Platelet-derived growth factor stimulates the phosphorylation of ribosomal protein S6

Junji Nishimura and Thomas F. Deuel*

Departments of Medicine and *Biological Chemistry, Washington University School of Medicine, The Jewish Hospital of St. Louis, St Louis, MO 63110, USA*

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The human platelet derived-growth factor (PDGF) is both a potent mitogen and a strong chemoattractant protein for cells involved in inflammation and repair. In seeking mechanisms by which PDGF might initiate specific activities in target cells, it was found that highly purified PDGF stimulates the phosphorylation of an M_r ~33000 protein in confluent Swiss mouse 3T3 cells [Biochem. Biophys. Res. Commun. (1981) 103, 355–361]. The M_r ~33000 protein has now been recovered in polysomes by differential centrifugation and identified as ribosomal protein S6 by two-dimensional polyacrylamide gel electrophoresis.

Platelet-derived growth factor *Protein phosphorylation* *S6 protein* *Ribosomal protein*

1. INTRODUCTION

The platelet-derived growth factor (PDGF) is a potent mitogen for mesenchymal cells [2–4] and has been shown to be a strongly chemoattractant for cells involved in inflammation [5] and in repair [6–8]. Tyrosine-specific protein kinase(s) are activated in membranes stimulated by EGF; this protein kinase activity may be involved in cell growth stimulated by polypeptide growth factors and by transforming viruses [9–14]. In [13] PDGF stimulated the tyrosine specific phosphorylation of M_r ~170000 protein by an endogenous protein kinase in membrane preparations of Swiss mouse 3T3 cells, similarly to [14]. ^{32}P incorporation into an M_r ~33000 protein was stimulated within 3 min after addition of PDGF to confluent Swiss mouse 3T3 cells [1]. Phosphoserine but not phosphotyrosine was identified in hydrolysates of this M_r ~33000 protein. We now report that the M_r ~33000 protein has been identified as the ribosomal protein S6 by two-dimensional electrophoretic analysis. This effect of PDGF on S6 phosphorylation is mimicked by serum and other polypeptide growth factors.

* To whom correspondence should be addressed

2. MATERIALS AND METHODS

PDGF was purified to apparent homogeneity (SDS–polyacrylamide electrophoresis) by established methods [15]. Mouse EGF and bovine pituitary FGF were purchased from Kor Biochemicals and bovine insulin from Sigma.

Swiss mouse 3T3 cells were grown to confluency, labelled with ^{32}P (100 μCi/ml, New England Nuclear) for 1 h at 37°C and incubated with or without PDGF (50 ng/ml) for 30 min, as in [1]. These cells were harvested and subjected to cellular fractionation for isolation of ribosomes [16]. Cell monolayers were rinsed twice with 0.15 M NaCl, 20 mM Tris–HCl (pH 7.4), and lysed in 0.1 M KCl, 40 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris–HCl (pH 7.6) containing 0.25% Triton X-100 at 4°C. Nuclei were removed by centrifugation at 10000 × g for 10 min. The supernatant was adjusted to 0.5% sodium deoxycholate and layered over a cushion of 15% sucrose. Ribosomes were pelleted by centrifugation at 200000 × g for 2.5 h.

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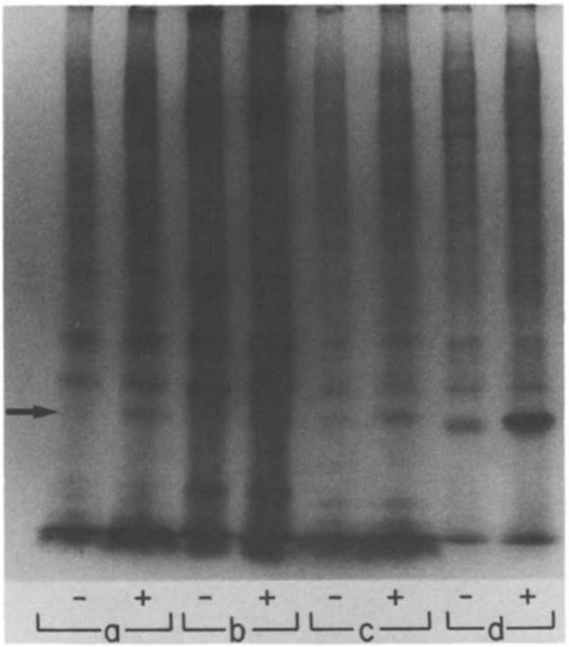

Fig.1. Subcellular localization of a PDGF-stimulated M_r ~33000 phosphoprotein. Cells were labelled with ^{32}P (100 μCi/ml), incubated with (+) or without (−) PDGF (50 ng/ml), lysed in 0.25% Triton X-100 and fractionated as in section 2. Protein (60 μg) from each fraction was solubilized in 2% SDS, 2% 2-mercaptoethanol, 10% glycerol, 0.004% bromophenol blue, 60 mM Tris–HCl (pH 6.8), and applied on 7–12.5% gradient SDS–polyacrylamide gels. Gels were dried and subjected to autoradiography using Kodak X-Omat AR film: (a) whole cell extract; (b) 10000 × g precipitate; (c) 10000 × g supernatant; (d) 200000 × g precipitate; (⟶) M_r ~33000 protein.

