluted with salt-free buffer. The eluted poly(A)RNA was precipitated, quantified, and aliquotted such that 4 μg of RNA from each sample was used per lane in 1% agarose–2.2 M formaldehyde gels. Gels were electrophoresed overnight at 20 V, then transferred to nylon membranes (Nytran, Schleicher & Schuell). The RNA was cross-linked to the membrane using UV irradiation; subsequently membranes were pre-hybridized in 50% formamide, 5X SSC, .05% bovine serum albumin (BSA), 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA for 1–2 h at 42°C. The membranes were hybridized for 12–20 h at 42°C in an identical buffer containing the denatured probe (see below). Probes were labeled with a random primer kit (Boehringer Mannheim) to a specific activity of 1–3 × 10^9 dpm/μg. Membranes were washed in buffer containing 1X SSC and 0.1% SDS at 37–42°C and autoradiographed overnight with intensifying screens. The following probes were used: a plasmid containing the Xba1/Sac1 fragment of pSVcmyc1 (ATCC #41029) which includes the second and third exons of mouse c-*myc* cDNA [29]; a plasmid (pSPD1fos) containing the Sal1/Pst1 insert comprising 688 bp from the coding region of v-*fos*, subcloned from pFBJ-2 [30]; a plasmid (pAMTK) containing the 1156 bp EcoR1 fragment of mouse TK cDNA; and the 700 bp BamH1/Pst1 fragment of plasmid SP65, containing the cDNA encoding rat cyclophilin (1B15) [31].

Immunoprecipitation Assays

Density-arrested Balb/c-3T3 fibroblasts in 100 mm dishes were incubated in methionine-deficient DMEM containing 10% fetal calf serum or either 10% PPP alone or 10% PPP and various concentrations of either PDGF-AA or BB for 1 h at 37°C. [^{35}S]methionine at a final concentration of 400 μCi/ml was then added and the cells were incubated for an additional 20 min at 37°C. Cells were rinsed in phosphate-buffered saline and lysed with lysis buffer (20 mM Tris pH 7.5, 100 mM NaCl, 0.5% Triton-X 100, 0.5% deoxycholate, 0.5% SDS, 0.1 TIU Trasylol Aprotinin, 1 mM EDTA, 50 mM sodium flouride, 100 μM $NaVO_3$, and 1.6 mg/ml of Iodo-acetic acid). The lysate was sonicated and spun at 10,000 RPM for 10 min at 4°C in a microfuge to remove nuclear membranes. Equal counts were precleared for 3–4 h at 4°C with Pansorbin (Calbiochem) and then incubated with polyclonal antisera to the mouse c-*myc* protein [32] overnight at 4°C and subsequently with Protein-A agarose (Gibco-BRL) for 3 h at 4°C. The precipitated antibody/c-*myc* complex was rinsed 4 times in radioimmune precipitation buffer (10 mM Tris pH 8.0, 1.5 mM $MgCl_2$, 150 mM NaCl, 1 mM EDTA, 1% Triton-X 100, 1% deoxycholate, 0.1% SDS, and 1 mM PMSF) and heated to 95°C in sample buffer [33] for 10 min. Immunoprecipitated proteins were analyzed by SDS-PAGE on a 12.5% polyacrylamide gel and visualized by autoradiography.

Materials

Recombinant PDGF-AA (rPDGF-AA) (> 95% pure by PAGE) and PDGF-BB (rPDGF-BB) (> 95% pure by PAGE) were obtained from Biosource Inc. (Westlake Village, CA). Platelet-poor plasma (PPP) was made as previously described [26]. Protein-A agarose (BRL) was pretreated with a blocking solution of 1% BSA (Pentex fraction V, Miles Pharmaceuticals) in PBS.

