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Rapid Modulation of a 64 K Dalton fibroblast protein: a PDGF mediated early cellular event

Jai Pal Singh*, Paul D. Bonin, and Lonnie D. Adams

Metabolic Diseases Research and Biotechnology-Biopolymer, The Upjohn Company
Kalamazoo, MI 49001

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The results presented here reveal a novel platelet derived growth factor (PDGF) mediated early cellular event. Treatment of growth arrested Balb/c3T3 fibroblasts with PDGF induces a specific and rapid modulation of a 64,000 Dalton (64 KD) protein preexisting in quiescent cells. The kinetics of 64 KD protein modulation indicate that, temporally, this PDGF mediated step lies between the membrane associated immediate events such as receptor autophosphorylation or ion mobilization and the earliest known transcriptional event, the activation of the proto-oncogene *c-fos*.

Platelet derived growth factor (PDGF) is a potent growth mediator for mesenchymal cells (1,2). Its biological effects are mediated by initial binding to specific high affinity cell surface receptors (3-6). Within 10-20 min after PDGF addition, a transcriptional activation of specific nuclear genes is initiated (7-9). The sequence of biochemical events that leads to the activation of nuclear gene transcription and subsequent DNA replication is currently a subject of intense investigation. Since PDGF mediated activation of gene transcription occurs in the absence of *de novo* protein synthesis, the catalytic events required for the signal transduction pathway are likely to be mediated by appropriate modifications of pre-existing proteins from an inactive to active form or *vice versa*. PDGF induced protein phosphorylation has been previously examined (10-12). More recently, using upstream inducible enhancer sequences of *c-fos*, it has been demonstrated that serum or purified PDGF induces transcription enhancing factors that directly interact with DNA and modulate *c-fos* transcription (13-15). To date, no other intermediate step representing specific modification of a pre-existing protein has

*To whom correspondence and reprint requests should be addressed at 7250-209-415, 301 Henrietta Street, The Upjohn Company, Kalamazoo, MI 49001.

Abbreviations: PDGF, platelet derived growth factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; IGF, insulin-like growth factor.

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been identified. In the present study we show a specific and rapid modulation of a fibroblast protein that occurs prior to the transcriptional activation of c-*fos*.

METHODS AND MATERIAL

[^{35}S]-Methionine Labeling of Cellular Proteins

Stock cultures of Balb/c3T3 cells were maintained in Dulbecco's Modified Eagle's Medium (DME) containing 10% calf serum as described before (16). Cells were plated in 35 mm culture dishes at a cell density of 7 X 10^4/dish in 3 ml of DME containing 10% calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Plates were incubated for five days in order to obtain a confluent monolayer of quiescent cells. The final cells density was about 4-5X10^5/dish. The spent medium was removed, cells were washed once in DME, and the medium was replaced with 1 ml of methionine free DME containing 0.5% platelet poor plasma and 34 µCi/ml [^{35}S]-methionine (specific activity 1151 Ci/mmol). Cells were incubated at 37°C over 5% CO_2, 95% air for 6 hr. At the end of the incubation, medium was removed and cell monolayer was washed twice in DME. This protocol was employed for the labeling of cellular proteins throughout these studies. Proteins were solubilized by extraction of labeled cells in 0.5 ml of urea lysis buffer that consisted of 9 M urea, 4% NP-40, 2% 2-mercaptoethanol, and 2% pH 9-11 ampholytes. Extracts were stored at -70°C until used for 2-D gel electrophoresis.

Two-Dimensional Gel Electrophoresis

Samples for 2-D gels were prepared by first removing the particulate materials by centrifugation at 230,000 g for 60 min. Clearl supernatants were then analyzed by 2-D gel electrophoresis as described previously (17,18). First dimension tube gels were run using pH 3.5-10 ampholytes. The second dimension, SDS-PAGE was performed using 10-20% gradient gels.

Scanning of Autoradiograms and Quantification

Autoradiography of the dried gels was performed using Kodak XAR 5 film and intensifying screens. The autoradiograms were scanned as 2048 X 2048 pixel digitized images, which were stored on disk in the Visage image analysis system (Bio Image Corp., Ann Arbor, MI). System software was used to automatically find the boundaries of each protein spot and determine its itegrated optical density.

