Phosphorylation sites for ribosomal S6 protein kinases in mouse 3T3 fibroblasts stimulated with platelet-derived growth factor

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Platelet release products and purified platelet-derived growth factor stimulated the phosphorylation of ribosomal protein S6 in cultured mouse Balb/c 3T3 fibroblasts. The post-nuclear fraction of the stimulated cells was enriched in S6 kinase activity specific for sites resembling those phosphorylated within intact cells in response to PDGF as determined by tryptic peptide mapping. 3T3-S6 sites closely resembled those phosphorylated in S6 of rat hepatocytes stimulated with insulin and included sites for both cAMP-dependent and independent kinases.

Platelet-derived growth factor *Ribosome* *Phosphorylation* *3T3 Fibroblast*

1. INTRODUCTION

Growth factors stimulate quiescent cells to divide by inducing a number of metabolic changes. Among the earliest events promoted by growth factors is the phosphorylation of a number of proteins including the ribosomal protein S6 [1–5]. Phosphorylation of S6 occurs within minutes and is correlated with increased polysome formation [6,7] and overall protein synthetic activity [4,8] suggesting a regulatory role of S6 phosphorylation in protein synthesis [4]. Several S6 kinases have been identified, including the cAMP- [9,10] and cGMP- [11] dependent kinases, a protease-activated kinase [11] and a Ca^{2+}-dependent kinase [12]. However, the nature of the growth-associated kinase has not been established although analyses of S6 phosphorylation sites indicate that the predominant activity during anabolic regulation is cAMP-independent [13–15].

Platelet derived growth factor (PDGF) is the principal mitogen in serum which stimulates quiescent connective tissue cells in culture to initiate protein and DNA synthesis [16]. PDGF is a two-chain polypeptide molecule of M_r ~30000 [17,18], whose amino acid sequence (partial) is closely related to that of the putative transforming protein ($p28^{sis}$) of Simian sarcoma virus [19]. The action of PDGF resembles that of epidermal growth factor and insulin in that it promotes membrane-associated phosphotyrosine kinase [20–22] and initiates a series of common intracellular events including the phosphorylation of S6 [23].

Here, we have studied the effects of PDGF preparations on S6 phosphorylation in cultured Balb/c 3T3 fibroblasts. Our findings confirm that S6 phosphorylation is enhanced by PDGF [23] and show that S6 kinase activity is elevated in the post-nuclear fractions of growth factor-stimulated cells. The S6 sites phosphorylated within intact cells in response to growth factors and in the cell-free post-nuclear kinase reaction appeared to be the same and closely resembled those phosphorylated within rat hepatocytes in response to insulin [14].

Abbreviations: PDGF, platelet derived growth factor; RP-HPLC, reverse phase high-performance liquid chromatography; SDS, sodium dodecyl sulphate; Bt_2cAMP, N^6,O^2-dibutyryl adenosine 3′:5′-monophosphate

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2. MATERIALS AND METHODS

2.1. *Platelet-derived growth factor preparations*

Platelet release products: Washed human platelets were aggregated in buffer containing bovine thrombin (Parke Davis, USA, 0.1 NIH U/ml and aprotinin (Bayer, 10 KIU/ml). The aggregated platelets were removed by centrifugation at 2200 × *g* for 5 min at 4°C for 48 h, lyophilised and reconstituted in 0.15 M NaCl at 10 mg protein/ml (platelet release products). Residual thrombin content was estimated at 0.05 NIH U/mg using the chromogenic substrate H–D-Phe–Pip–Arg–NHA [24].

Purified PDGF: PDGF was prepared from outdated platelet concentrates as in [25] and further purified by RP-HPLC on a column of Lichrosorb C_8 dp 10 (Merck) using a linear gradient of isopropanol in water in the presence of 0.1% trifluoroacetic acid. The preparation was free of detectable thrombin but exhibited several bands on SDS–polyacrylamide gel electrophoresis and could not be considered homogeneous. The amount of PDGF was estimated by amino acid analysis.