RESULTS AND DISCUSSION

Previous studies have shown that quiescent Balb/c-3T3 fibroblasts must be exposed to PDGF-AB for approximately 4–6 h after which time the presence of plasma factors is sufficient for fibroblasts to traverse G_1 and complete the cell cycle. To compare the time of exposure required for PDGF isoforms to induce the proliferation of quiescent cells, we incubated density-arrested Balb/c-3T3 fibroblasts in the presence of PPP and either PDGF-AA or PDGF-BB for various times. Following the removal of PDGF, we continued the incubation of the cells in medium containing 10% PPP and [^{3}H]thymidine for 30 h. The percent of [^{3}H]thymidine-labeled nuclei was determined. As indicated in Figure 1, both PDGF-AA and PDGF-BB were capable of inducing a full mitogenic response in fibroblasts. However, whereas PDGF-AA must be present for a period of at least 16 h to stimulate 90% of the cells to initiate DNA synthesis, PDGF-BB induced the proliferation of the same number of cells following a 4 h treatment. In contrast to PDGF-BB, a 4 h exposure to PDGF-AA only stimulated 20% of the quiescent population of fibroblasts to undergo DNA synthesis. We have used varying amounts of PDGF-BB ranging from subnanogram to 50 ng/ml to induce a range of mitogenic responses from 15% to 100%. At the lower concentrations of PDGF-BB tested, we observed a decreased percentage of cells entering the cell cycle; however, a 4 h PDGF-BB exposure period stimulated the same percentage of cells to enter the cell cycle as did continuous exposure of cells to the same concentration of PDGF-BB. These results agree with the data depicted in Figure 1, in that a 4 h exposure to PDGF-BB was sufficient for maximal stimulation of fibroblasts.

One possible explanation for the longer exposure time required for PDGF-AA to induce growth (Fig. 1) is that fewer receptors are activated (PDGF α receptors only) and therefore more time is needed to initiate competence and the immediate early responses. If this is so the rate of entry of PDGF-AA–treated cells into S phase would be slower than that of PDGF-BB–stimulated cells. To compare rates of entry into S phase, density-arrested Balb/c-3T3 fibroblasts were placed into medium containing 10% PPP and either PDGF-AA or PDGF-BB at concentrations sufficient to stimulate 95–100% of the cells. At the appropriate times cultures were fixed and processed for autoradiography to determine the percentage of cells in S phase. Figure 2 shows that cultures stimulated with PDGF-AA and PDGF-BB entered S phase after a lag of 12 h with the same kinetics. We conclude that PDGF-AA initiates cellular proliferation at the same rate as PDGF-BB even though PDGF-AA activates only 20–25% of the total PDGF receptor population.

To ascertain whether differences exist in the expression pattern of two of the immediate early genes, c-*myc* and c-*fos*, we exposed fibroblasts to PDGF-AA or BB in PPP-supplemented medium and then examined the level of mRNA expression of these genes at the times indicated in Figure 3. The time required for induction of

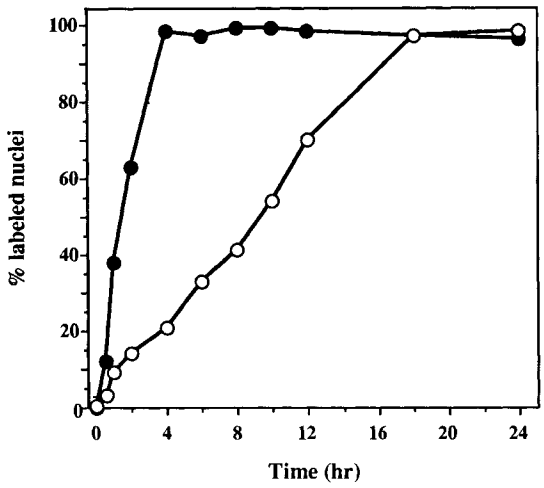

Fig. 1. Time of exposure of cells to PDGF-AA or PDGF-BB to induce DNA synthesis. Density-arrested Balb/c-3T3 fibroblasts in 35 mm dishes were treated with Dulbecco's modified Eagle's medium (DMEM) containing 10% PPP and either 50 ng/ml PDGF-AA (○) or 25 ng/ml PDGF-BB (●) for the times indicated. Cells were then rinsed and incubated in medium containing 10% PPP and [^{3}H]thymidine (5 μCi/ml) for an additional 30 h at which time percent labeled was determined as described in Materials and Methods. Percent labeled nuclei was determined as compared to the total number of nuclei present. The data points represent the average of three separate experiments. The standard deviation was ± 10%.

