Volume 283, number 2, 219–222 FEBS 09762 June 1991

ADONIS 001457939100471W

Platelet-derived growth factor stimulates phosphorylation of the 25 kDa mRNA cap binding protein (eIF-4E) in human lung fibroblasts

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Received 4 March 1991

Platelet-derived growth factor exerts rapid effects on protein synthesis and polysome formation in cultured cells. We report that platelet-derived growth factor stimulates a rapid phosphorylation of eIF-4E in WI-38 human lung fibroblasts. The effect was dependent on both time and PDGF concentrations. Phosphoserine was the sole phosphoamino acid identified and tryptic phosphopeptide maps showed a single phosphopeptide under both control and PDGF conditions. Phosphorylation of eIF-4E may be one of the events required for initiating entry into G_1 and commitment into S phase of the cell cycle.

Initiation factor; Platelet-derived growth factor; Protein biosynthesis; RNA caps

1. INTRODUCTION

The phosphorylation of at least several translation initiation factors is known to regulate mRNA translation rates [1]. Platelet-derived growth factor (PDGF) is a potent mitogen for fibroblasts and smooth muscle cells that has been shown to stimulate the accumulation of polysomes in cultured cells [2–5]. PDGF and other mitogens have been shown to stimulate the phosphorylation of the ribosomal protein S6 which has been implicated in the control of mRNA initiation rates [6]. The 25 kDa mRNA cap binding protein, eIF-4E, exists in both phosphorylated and dephosphorylated forms in mammalian cells [7–10]. eIF-4E also exists in a multi-protein complex designated eIF-4F which contains a p220 subunit that is modified in cells infected by picornaviruses [11,12]. Several lines of evidence suggest that phosphorylation of eIF-4E facilitates mRNA initiation. Physiologic conditions that promote eIF-4E phosphorylation in general stimulate polysome formation and conditions that diminish eIF-4E phosphorylation are accompanied by disaggregation of polysomes [8,10,13,14]. In addition, phosphorylated forms of eIF-4E have been reported to preferentially appear in 43S initiation complexes [15]. The role of phosphorylation of the p220 subunit of eIF-4E and the effects that eIF-4E or p220 phosphorylation have on the eIF-4F complex remain unknown [16,17]. Recent studies have demonstrated that overexpression of eIF-4E in NIH 3T3 cells and Rat 2 fibroblasts results in malignant transformation by an unknown mechanism [18]. On the other hand, overexpression of mutant eIF-4E that lacks the constitutive phosphorylation site does not result in malignant transformation of cells. These observations suggest a possible role for eIF-4E phosphorylation in the control of protein synthesis and possibly some other function that can initiate malignant transformation.

In this report we demonstrated that PDGF stimulates the rapid phosphorylation of eIF-4E in WI-38 human lung fibroblasts. In addition, we provide evidence that PDGF stimulates the phosphorylation of eIF-4E at the constitutive serine phosphorylation site.

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Abbreviations: m^7GTP, 7-methylguanosine triphosphate; eIF-4E, eukaryotic initiation factor 4E; PDGF, platelet-derived growth factor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Reagents*

m^7GTP sepharose was purchased from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Phosphorus-32 was from ICN Biomedicals Inc. (Costa Mesa, CA). Human PDGF (type BB) was either a generous gift from W. Jack Pledger or obtained from Biosource International Co. (Westlake Village, CA). Fetal calf serum was from Hyclone Lab. Inc. (Logan, UT) and tissue culture medium from Gibco (Grand Island, NY). All other reagents were from Sigma Chemical Co. (St. Louis, MO) unless specified otherwise.

2.2. *Cell culture*

WI-38 human lung fibroblasts were obtained from W. Jack Pledger. Monolayer cells were grown in 6 well (9.6 cm) Falcon plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with

10% heat-inactivated fetal calf serum. Cells were grown to confluence and used after they became quiescent in spent growth medium [19]. Prior to metabolic labeling density-arrested cells were washed with DMEM without phosphate and incubated in 1 ml of the same medium for 2 h. After preincubations cells were labeled with 0.2 mCi phosphorus-32/ml for 1 h. The labeling medium was removed, and cells were rinsed with phosphate-buffered saline and incubated in DMEM with PDGF for the times indicated in figure legends.