2.2. *3T3 Fibroblast cultures*

Balb/c 3T3 fibroblasts (from Commonwealth Serum Laboratories (CSL), Melbourne) were grown to confluence in Dulbecco's modified Eagle's medium (CSL) containing 10% foetal calf serum in an atmosphere of 5% CO_2 in air. The serum supplement was changed to 5% human plasma-derived serum 2 days prior to stimulation with growth factors. Plasma-derived serum was prepared from platelet-poor plasma (prepared in the presence of prostaglandin E_1 to reduce granule release) by heating at 56°C for 30 min, centrifuging at 2200 × *g* for 30 min and dialysing the serum against 0.15 M NaCl–0.01 M phosphate buffer (pH 7.4) for 48 h at 4°C (thrombin content, 0.04 NIH U/ml). 3T3 cells maintained in this way were stimulated to incorporate [^{3}H]thymidine (Amersham International, Bucks; 4 μCi/ml) into DNA during 40 h cultures by the addition of growth factors. Purified PDGF and platelet release products were maximally active at 1.5 μg/ml and 100–250 μg/ml, respectively. Under the same conditions, thrombin was only effective in promoting [^{3}H]thymidine incorporation at 10 NIH U/ml.

2.3. *^{32}P-Labelling of 3T3 cells and subcellular fractionation*

Carrier-free $^{32}P_i$ (2 mCi, Amersham) was added to cultures of 3T3 cells (about 10^7 cells/dish) in 10 ml medium supplemented with plasma-derived serum (see section 2.2). Growth factors were added after 2.5 h and the cultures continued for 30–60 min before harvesting the cells by rinsing away the medium, dislodging the cells with a rubber spatula and centrifuging at 100 × *g* for 2 min and homogenising the cells in detergent buffer [14]. The nuclear fraction was obtained by centrifugation at 1000 × *g* and the ribosomes by centrifugation of the post-nuclear supernatant for 5 h at 120000 × *g* at 4°C.

2.4. *Analysis of ^{32}P-labelled S6*

Ribosomal pellets were resuspended in 2% SDS–20 mM tricine and analysed by SDS–polyacrylamide gel electrophoresis (12% gels) [8]. Segments of the electrophoretograms containing S6 were excised, homogenised in 100 mM NH_4HCO_3, digested with trypsin (30 μg/ml) for 16 h at 33°C, the gel fragments removed by centrifugation and the phosphopeptides recovered by lyophilisation (>80% of ^{32}P-radioactivity in the original gel segment). Tryptic maps were obtained by isoelectric focussing of the S6 phosphopeptides in polyacrylamide gels [14].

2.5. *Post-nuclear S6 kinase activity*

3T3 cells were cultured in plasma-derived serum (see section 2.3) in the presence and absence of platelet release products for 30 min, harvested and homogenised [26] in Tris–HCl buffer (pH 7.6 containing 10 mM KCl and 3.5 mM $MgCl_2$ (0.2 ml/10^7 cells). The homogenates were added to 1/10 vol. 100 mM Tris–HCl buffer (pH 7.6) containing 0.6 M KCl, 25 mM $MgCl_2$, 10 mM dithiothreitol and 2 M sucrose and centrifuged for 10 min at 100 × *g* to obtain the post-nuclear supernatant fractions. The S6 kinase activity was assayed at 30°C in a reaction mixture (50 μl) consisting of 10 μl post-nuclear fraction containing 11 μg protein and 0.9 μg RNA, 0.5 mM [γ-^{32}P]ATP (1–5 μCi), 40 S rat liver subunits (30 μg RNA) [27] or 80 S rat liver ribosomes (50 μg RNA) [10] in 20 mM Tris–HCl buffer (pH 7.6) containing 50 mM KCl, 5 mM $MgCl_2$, 25 mM NaF, 1 mM EGTA, 0.1 mM EDTA and 1 mM

dithiothreitol. The reaction was stopped with 10% trichloroacetic acid (w/v) and the precipitate dissolved in 2% SDS–20 mM tricine and analysed by SDS gel electrophoresis (12% gels).

3. RESULTS

3.1. *Effects of growth factors on $^{32}P_i$ incorporation into 3T3 cells*

Platelet release products and purified PDGF enhanced $^{32}P_i$ incorporation into ribosomes and another subcellular fraction of 3T3 cells several fold (not shown). Analysis of the ribosomal fraction by SDS–polyacrylamide gel electrophoresis showed that the ^{32}P-labelling of several polypeptides was selectively enhanced by the growth factors (fig.1). The most prominent of the affected species was identified as ribosomal protein S6 on the basis that it comigrated with [^{32}P]S6 from rat liver ribosomes [10]. The increase in the phosphorylation of S6 was accompanied by a selective enhancement of the slowly migrating derivatives characteristic of multiply phosphorylated forms of S6 (fig.1, track 2). The effects of purified PDGF on $^{32}P_i$ incorporation into S6, as well as the major RNA containing fractions, were generally smaller than those of platelet release products (fig.1), despite the fact that the two preparations were employed at concentrations maximally active in the stimulation of [^{3}H]thymidine incorporation during 40 h cultures (not shown).