Purified human natural PDGF was obtained from R&D Systems Inc., Minneapolis, MN. Recombinant PDGF (c-*sis*) and IGF-1 were from Am Gen Corporation, Thousand Oak, CA. EGF and FGF were obtained from Collaborative Research Inc., Bedford, MA.

RESULTS AND DISCUSSION

In order to detect low abundance proteins and their potential modification upon PDGF treatment, cellular proteins were first labeled by incubation of quiescent cultures of Balb/c3T3 fibroblasts in [^{35}S]-methionine containing medium for 6 h. Cells were then washed and treated with PDGF for varying lengths of time. At the indicated intervals, medium was removed and proteins were extracted in urea lysis buffer containing 9 M urea and 4% NP-40. Protein extracts were subjected to high resolution 2-dimensional gel electrophoresis followed by autoradiography. Fig. 1A shows the 2-D gel electrophoresis pattern of [^{35}S]-methionine labeled total cell proteins extracted from a growth arrested culture of Balb/c3T3 cells. For comparisons and convenience of data presentation, the protein profile shown in Fig. 1A is from a culture that was incubated for 30 minutes after the removal of the free radiolabeled [^{35}S]-methionine. The 2-D gel

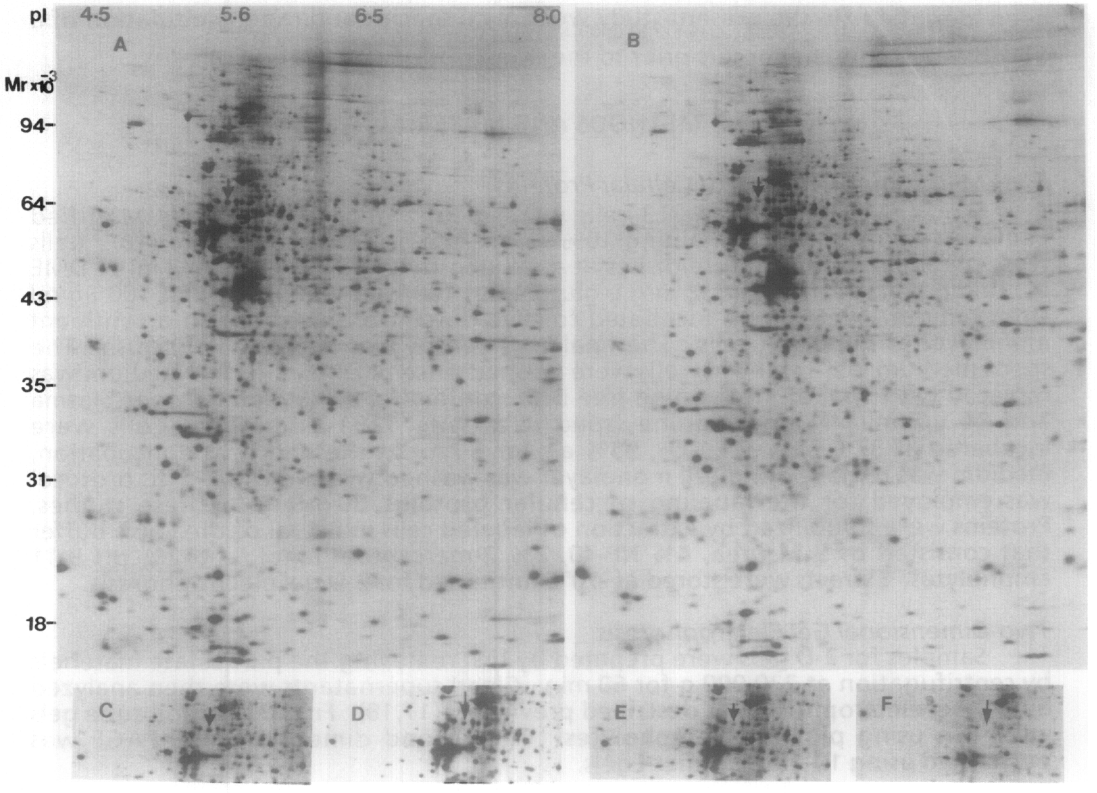

Figure 1. Two-dimensional polyacrylamide gel electrophoresis patterns of [^{35}S]-methionine labeled proteins extracted from control and PDGF treated Balb/c3T3 cells. Confluent cultures of Balb/c3T3 cells (4-5x10^5 cells/35 mm/dish) were labeled as described under Methods. The cells were then washed and incubated in medium containing 5 ng/ml PDGF (B-E) for 30, 5, 10, 20 min respectively. Control cells without PDGF were incubated in parallel for the same length of time. For convenience of data presentation, only the 30 min data point from the controls is shown (A). Fig. (F) is for cells that were treated with PDGF and 100 µg/ml cyclohexamide for 30 min. Actinomycin D treatment also produced similar results (not shown here). Total cell proteins were solubilized in 0.5 ml of urea lysis buffer that consisted of 9 M urea, 4% NP-40, 2% 2-mercaptoethanol, and 2% pH 3-10 ampholytes. Clear supernatants were then analyzed by 2-D gel electrophoresis as described before (17-18). Autoradiography of gels was performed under identical conditions and exposure times. The data shown here are representative from an experiment in which three independent incubations for each time point were performed.