2.3. *Isolation of eIF-4E*

Labeled cells were washed with cold phosphate-buffered saline and lysed by gently rocking flasks on ice for 30 min with 1 ml of lysis buffer (20 mM Hepes-pH 7.5, 100 mM KCl, 50 mM β-glycerol phosphate, 10% glycerol, 0.2 mM EDTA, 0.2 mM Na_3VO_4, 0.5% Triton X-100, 7 mM BME, 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml aprotinin). Lysates were centrifuged for 10 min at 15000 rpm in a Sorvall SS-34 rotor at 2°C. The supernatants were mixed with 25 μl (packed volume) of m^{7}GTP sepharose at 4°C for 30 min. Samples were microfuged for 10 s, supernatants removed and the pellet washed three times in lysis buffer. eIF-4E was eluted from the m^{7}GTP sepharose by incubating samples in 50 μl of lysis buffer containing 200 μM m^{7}GDP for 30 min at 4°C. m^{7}GTP sepharose was pelleted by centrifugation, supernatants added to Laemmli sample buffer and analyzed by 10% SDS-PAGE and autoradiography as described in detail elsewhere [20].

2.4. *Phosphoaminoacid analysis and phosphopeptide maps*

Gel slices containing eIF-4E were excised, rehydrated in water and transferred to 1 ml of 200 mM ammonium carbonate (pH 8.6) containing 50 μg of DPCC-treated trypsin. Gel slices are incubated at 37°C for 6 h after which time 20 μg of additional trypsin was added. After 22 h of incubation supernatants were removed and lyophilized. This treatment resulted in the release of 95% of the counts from the gel slices. Phosphoamino acid analysis of unseparated phosphopeptide samples were performed as described elsewhere [20]. The phosphopeptides recovered were dissolved in water and analyzed on thin layer cellulose plates (Kodak) by electrophoresis at 800 V in pyridine acetic acid: water (10:0.4:90, v/v) pH 6.5 for 1.5 h followed by ascending chromatography in the second dimension in butanol:acetic acid:water (3:1:1, v/v). Tryptic phosphopeptides were identified by autoradiography.

3. RESULTS AND DISCUSSION

To study the effect of PDGF on the phosphorylation state of eIF-4E, we metabolically labeled confluent WI-38 fibroblasts with phosphorus-32 as described in section 2 and incubated cells with different concentrations of PDGF for 30 min. eIF-4E was isolated from cell lysates and its phosphorylation status determined by autoradiography of samples analyzed by SDS-PAGE. An autoradiogram of a representative gel is shown in Fig. 1. Quantitation of eIF-4E phosphorylation by densitometry demonstrated a 2–4-fold increase in the phosphorylation of eIF-4E in response to PDGF treatment with the maximal stimulation occurring at 60 ng/ml of PDGF (Fig. 1B). Studies examining the time required to observe the PDGF initiated stimulation of eIF-4E phosphorylation demonstrated a clear 2–3-fold stimulation after 15 min which was very close to the maximal stimulation observed at 30 min (Fig. 2). This indicates that phosphorylation of eIF-4E is a relatively early event that occurs after addition of PDGF to quiescent WI-38 cells.