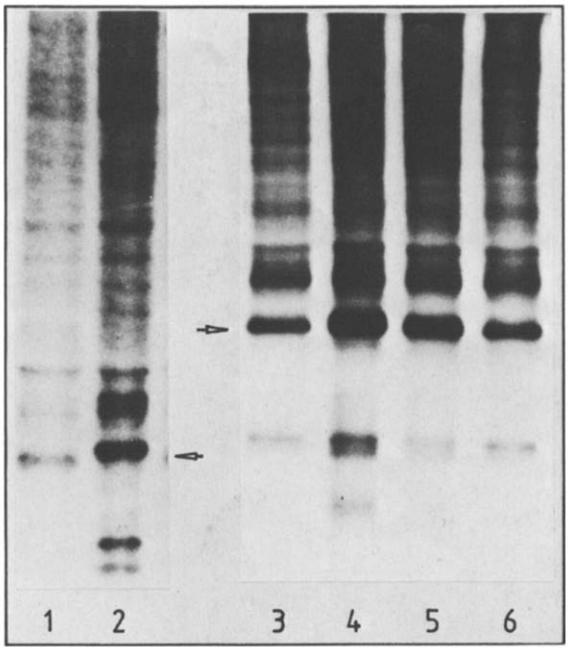

Fig.1. Analysis of ribosomes from ^{32}P-labelled 3T3 cells by SDS–polyacrylamide gel electrophoresis and autoradiography: (1,2) 3T3 cells cultured for 2.5 h in $^{32}P_i$-medium, followed by culture in the presence (2) and absence (1) of platelet release products for 30 min; 9% polyacrylamide gel; (3–6) control cells after 2.5 h (3) and 3.5 h (6) and cells cultured with platelet release products (4) or PDGF (5) for 1 h following culture for 2.5 h in $^{32}P_i$ medium; 12% gels; (⟶) equivalent position of [^{32}P]S6 from rat liver [10].

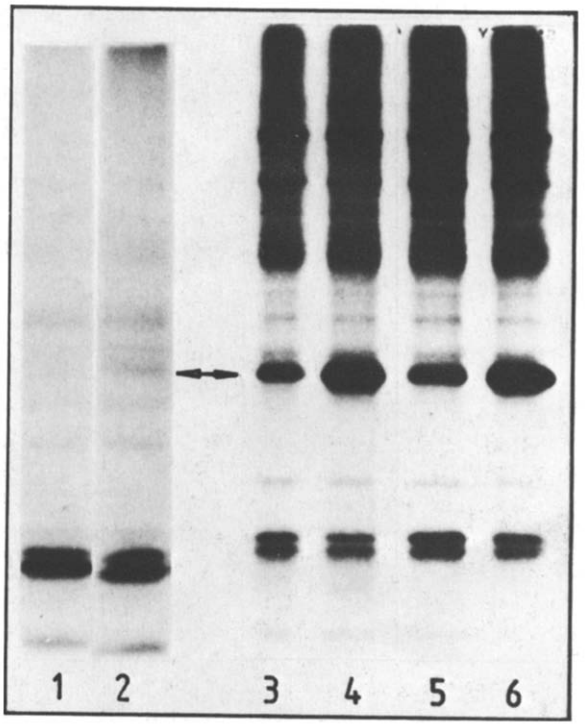

Fig.2. SDS gel analysis of ribosomes phosphorylated in cell-free systems: Post-nuclear fractions from control 3T3 cells (1,3,4) or cells cultured with platelet release products (2,5,6) were incubated with exogenous whole ribosomes (1,2) or 40 S subunits (3–6). Post-nuclear fractions were freshly prepared in 1 and 2 and stored frozen (−70°C) in 3–6. Bt_2cAMP (10^{-6} M) was included in reactions 4 and 6. The specific radioactivity of [^{32}P]ATP in 1 and 2 was 1/10th that in 3–6. The position of S6 is denoted by arrows.

3.2. *S6 kinase activities in 3T3 cells*

The kinase activities in post-nuclear fractions of 3T3 cells cultured in the presence and absence of platelet release products were assayed. The incorporation of ^{32}P into S6 was only evident when exogenous ribosomes (fig.2, track 1,2) or 40 S ribosomal subunits (fig.2, track 3–6) were included in the kinase reaction mixtures. Rat liver ribosomes, which are structurally similar to mouse ribosomes [2] were employed because of their ready availability. The S6 kinase activity was greatest in freshly prepared post-nuclear fractions from growth factor-stimulated cells, with only a trace of activity being detected in control preparations (fig.2, tracks 1,2). On storage at −70°C, the post-nuclear fractions from control cells developed additional S6 kinase activity. The overall activity of the preparations from growth factor-treated cells stored under identical conditions remained higher by 30–40% (fig.2, track 3 *vs* 5).