Acetic acid extraction of ribosomal proteins followed the procedure in [17]. Ribosomes were suspended in 100 mM $MgCl_2$, 10 mM Tris–HCl (pH 7.7). Glacial acetic acid (2 vol.) was added and the mixture stirred for 1 h at 4°C. The RNA precipitate was removed by centrifugation. The

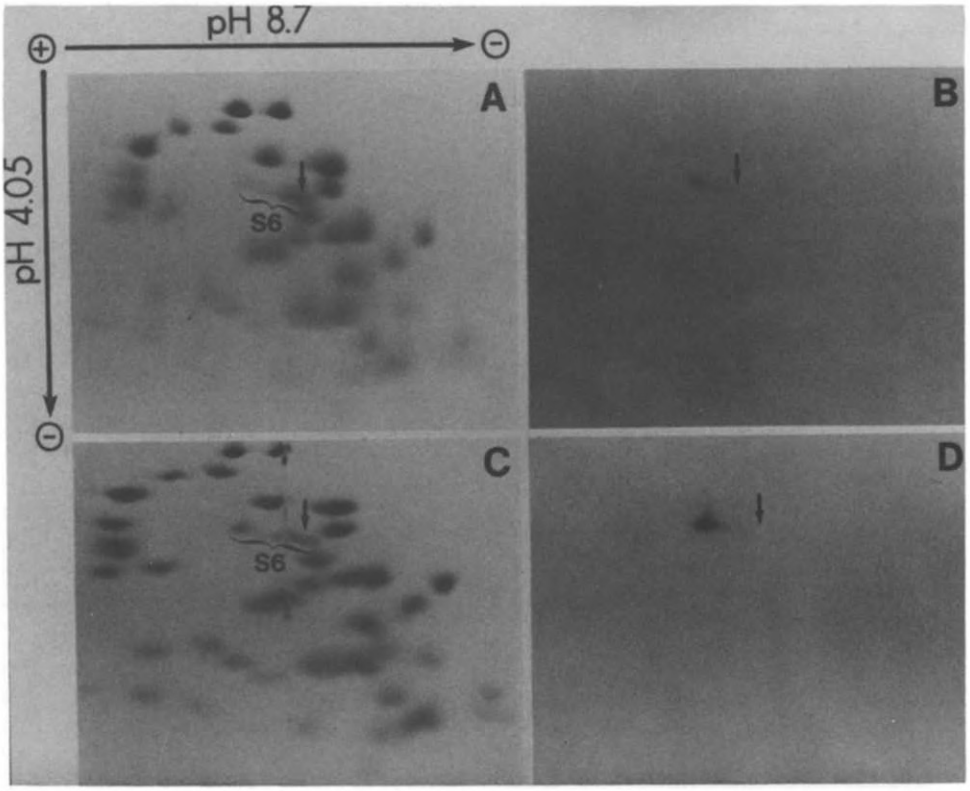

Fig.2. Coomassie blue staining patterns (A,C) and autoradiograms (B,D) of two-dimensional polyacrylamide gels of ribosomal proteins. Ribosomal protein (50 μg) from ^{32}P-labelled cells stimulated without (A,B) or with 50 ng PDGF/ml (C,D) was mixed with 100 μg carrier ribosomal protein from non-labelled cells, assuming that 1 A_{280} corresponds to 0.6 mg protein/ml. Migration in the first dimension (pH 8.7) is from left to right (toward the cathode). Migration in the second dimension (pH 4.05) is from top to bottom (toward the cathode). Arrows mark the location of cathodal side of S6.

supernatant was dialysed against 1% acetic acid for 24 h and lyophilized. Ribosomal proteins were dissolved in 8 M urea, 20 mM Tris, 1 mM EDTA, 26 mM boric acid, 5% 2-mercaptoethanol (pH 8.7) and separated by two-dimensional polyacrylamide gel electrophoresis as in [18].

Protein content was determined as in [19]. SDS–polyacrylamide gel electrophoresis for separation of cellular proteins was done as in [20].

3. RESULTS

When confluent Swiss mouse 3T3 cells were stimulated for 30 min in the presence of PDGF (50 ng/ml), a sharp increase in ^{32}P incorporated into an M_r ~33000 protein is found (fig.1). Cells were then lysed in 0.25% Triton X-100 and the lysates were subjected to cell fractionation as in section 2. The M_r ~33000 phosphoprotein was predominantly localized in the 200000 × *g* precipitate, the crude ribosome fraction. Neither the 10000 × *g* precipitate (nuclear fraction) nor the 200000 × *g* supernatant (cytosol fraction) contained significant amounts of this labelled protein. The 200000 × *g* pellet, however, contains many other non-ribosomal proteins [16].