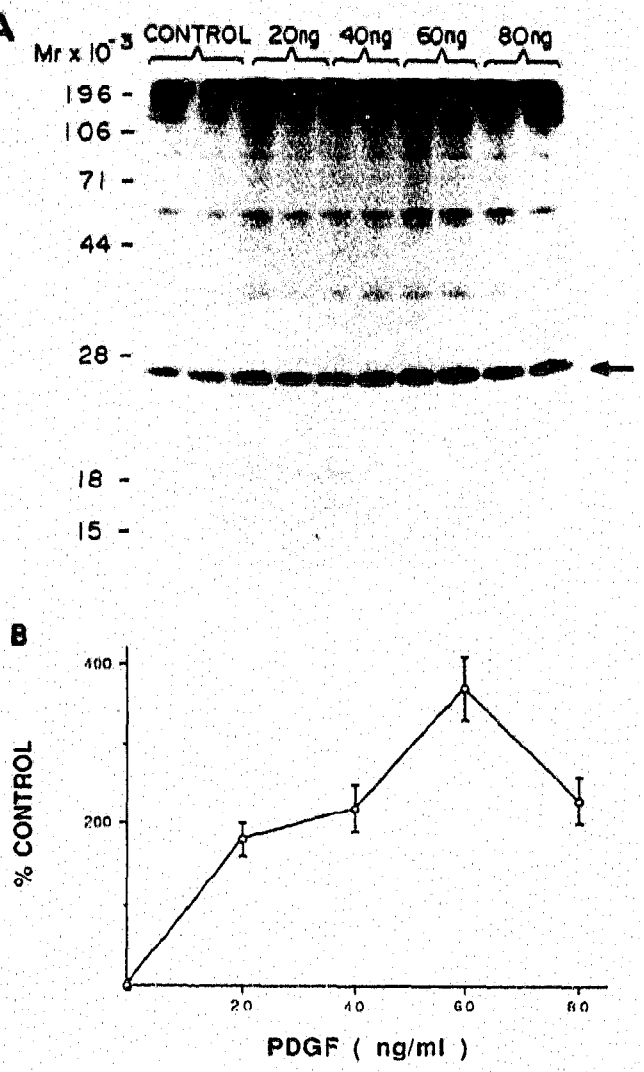

Fig. 1. Dose response of PDGF-stimulated eIF-4E phosphorylation. An autoradiogram (18 h exposure) of eIF-4E (arrow) isolated from WI-38 cells treated with different concentrations of PDGF under the conditions described in section 2 is shown in A. B shows the relative percent increase in eIF-4E phosphorylation following PDGF treatment of cells as compared to controls as determined by densitometric analysis of autoradiograms. Data points represent the mean ± SD of two separate experiments done in duplicate.

The effect of PDGF on the phosphorylation state of eIF-4E was further characterized by phosphoamino acid analysis and phosphopeptide map analysis. The sole phosphoamino acid identified in eIF-4E isolated from control as well as PDGF-treated cells was serine (Fig. 3). Tryptic digestion of eIF-4E isolated from control and PDGF treated cells followed by two-dimensional phosphopeptide map analysis demonstrated the presence of a single phosphopeptide under both conditions. The location of both of these tryptic phosphopeptides was in the same region as the tryptic phosphopeptide identified in HeLa cells and