3.3. *Isoelectric focussing analysis of tryptic phosphopeptides of S6*

The site specificities of S6 kinase activities in intact 3T3 fibroblasts were investigated by peptide mapping. Segments of SDS–polyacrylamide gels containing [^{32}P]S6 were homogenised and digested with trypsin and the resulting phosphopeptides analysed by isoelectric focussing in polyacrylamide gels (fig.3). Two of the major phosphopeptides from S6 of control cells comigrated with the mono- (band 1) and diphospho- (band 8) derivatives of the Arg–Leu–Ser–Ser–Leu–Arg region of rat S6 [10] (fig.3, track 1). Stimulation of cells with either platelet release products or purified PDGF greatly enhanced most of the phosphopeptides evident in control digests and caused the appearance of several new species particularly in the acidic region of the gel (fig.3, track 2,3). Overall, at least 13 species were promoted and only one of the major species (band 10) in control digests was relatively

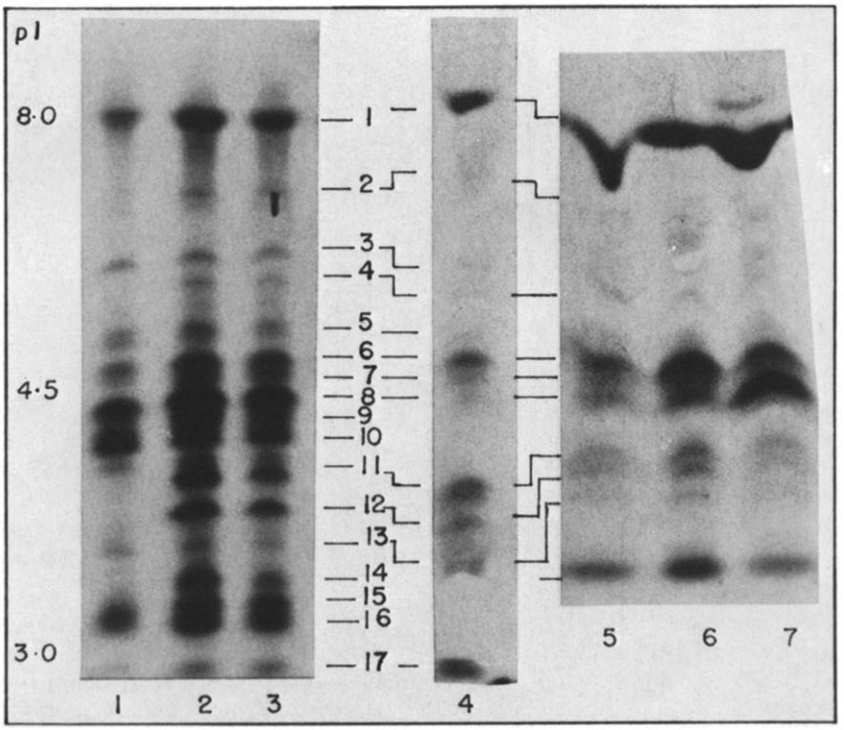

Fig.3. Isoelectric focussing of ^{32}P-labelled tryptic peptides of S6: (1–3) peptides from S6 of 3T3 cells cultured in the absence (1) and presence of either platelet release products (2) or PDGF (3); (4) peptides from S6 phosphorylated in a cell-free system based on freshly prepared post-nuclear fraction from growth factor-treated cells; (5–7) S6 from systems based on post-nuclear fractions of control (5) or growth factor-treated cells (6,7). Bt_2cAMP was included in expt 7. pH gradients were estimated with a surface electrode after completion of the isofocussing.

unaffected. The release products and purified PDGF promoted the same phosphopeptides, although the size of the effects with PDGF were less, particularly in the case of band 14 (fig.3, track 3).

A number of phosphopeptides were also generated from S6 phosphorylated in reactions based on post-nuclear fractions of cells stimulated with platelet growth factors (i.e., fig.3, tracks 4,6). Species comigrating with each of the major derivatives of mouse S6 other than bands 9 and 10 were apparent, including equivalents of bands 14–16 although these were not resolved in the experiment shown. However, the intensities of many of the species, particularly the acidic bands 8–12 and 14–16, were relatively weak. Insufficient radioactivity was incorporated into S6 in reactions based on freshly prepared post-nuclear fractions from control cells for peptide map analyses. The S6 kinase activity acquired by the control fractions on storage at −70°C phosphorylated sites similar to those phosphorylated by the activity in fractions from growth factor-treated cells (fig.3, track 5). However, a difference was apparent in the two activities in that the phosphorylation of the band 6 peptide was selectively enhanced in the growth factor-treated system.