profile of cell extracts prepared immediately after the 6 h labeling period were virtually identical to that shown here. In general, about 900 radiolabeled proteins spots were observed. Visual and computer aided densitometric analysis and comparison of protein maps from the control and the PDGF treated cultures showed that >99% of the protein spots were qualitatively matched. During this early period, the most dramatic difference between the control and the PDGF treated cells was a rapid decrease in a 64 K Dalton protein indicated by the arrow in Fig. 1. By 30 min, the 64 KD protein was barely detectable in PDGF treated cultures (Fig. 1B). In control cultures, incubation up to 30 min produced only a slight reduction in

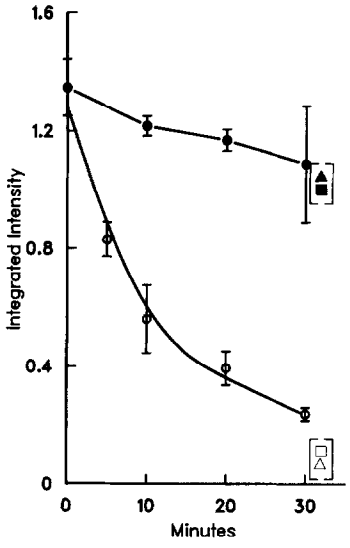

Figure 2. Quantification of CP 64 modulation in response to PDGF. The autoradiograms from controls (●-●) and PDGF treated (o-o) cells were scanned as 2048 x 2048 pixel digitized images, which were stored on disk in the Visage™ image analysis system (Bio Image Corp., Ann Arbor, MI). System software was used to automatically find the boundaries of each protein spot and to determine its integrated optical density. The values shown here are in arbitrary units derived by taking the ratio of the integrated intensity of CP 64 and a reference protein that did not exhibit a significant change in response to PDGF treatment. Data shown represent mean values (n = 3) with standard deviation indicated for each data point. Data in parenthesis show the effect of cyclohexamide (△) or actinomycin D (□) on the control (▲■) and PDGF treated (△□) cells.

the intensity of this protein spot. In a parallel experiment when the gels were stained with silver reagents instead of autoradiography, the 64 KD protein was detectable and showed similar kinetics of actual loss of protein mass upon treatment with PDGF (data not shown). Fig. 1F also shows that PDGF modulation of 64 KD protein was not affected by inhibitors of transcription or translation, suggesting that this PDGF mediated event does not require new protein synthesis. From the intensity of silver staining or the intensity of the 64 KD protein spot relative to the integrated intensity of all labeled proteins in the autoradiograms shown in Fig. 1A, it is apparent that the 64 KD protein spot represents a low abundance protein of Balb/c3T3 fibroblasts. Previous approaches to define PDGF modulation of cellular proteins have focused on the PDGF inducible synthesis of new proteins (19-22). The discovery of a specific modulation of a protein constitutively expressed in growth arrested cells by PDGF is novel and may represent an important step in the signal transduction pathway. For the time being this protein will be referred as cell protein (CP) 64.

