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STIMULATION OF PROTEIN PHOSPHORYLATION IN SWISS MOUSE 3T3 CELLS BY HUMAN PLATELET DERIVED GROWTH FACTOR

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SUMMARY: Purified human platelet derived growth factor stimulates the phosphorylation of an ≃ 33,000 M.W. protein in Swiss mouse 3T3 cells. Phosphorylation can be detected 3 min after ^{32}P-labelled cells are stimulated by PDGF and is marked after 10 min. The $S_{0.5}$ of PDGF for phosphorylation is 3 ng/ml. Phosphoserine but not phosphotyrosine has been identified in hydrolysates of this ≃ 33,000 M.W. protein.

INTRODUCTION

Rapid and profound changes in overall cell metabolism, in DNA synthesis, and in cell division have recently been shown to result from the addition of specific polypeptide growth factors to quiescent cultured mammalian cell lines (1-5). The diversity of cellular responses to specific growth factors suggests that these growth factors may initiate responses of a pleotrophic nature, although the mechanisms whereby growth factors regulate mammalian cell division and other cellular responses have not been biochemically identified.

One possible mechanism to initiate multiple cellular metabolic activities is the activation of specific protein kinases. Phosphorylation of a 33,000 M.W. membrane protein occurs rapidly when fibroblast growth factor (FGF) or serum are added to ^{32}P-labeled 3T3 cells (6). Epidermal growth factor (EGF) stimulates the cyclic AMP-independent protein phosphorylation of a membrane protein in A-431 cells. Membrane preparations solubilized by non-ionic detergents retain EGF-binding capacity and the capacity for EGF to stimulate phosphorylation of specific membrane proteins (7-12). Phosphotyrosine has been identified in both endogenous membrane proteins and in added histones after stimulation by EGF. Phosphotyrosine has been identified also in phosphorylated proteins in cells specifically transformed by src-gene

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containing transforming viruses (13-17).

We have investigated protein phosphorylation as an early event after purified platelet derived growth factor is added to confluent mouse 3T3 cells in culture. Using preparations of PDGF purified to homogeneity (18), we have demonstrated a phosphorylated protein which appears in extracts of 3T3 cells harvested shortly after the addition of the platelet derived growth factor to resting cells. This protein has an approximate molecular weight of 33,000.

METHODS

Swiss mouse 3T3 cells were grown until they reached confluency in Dulbecco's Modified Eagle's medium (GIBCO) containing 10% fetal calf serum. PDGF was purified to apparent homogeneity before use in all experiments and was assayed as described previously (18). For experiments seeking phosphorylation, the medium was changed to 0.1 mM phosphate (10% of normal phosphate content). Cells were then preincubated with ^{32}P (100 uCi/ml, New England Nuclear) at 37° C for 1 hr to avoid the effect of an increase in the specific activity of phosphate pools caused by the early stimulation of phosphate uptake by mitogens (4, 5). The labeling medium was removed and the cell monolayers were rinsed twice with low phosphate medium. The cells were then incubated in low phosphate medium in the absence or presence of PDGF (25 ng/ml) at 37° C. The reaction was terminated by the addition of cold 10% TCA after the cells were rinsed with cold 0.15 M NaCl, 20 mM Tris-HCl, pH 7.4. The TCA precipitate was washed twice and solubilized in gel buffer (2% SDS, 2% 2-mercaptoethanol, 10% glycerol, 0.004% Bromophenol Blue, 60 mM Tris-HCl pH 6.8). After heating at 100° C for 2 min, the proteins were separated in a gradient of 7.5-12% polyacrylamide with 0.1% SDS, according to the method of Laemmli (19). Protein concentration was determined by the method of Lowry (20).

Autoradiography was performed using Kodak X-Omat R film after gels were stained with Coumassie blue and dried under vacuum.

To analyze the phosphoamino acid formed, the 33,000 M.W. protein band was cut from the gel, homogenized in Dounce homogenizer and the protein was extracted in 0.05 M NH_4HCO_3 and 0.1% SDS. Forty μg of bovine serum albumin was added as carrier, the protein precipitated at 4° C with 20% trichloroacetic acid, washed with ethanol:ether (1:1), suspended in 6 N HCl, and partially hydrolized at 100° C for 2 hrs in tubes sealed under vacuum. After evaporation, the hydrolysates were dissolved in buffer containing 1 mg/ml of the standards phosphoserine, phosphothreonine, and phosphotyrosine. Acid hydrolysates were analyzed on thin layer plates by electrophoresis at pH 3.5 and at pH 1.9.