3.4. *Cyclic AMP-dependent S6 kinase activity*

The extent to which cAMP-dependent kinase contributed to the S6 kinase activities of the post-nuclear fractions was investigated by studying the effects of adding Bt_2cAMP (10^{-6} M). S6 phosphorylation was stimulated by >3-fold in both the control and growth factor-stimulated systems (fig.2, tracks 4,6). Peptide map analysis showed that the 3T3 cAMP-dependent kinase activity phosphorylated the Arg–Leu–Ser–Ser–Leu–Arg segment of S6 [10] forming both the mono- (band 1) and diphospho- (band 8) derivatives (fig.3, track 7). Traces of the triphosphopeptide Tla [10,14] also appeared in response to Bt_2cAMP. The formation of other tryptic phosphopeptides was relatively unaffected by the cyclic nucleotide, although there may have been a small stimulation of band 6.

4. DISCUSSION

A role of PDGF in the regulation of S6 phosphorylation [23] has been confirmed using purified PDGF. PDGF was also likely to have been a major contributor to the effects of platelet release products on S6 phosphorylation, possibly in conjunction with other putative growth factors [28–30] and residual thrombin [23]. Thrombin was unlikely to have been solely responsible for the observed effects since the concentration in culture medium did not exceed 0.013 NIH U/ml, 10% of that reported to stimulate S6 phosphorylation and ≤1% of that required to enhance [^{3}H]thymidine incorporation (section 2.4; [23]). However thrombin could have acted synergistically with PDGF [23], possibly explaining the larger effects of release products compared with the effects of purified PDGF (fig.1).

A close structural relationship between the major S6 phosphopeptides from PDGF-stimulated mouse fibroblasts (fig.3) and insulin-stimulated rat hepatocytes [14] was apparent from their similar isoelectric focussing characteristics. The intense labelling and low pI-values of some of the peptides suggested multiple phosphorylation. The clustering of phosphorylation sites within tryptic peptides has also been suggested by peptide map analysis of the different phosphorylated forms of S6 [15,31]. Many of the tryptic peptides in digests of rat S6 appear to originate from a region having the sequence:

Arg–Arg–Leu–Ser_4–Ser_5–Leu–Arg_7–Ala–Ser–Thr–Ser–Lys–Ser–Glu–Glu–(Ser)–Gln–(Lys)

containing 7 potential phosphorylation sites [10,14] (submitted). Ser_4 constitutes the major S6 site for cAMP-dependent kinases within hepatocytes [10,14]; Ser_5 and to a lesser extent Ser_9 can also be phosphorylated by the same enzyme in vitro [10]. Phosphorylation of Ser_4 and Ser_5 gives rise to the mono and diphospho forms of the Arg–Leu–Ser–Ser–Leu–Arg peptide [10] both of which were prominent in tryptic digests of S6 phosphorylated by the 3T3 post-nuclear kinases in response to Bt_2cAMP (bands 1,8; fig.3). Apparently, equivalent peptides were also detected in digests of S6 labelled within 3T3 cells in response to PDGF (fig.3), indicating that cAMP-dependent S6 kinase may have contributed to the growth factor response. The cAMP-dependent kinase activity may have been due to the accumulation of cAMP

elevating prostaglandins synthesised in response to PDGF (e.g., [32] cf. [5]).

Phosphorylation sites distinct from the major cAMP-dependent sites were present in the majority of S6 phosphopeptides enhanced by PDGF, strongly suggesting the existence of a PDGF-regulated S6 kinase in 3T3 cells unrelated to the cAMP-dependent kinase. The cAMP-independent kinase appeared to be activated following storage at −70°C and subsequent thawing of the post-nuclear fraction. This phenomenon suggests an S6 kinase that may be activated by an endogenous protease in a manner similar to the activation of a reticulocyte S6 kinase [11]. An enzyme having a similar site specificity to the reticulocyte kinase is present in mouse 3T3 fibroblasts and is subject to regulation by insulin [33]. Regulation of the same kinase by PDGF could explain the apparent similarity in the S6 phosphorylation sites regulated by PDGF and insulin in 3T3 cells and hepatocytes, respectively (see above).

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