Fig. 2 shows the kinetics of modulation of CP 64 in response to PDGF. It should be noted that the cultures were treated with PDGF after a wash out of the free [35S] methionine and the data represent a change in the amount of CP 64 from a steady state level. A quantitative change in the amount of CP 64 was detected as early as

3-5 min after PDGF treatment. By 30 min, the cellular concentration of CP 64 in PDGF treated cells had declined by about 79% as compared to the controls. Similarly, PDGF treatment resulted in about 13-fold change in the half life of CP 64. In growth arrested cells, the estimated half life of CP 64 was about 110 min. PDGF treatment induced a rapid decline in CP 64 resulting in a half life of about 7-8 min. Figure 2 also shows that cyclohexamide or actinomycin D did not significantly effect either the steady state levels or the PDGF modulation of CP 64. Taken together, the time course data and the metabolic inhibitor data clearly show that PDGF mdoulation of CP 64 is an active and specific event that occurs prior to the transcriptional activation of the earliest PDGF inducible proto-oncogene c-*fos*. Increased levels of c-*fos* mRNA are detectable at 10 min and peak at 30 min after PDGF treatment (8).

Modulation of CP 64 occurred at PDGF concentrations similar to those required for the stimulation of DNA synthesis in Balb/c3T3 cells. PDGF was the most potent stimulator of CP 64 modulation. Fig. 3 shows that polypeptide growth factors

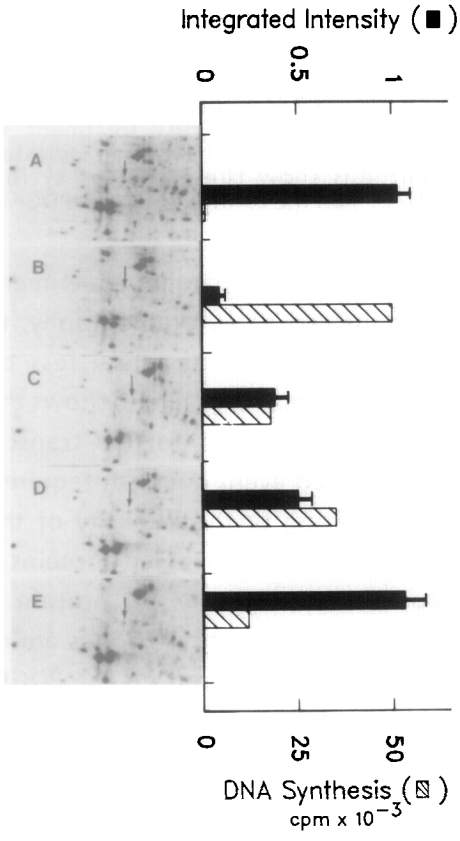

Figure 3. Effect of other growth factors on CP 64 modulation. Confluent cultures of Balb/c3T3 fibroblasts (in triplicate) were labeled with [^{35}S]-methionine as in Fig. 1 and then incubated for 30 min in the absence (A) or presence of 5 ng/ml of growth factor, PDGF (B); EGF (C); acidic FGF (D) or IGF-1 (E). Quantification of CP 64 was performed as in Fig. 2. For determination of DNA synthesis in response to growth factors was determined as described previously (16).