RESULTS

After 3T3 cells were preincubated with ^{32}P for 1 hr, cultures were washed (0 time) and incubated with and without PDGF (25 ng/ml) for an additional 30 min. Cells harvested at 0 time and after 30 min of incubation were processed as described in Methods. Figure 1 presents radioautograms of SDS polyacrylamide gels prepared from cell extracts of these cultures. The

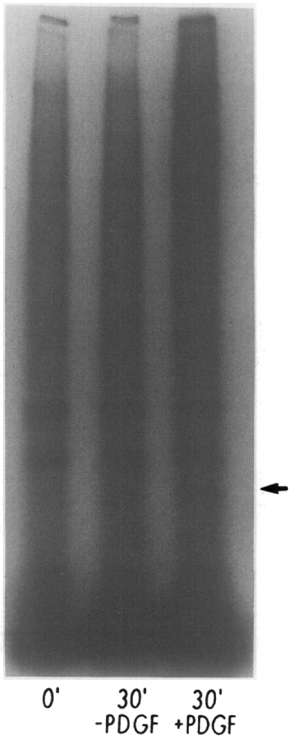

Figure 1. Radioautograms of SDS polyacrylamide gels of cell extracts incubated with (+) or without (-) PDGF at 25 ng/ml for 30 min. A 0 time control cell extract is included also. The arrow points to proteins migrating at $M_r \simeq 33,000$.

arrow points to an ≃ 33,000 M.W. protein. This protein is minimally phosphorylated at 0 time and after 30 min incubation without PDGF. Cell extracts showed essentially no increase in phosphorylation of this protein incubated without PDGF. In contrast, a striking increase in radiolabeling of this protein is shown in radioautograms prepared from cells incubated with 25 ng/ml PDGF. PDGF thus stimulates phosphorylation of a 33,000 M.W. protein in confluent 3T3 cells. Other proteins are shown to be phosphorylated but to a far less degree than the 33,000 M.W. protein identified in the gels.

The time course of protein phosphorylation in response to PDGF was then examined. Figure 2 shows radioautograms from SDS gels of extracts of cells exposed to PDGF for 0, 1, 3, 10, and 30 min. A modest increase in radiolabelling is noted 3 min after exposure to PDGF, with very significant

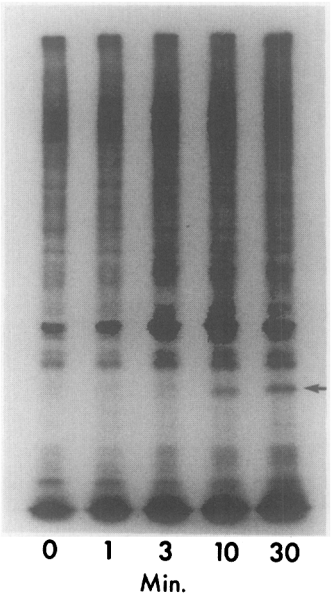

Figure 2. Time course of the phosphorylation reaction. Radioautograms were prepared from cell extracts from cultures obtained at 0, 1, 3, 10 and 30 min after PDGF (25 ng/ml) was added. The arrow points to proteins migrating at $M_r \simeq 33,000$.

increases found at 10 and at 30 min. Because PDGF itself (M.W. 28-31,000) is reduced quantitatively by mercaptoethanol into two low molecular weight peptides (21, 22), the 33,000 M.W. phosphoprotein identified in SDS gels cannot be PDGF itself.

Figure 3 shows the response of increasing PDGF from 1 ng/ml to 50 ng/ml or radiolabeling of the 33,000 M.W. protein. Cells were incubated with PDGF at the concentrations shown for 30 min and extracts prepared and processed as above. The radiolabelled 33,000 molecular weight band was cut from the gels, solubilized, and counted. As can be seen in Figure 3, half maximum phosphorylation occurrs at a PDGF concentration ($S_{0.5}$) of $\simeq$ 3 ng/ml ($\simeq 10^{-10}$ M).