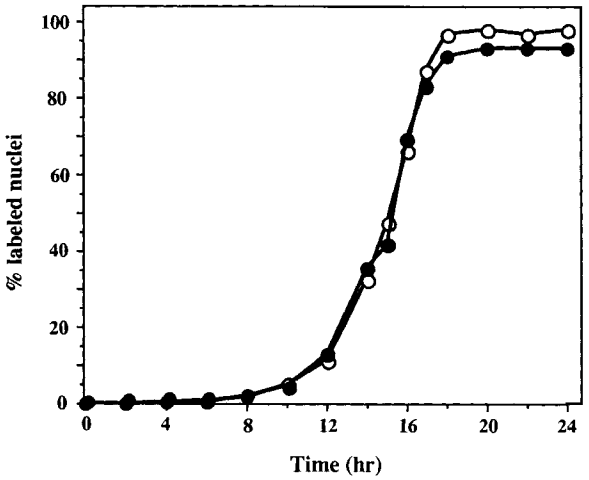

Fig. 2. Rate at which Balb/c-3T3 fibroblasts enter S phase in the presence of either PDGF-AA or BB. Density-arrested Balb/c-3T3 fibroblasts in 35 mm dishes were treated with DMEM containing 10% PPP and [^{3}H]thymidine (5 μCi/ml) and either 50 ng/ml PDGF-AA (○) or 25 ng/ml BB (●) for the times indicated. Results are expressed as percent labeled nuclei as compared to the total number of nuclei present. The data points represent the average of three separate experiments. The standard deviation was ± 10%.

these genes and the level of expression was comparable for PDGF-AA– and PDGF-BB–treated fibroblasts (Fig. 3). The autoradiogram depicting c-*fos* and TK mRNA levels was intentionally overexposed to intensify TK expression. These data, like those in Figure 2, show that PDGF-AA and PDGF-BB equally induce early growth responses. The expression of a late G_1 gene, TK (Fig. 3), was also equivalent in cells treated with either PDGF-AA or BB and thus correlates with the equal rate of entry of cells into S phase (Fig. 2). The difference in the number of PDGF β versus α receptors, therefore, does not affect the ability of PDGF-AA and PDGF-BB to equivalently activate the genes examined or produce similar rates of entry into S phase.

Since c-*myc* expression has been correlated with competence formation we examined the induction of the MYC protein, as it is possible that a PDGF isoform-specific event may control the translation of the c-*myc* mRNA. The data in Figure 4, however, show that similar amounts of MYC protein were synthesized in the presence of PPP and either PDGF-AA or PDGF-BB. These data demonstrate that similar amounts of MYC protein were made in response to concentrations of PDGF-AA and PDGF-BB that induced similar amounts of DNA synthesis. While our data do not exclude the possibility that other early genes may be differentially regulated by PDGF-AA and PDGF-BB, the data suggest that a mechanism other than control of c-*myc* and c-*fos* is responsible for the differences observed

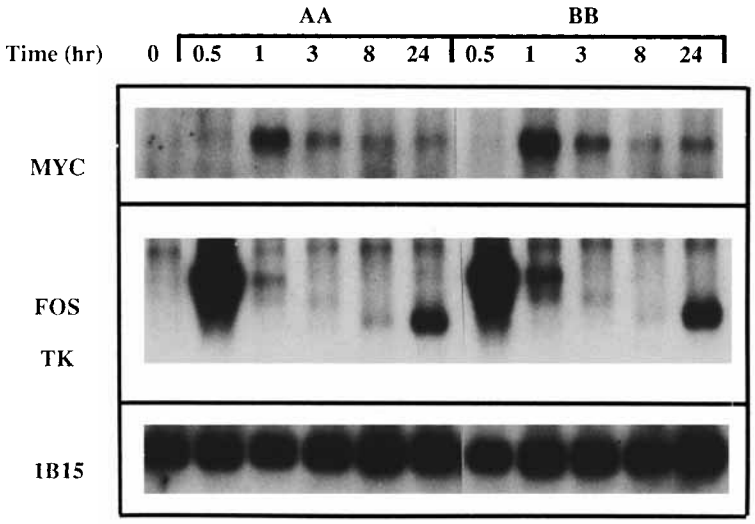

Fig. 3. Expression of the c-*fos*, c-*myc*, and TK genes in Balb/c-3T3 fibroblasts following exposure to either PDGF-AA or BB. Density-arrested Balb/c-3T3 fibroblasts in 100 mm dishes were exposed to medium containing 10% PPP and either 50 ng/ml PDGF-AA or BB for the times indicated. Cells were then harvested for poly(A)RNA as described in Materials and Methods and northern analysis was performed. The autoradiogram depicting c-*fos* and TK mRNA levels was intentionally overexposed to intensify TK expression. The lower panel depicts the constitutive expression of cyclophilin (1B15) to normalize for loading efficiency.