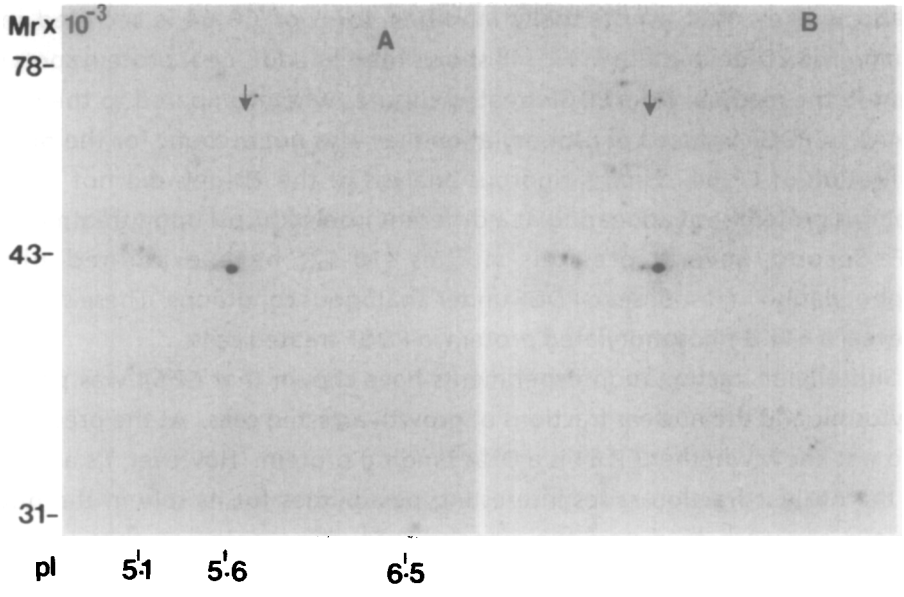

Figure 4. 2-D gel profile of proteins appearing in the culture medium of Balb/c3T3 cells during 30 min incubation in the absence or presence of PDGF. Supernatants from 6 plates of each of the control (A) and PDGF (B) cells were pooled separately and then concentrated to 0.5 ml by diaflo filtration. A sample of culture medium equal to 10-fold equivalent of the cell extract was used for 2-D gel electrophoresis. Also, in order to further increase the sensitivity of detection, the films for the autoradiography of the proteins in the culture medium were exposed twice the length of time employed for the cell extracts. Arrow indicates the expected location of CP 64.

epidermal growth factor (EGF) and fibroblast growth factor (FGF) also induced changes in the concentration of CP 64 but to a lesser degree than PDGF. Treatment with IGF-1 did not produce a significant change in CP 64. Fig. 3 also shows the stimulation of DNA synthesis in Balb/c3T3 cells in response to an optimum concentration of each of the above growth modulators. It is interesting to note (Fig. 3), that the ability of PDGF, FGF, EGF, and IGF to modulate CP 64 as shown here exhibits a striking parallelism with their ability to stimulate DNA synthesis in Balb/c3T3 cells. A similar parallelism seems to exist between the ability of these growth factors to induce proto-oncogene c-*fos* (7-9) and the modulation of CP 64.

We next investigated the nature of PDGF mediated alteration in CP 64. Our initial studies suggest that secretion into the medium may not explain the reduced levels of CP 64 in the cell extracts. CP 64 was not detected in 2-D gels of culture supernatants obtained from the PDGF treated cells. For these experiments, culture supernatants from PDGF treated cells were concentrated and a sample equal to 10-fold the cell extract equivalent used in Fig. 1 was applied to the gels. As shown in Fig. 4, several [^{35}S]-methionine labeled proteins were detected in the culture supernatant. However, a protein corresponding to CP 64 could not be detected even when a highly enriched sample of the culture medium was applied to the gels.

It is also unlikely that a potentially modified form of CP 64 is secreted in the medium. The 2D-gel pattern in Fig. 4B shows that no additional protein spots were present in the medium from PDGF treated cultures when compared to the controls (Fig. 4A). A PDGF induced phosphorylation may also not account for the observed modification of CP 64. First, a rigorous analysis of the 2D-gels did not reveal an analogous protein spot appearing at a different isoelectric pH upon treatment with PDGF. Second, several previous studies (10-12) have examined protein phosphorylation in response to PDGF under analogous conditions. These studies did not reveal a 64 KD phosphorylated protein in PDGF treated cells.

Subcellular fractionation experiments have shown that CP 64 was present in the cytosolic and the nuclear fractions of growth arrested cells. At the present time, we do not know whether CP 64 is a DNA binding protein. However, its association with the nuclear fraction raises interesting possibilities for its role in the nucleus. Since our approach to dissect the signal transduction pathway that led to the discovery of CP 64 modulation was based on the detection of events that follow PDGF addition (as opposed to a backward approach starting from gene level and define factor involved in transcription (13-15)), it would now be interesting to determine whether CP 64 belongs to the family of DNA binding proteins. While no casual relationship between CP 64 and the activation of gene transcription has yet been demonstrated, our findings have provided an important intermediate step to study the cellular events that precede and follow the CP 64 modulation in response to PDGF. Clearly, the rate and the degree of CP 64 modulation and the temporal position of these changes (between the membrane associated immediate events and the activation of nuclear gene transcription) are quite intriguing with regards to a potential role of CP 64 in the PDGF mediated signal transduction pathway.

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