Attempts were made to identify the amino acids phosphorylated in the 33,000 M.W. protein. As shown in Figure 4, phosphoserine has been identified in the partial acid hydrolysates. Evidence for phosphotyrosine was not found.

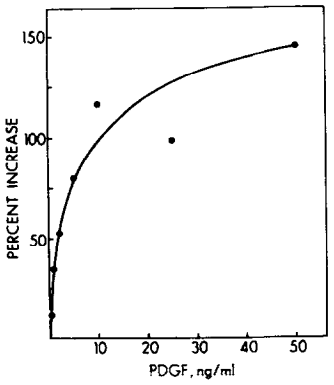

Figure 3. Effect of PDGF concentration on the phosphorylation of an $M_r \simeq 33,000$ protein in cell extracts of Swiss 3T3 cells. Quiescent cells were incubated with PDGF at the concentrations shown. After 30 min, cell extracts were prepared and subjected to SDS polyacrylamide gel electrophoresis. The $M_r \simeq 33,000$ protein was extracted and radioactivity assessed as described in Methods above. The relative percent increase was determined by comparison with control cultures in the absence of PDGF.

DISCUSSION

These experiments provide evidence that phosphorylation of a 33,000 M.W. protein occurs shortly after the addition of purified PDGF to quiescent 3T3 cells in culture. The phosphorylation can be demonstrated as early as 3 min. after the addition of PDGF to quiescent cells and appears to be nearly maximum 30 min. after the addition of PDGF to the cells. Without PDGF, increased phosphorylation of this protein is not seen within limits of detection.

Analysis of the phosphoamino acid generated was performed by extracting the protein from SDS gels, by hydrolysing the protein, and by separating the constituent amino acids by high voltage electrophoresis in the presence of phosphoamino acid standards. The protein was found to contain phosphoserine as the sole phosphorylated amino acid, in contrast to the protein phosphorylated with EGF, which contains phosphotyrosine (7). Efforts to identify this protein are ongoing as are experiments to see if increased phosphorylation of this protein is found in response to other growth factors.

This protein phosphorylation has provided a potent tool for identifying an interaction of PDGF with the 3T3 cells surface as early as 3 min after addition of PDGF to cell cultures. The phosphorylation of this protein also

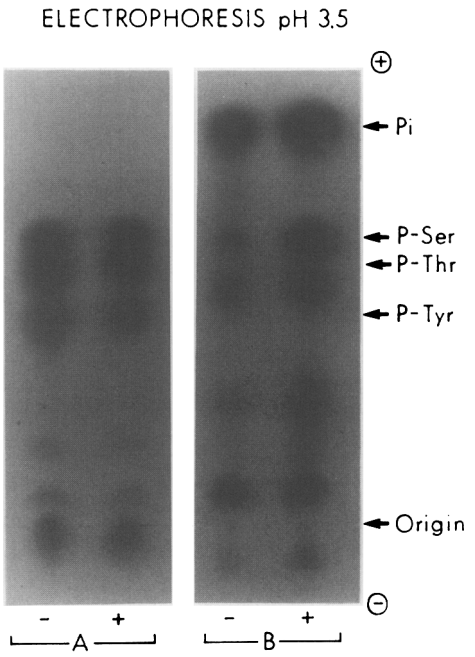

Figure 4. Analysis of phosphoamino acids in 33,000 M.W. protein. Quiescent cells were incubated with (+) or without (-) 50 g/ml PDGF. After 30 min, cell extracts were subjected to SDS polyacrylamide gel electrophoresis. The $M_r \simeq 33,000$ protein was extracted and subjected to partial acid hydrolysis (See Methods). The products were subjected to electrophoresis at pH 3.5 (left) in glacial acetic acid/pyridine/H_2O, 50:5:945 (vol/vol) and at pH 1.9 (right) in glacial acetic acid/formic acid/H O, 78:25:897 (vol/vol). (A) Ninhydrin stain. (B) Autoradiography.

provides an early biochemical event which may be found in common with other growth factors as they interact with specific target cells.

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