To further identify the labelled protein, we extracted the ribosomal proteins with acetic acid and examined the phosphorylation of the ribosomal fractions by two-dimensional polyacrylamide gel electrophoresis. The phosphorylated protein in these preparations was identified as S6 protein by comparison of Coomassie blue staining patterns with gel patterns as in [17] (fig.2). No phosphorylation of other proteins was found in the gels. There was a 3-fold increase in the ^{32}P incorporation into S6 from PDGF-stimulated cells compared to control cells when S6 portions were cut from gels, solubilized, and counted. The position of S6 protein shifted toward the anode after addition of PDGF, as protein was progressively phosphorylated [21].

Effects of other polypeptide growth factors on phosphorylation of this protein were examined. Fig.3 shows that EGF, FGF, and insulin as well as PDGF stimulate the phosphorylation of the M_r ~33000 protein which has been identified as ribosomal protein S6. Serum also stimulates phosphorylation of this protein (not shown).

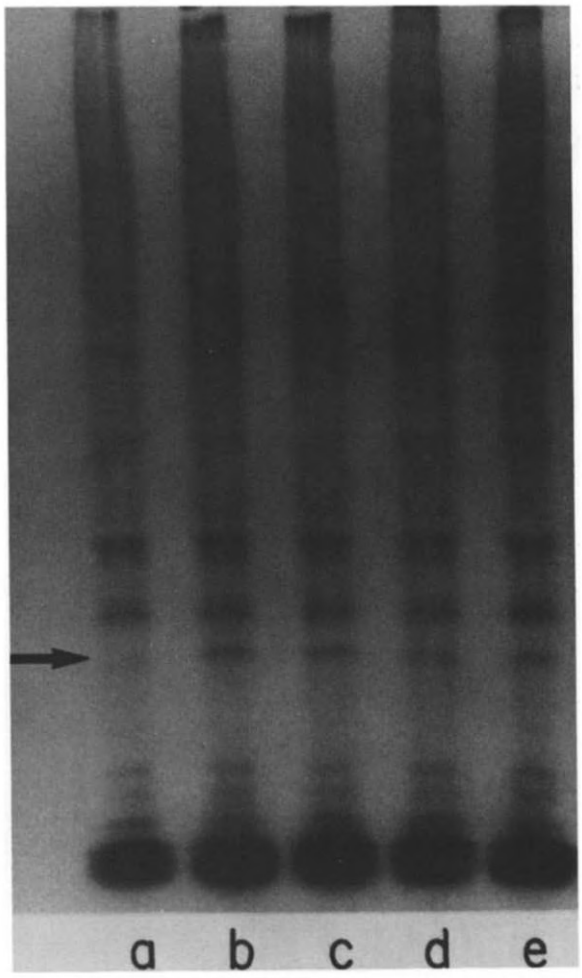

Fig.3. Effects of polypeptide growth factors on phosphorylation of M_r ~30000 protein. Cells were labelled with ^{32}P (100 μCi/ml) and incubated with growth factors for 30 min at 37°C. Protein (40 μg) from whole cell extracts was applied on 7–12.5% gradient SDS–polyacrylamide gels: (a) no additions; (b) PDGF (50 ng/ml); (c) EGF (100 ng/ml); (d) FGF (100 ng/ml); (e) insulin (100 ng/ml); (⟶) M_r ~33000 protein.

4. DISCUSSION

These investigations were undertaken to seek mechanisms whereby PDGF stimulates cells to initiate DNA synthesis [4,22] and induces cells to migrate towards a positive PDGF chemical gradient (chemotaxis) [5–8]. These biological activities of PDGF suggest PDGF may be a uniquely active protein in vivo in initiating aspects of in-

flammation and repair [5–8] and perhaps also in the pathological process of atherosclerosis [23]. In this report, the M_r ~33000 protein whose phosphorylation is stimulated as early as 3 min after exposure of Swiss mouse 3T3 cells to PDGF [1] has been identified as ribosomal protein S6 by two-dimensional electrophoretic analysis in SDS gels. The anodal shift of S6 on autoradiograms suggests a quantitative increase in the amount of phosphate moieties or groups into S6 after addition of PDGF.

Phosphorylation of S6 has been increased under a variety of growth conditions [24]. The stimuli include hormones, cyclic AMP, viral transformation, changes in growth conditions of cells in cultures, and the polypeptide growth factors [21,24–30]. The effect of PDGF and other growth promoting peptides on phosphorylation of S6 suggest these factors share a common mechanism for this early response of cells to growth stimuli. Increased phosphorylation of S6 occurs when protein synthesis is increased, although this correlation is not observed in all instances [24]. It has been suggested that phosphorylation of S6 provides a site for the attachment of certain classes of mRNA [31]. PDGF has been shown to stimulate selective synthesis of cytoplasmic proteins also [32]. Chinese hamster lung fibroblasts have been shown to possess an amiloride-sensitive Na^+/H^+ exchange system that is activated by growth factors. Amiloride blocks both growth factor-stimulated Na^+ influx and growth factor-stimulated ribosomal protein S6 phosphorylation, suggesting a coupling of these two processes [33].

PDGF also stimulates a tyrosine-specific protein kinase in membrane preparation of fibroblasts [13,14]. However, S6 phosphorylation is not due to direct action of the tyrosine specific protein kinase because the phosphorylation of S6 takes place at serine residues [1].

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