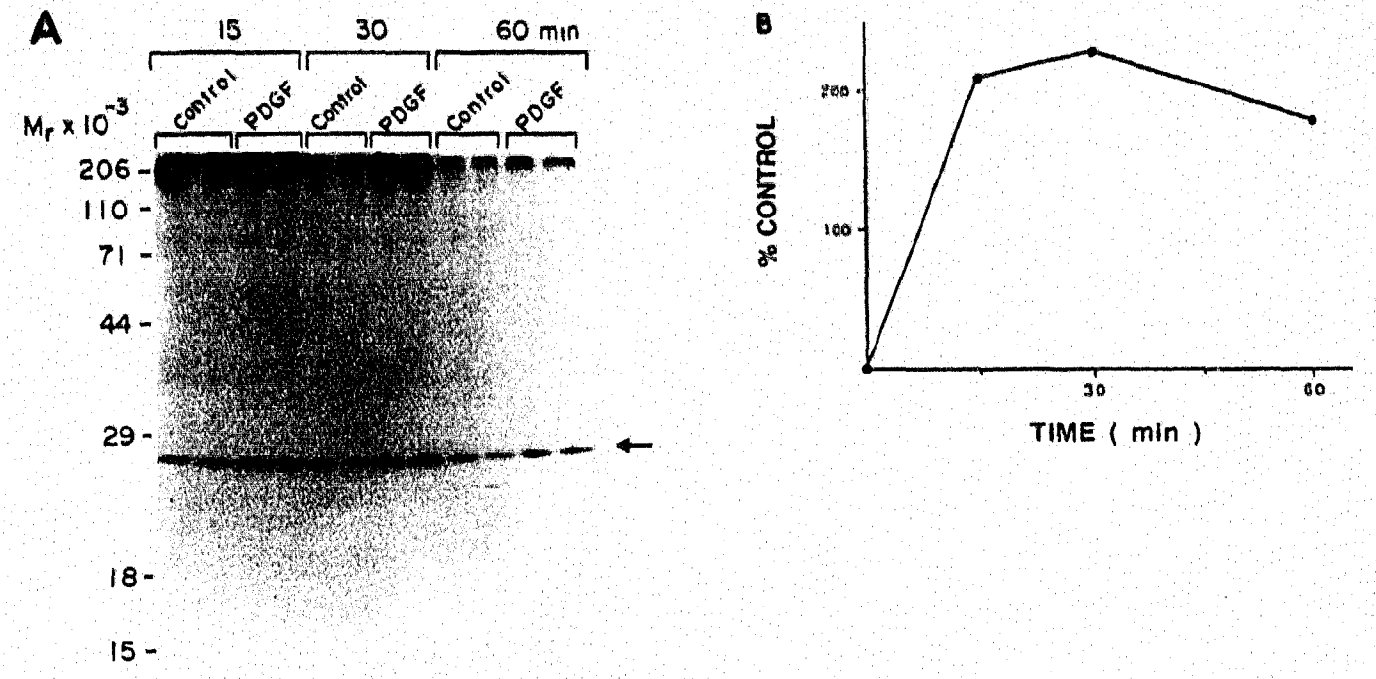

Fig. 2. Time of PDGF treatment required to observe a stimulation in eIF-4E phosphorylation. Cells were incubated with phosphorus-32 (0.2 mCi/ml) and 60 ng/ml PDGF for 15, 30, 60 min as indicated. eIF-4E was isolated and analyzed as described in section 2. A shows an autoradiogram (48 h exposure) of samples analyzed by SDS-PAGE where eIF-4E is indicated by an arrow. B shows the relative phosphorylation of eIF-4E at the indicated times as determined by densitometry of autoradiograms.

reticulocytes which corresponds to a KSK peptide containing serine-53 of eIF-4E [9]. We conclude from these results that PDGF stimulates the phosphorylation of eIF-4E at the major constitutive phosphorylation site which has been serine-53 in the cell types studied to date. Although protein kinase C has been shown to phosphorylate the constitutive phosphorylation site of eIF-4E in vitro at least one other enzyme, with a different substrate specificity, has been identified that phosphorylates eIF-4E [7,13]. Although casein kinase I is able to phosphorylate eIF-4E in vitro it is unlikely to account for the constitutive or PDGF stimulated phosphorylation reported here [20]. The tryptic phosphopeptide map of eIF-4E phosphorylated by casein kinase I was distinctly different than those reported here. The protein kinase(s) responsible for the PDGF stimulated phosphorylation of eIF-4E remain to be identified.