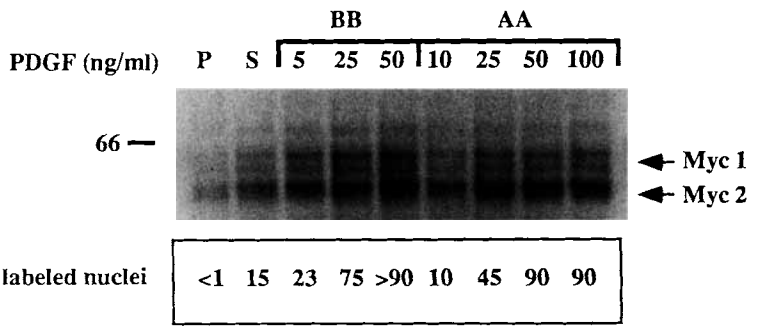

Fig. 4. Induction of c-*myc* protein following exposure of cells to either PDGF-AA or BB. Density-arrested Balb/c-3T3 fibroblasts in 100 mm dishes were incubated in methionine-deficient DMEM containing 10% fetal calf serum (S) or either 10% PPP (P) alone or 10% PPP and various concentrations of either PDGF-AA or BB for 1 h at 37°C. [^{35}S]methionine at a final concentration of 400 μCi/ml was then added and the cells were incubated for an additional 20 min at 37°C. Cells were then immunoprecipitated with polyclonal antisera to a murine c-*myc* peptide as described in Materials and Methods. Immunoprecipitated proteins were analyzed by SDS-Page on a 12.5% polyacrylamide gel and visualized by autoradiography. The positions of the 66 kD molecular weight marker and Myc-1 and Myc-2 proteins are indicated. The bottom panel depicts % labeled nuclei in density-arrested Balb/c-3T3 fibroblasts exposed to the various agents for 24 h at 37°C.

between PDGF-AA and PDGF-BB to induce stable competence formation.

Our data indicate that genes expressed within the 4 h PDGF-BB treatment period are sufficient to stimulate the entry of cells into G_1 and S phase. While PDGF-AA, in the presence of PPP, is also capable of fully stimulating cells to proliferate, it does not produce a competent cell population within 4 h. The continuous exposure of cells to PDGF-AA may be required for expression of a gene or activation of a biochemical process that PDGF-BB either does not require or activate, following a transient exposure, in a sustained fashion. The data in Figure 4 suggest that PDGF-AA-mediated expression of the MYC protein is not sufficient for the stable initiation event. Reports by Armelin et al. [12] and Sorrentino et al. [14] suggest that c-*myc* expression functions with an additional cellular event or events to induce competence. Our data indicate that PDGF-AA and therefore the PDGF α receptor are unable to stimulate this additional component of competence formation.

While there are more PDGF β than α receptors in Balb/c-3T3 fibroblasts, it is unlikely that the stimulation of a greater number of PDGF receptors by PDGF-BB as compared to PDGF-AA is responsible for the rapid induction of competence. We have used various concentrations of PDGF-BB, and therefore activated different numbers of PDGF β and α receptors, and found that a 4 h PDGF-BB exposure period maximally stimulated DNA synthesis. In addition, the rates of entry into S phase of cells treated with PDGF-AA and PDGF-BB are the same and early gene expression is identical. These data demonstrate that PDGF-AA initiates the proliferation of density-arrested Balb/c-3T3 fibroblasts as efficiently as does PDGF-BB. We conclude that while activation of PDGF α receptors initiates growth, this initiation process differs from the activation of PDGF α and β receptors by PDGF-BB because only PDGF-BB can rapidly induce competence. Our data strongly support the hypothesis that PDGF α and β receptors have some regulatory control differences.

Recent reports have indicated that PDGF-AA and PDGF α receptors are expressed early in development and may mediate some aspects of cell differentiation [34]. Perhaps PDGF-BB and PDGF-AB, which are capable of activating β receptors in the absence of α receptors [35], control the proliferation of cells in a wound repair response while α receptors are involved during development and cell differentiation. Therefore, unlike PDGF-BB, PDGF-AA may not induce stable competence which is more likely required in fibroblasts during wound repair. While PDGF-AA is capable of activating a number of the same genes as BB, there may be additional genes that are only expressed following β receptor activation. The expression of these PDGF β receptor-specific genes may be sufficient to stably sensitize the cells to the progression factors required during G_1.

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