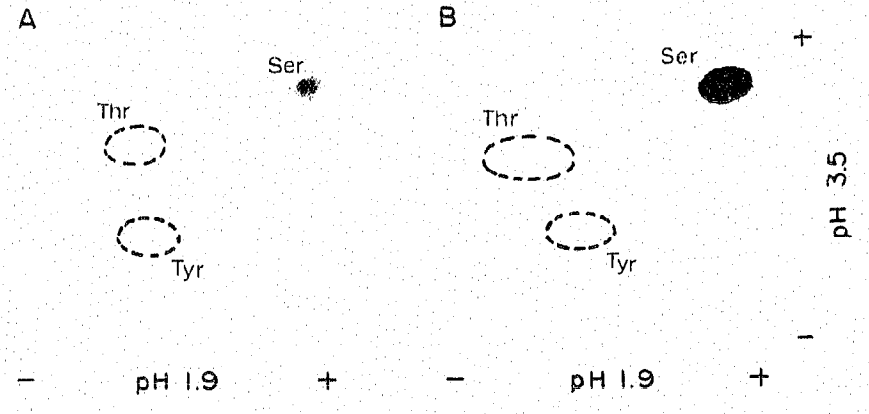

Fig. 3. Phosphoamino acid analysis of eIF-4E isolated from control and PDGF treated cells. [^{32}P]eIF-4E was hydrolyzed in 6 N HCl and analyzed by two-dimensional thin layer electrophoresis as described in section 2. Autoradiograms of eIF-4E phosphoamino acids from control (A) and PDGF treated cells (B) are shown.

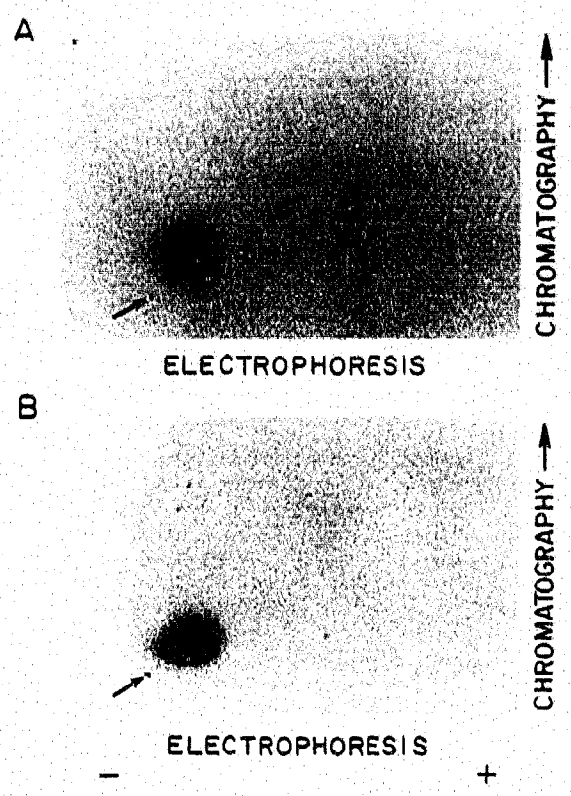

Fig. 4. Phosphopeptide map analysis of eIF-4E isolated from control and PDGF treated cells. [^{32}P]eIF-4E was isolated, digested with trypsin and analyzed by thin layer electrophoresis and chromatography as described in section 2. Autoradiograms of eIF-4E phosphopeptide maps are shown from control cells (A) and cells treated with 60 ng/ml PDGF for 30 min (B). The origin is indicated by an arrow.

Two model growth regulatory peptides, EGF and PDGF, stimulate the rapid phosphorylation of eIF-4E [21]. Both peptides are known to stimulate protein synthesis and the accumulation of polysomes in cultured cells [5,22]. Past studies provided convincing evidence that the rate of protein synthesis and the duration of the G_1 interval of the cell cycle are tightly coupled [23]. Modest decreases in the rate of protein synthesis greatly extended the time interval between the addition of serum to quiescent fibroblasts and the onset of DNA replication. In addition, a mutant cell line has been described that exhibits elevated rates of protein synthesis during mitosis and no measurable G_1 phase [24]. A partial inhibition of protein synthesis in these cells induced a measurable G_1 without effecting S, G_2 or M phases. These studies, in conjunction with the results reported here, provide evidence that specific growth peptide-initiated modifications in the translational apparatus play an important role in the regulation of cellular replication rates. We suggest that eIF-4E is one of the critical translational components targeted for modification following stimulation of the rate of cell replication.

Acknowledgements: We thank W. Jack Pledger for his suggestions, Ed Leof for reading the manuscript and Lisa Hall for her typing. This work was supported by NIH Grant GM 40219.

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