Treatment of vascular smooth muscle cells with antisense phosphorothioate oligodeoxynucleotides directed against p42 and p44 mitogen-activated protein kinases abolishes DNA synthesis in response to platelet-derived growth factor

Caspar J. M. ROBINSON*‡, Pamela H. SCOTT*, Andrew B. ALLAN*, Thomas JESS†, Gwyn W. GOULD† and Robin PLEVIN*

*Department of Physiology and Pharmacology, University of Strathclyde, Royal College, 204 George St., Glasgow G1 1XW, Scotland, U.K.,

and †Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

We have investigated the requirement for mitogen-activated protein (MAP) kinase in the stimulation of DNA synthesis by platelet-derived growth factor (PDGF) in rat aortic smooth muscle cells using a phosphorothioate-modified oligodeoxynucleotide (ODN) to deplete MAP kinase. Treatment for 72 h with MAP kinase antisense ODN directed against both the p42 and p44 isoforms of MAP kinase abolished the expression of MAP kinase and reduced agonist-stimulated MAP kinase activity by approx. 95%. The scrambled control ODN was without effect, but the sense control ODN slightly enhanced the expression of both isoforms. Abolition of MAP kinase activity by antisense

ODN treatment prevented angiotensin II- and PDGF-stimulated activation of p90 ribosomal S6 kinase activity, but did not affect activation of MAP kinase. In addition, antisense ODN pretreatment reduced PDGF-stimulated [³H]thymidine incorporation to < 5% of control, and decreased basal incorporation by approx. 90%. In contrast, basal [³H]thymidine incorporation was enhanced approx. 60% by control sense ODN treatment. These results indicate an obligatory role for MAP kinase in the activation of a number of early events in mitogenesis, including DNA synthesis, in vascular smooth muscle cells.

INTRODUCTION

A major cell signalling pathway involved in the regulation of cell growth and division in response to both growth factors and G-protein–receptor-coupled agonists is the mitogen-activated protein (MAP) kinase cascade [1]. The classical p42 and p44 MAP kinases are believed to play a key role in initiating early events in mitogenesis, such as phosphorylation and activation of phospholipase A₂ and Elk-1, and the stimulation of glucose uptake [2–5]. The use of antisense RNA approaches and the expression of dominant-negative mutants has indicated that MAP kinase is essential for the initiation of mitogenesis in response to growth factors in fibroblast cell lines [6,7].

Previously, a number of growth factors and constrictor peptides such as angiotensin II (AII) have been shown to stimulate the activation of MAP kinase in cultures of vascular and tracheal smooth muscle cells [8–11]. However, although it has previously been suggested that sustained activation of MAP kinase is obligatory for the initiation of smooth muscle cell division, no direct evidence in support of this hypothesis has been provided.

In the present study we have utilized a 17-mer phosphorothioate-modified oligodeoxynucleotide (ODN) directed against both the p42 and p44 MAP kinase isoforms [12] to investigate the role of MAP kinase in platelet-derived growth factor (PDGF)-stimulated DNA synthesis in rat aortic smooth muscle cells. We find that pretreatment of cells with a MAP kinase antisense ODN results in the specific depletion of MAP kinase expression

and the abolition of MAP kinase activity. In addition, activation of the putative downstream target p90 ribosomal S6 kinase (p90^{S6k}) was also substantially inhibited. Under these conditions, growth factor-stimulated DNA synthesis was essentially abolished. The findings of this study implicate MAP kinase as an essential component in growth factor-stimulated vascular smooth muscle cell DNA synthesis.

EXPERIMENTAL

Materials

All materials were of the highest commercial grade available. ECL detection reagents and the BIOTRAK[®] MAP kinase assay kit were obtained from Amersham International. Antibodies against MAP kinase kinase (MEK)-1 and -2, MAP kinase and p90^{S6k} were obtained from Affiniti Research Products Ltd. (Exeter, U.K.). Cell culture materials were purchased from Gibco (Paisley, Scotland, U.K.). The transfection reagent *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulphate (DOTAP) was purchased from Boehringer Mannheim (Lewes, E. Sussex, U.K.), and the Ni-nitrilotriacetic acid matrix was provided by Qiagen (Hilden, Germany). The pRSETB plasmids containing wild-type and inactive MAP kinase were kindly donated by Dr. G. Johnson (University of Denver, CO, U.S.A.). Antibodies directed against p38 MAP kinase and *c-jun*

Abbreviations used: AII, angiotensin II; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulphate; EGF, epidermal growth factor; JNK, *c-jun* N-terminal kinase; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase kinase; ODN, oligodeoxynucleotide; PDGF, platelet-derived growth factor; p90^{S6k}, p90 ribosomal S6 kinase.

‡ To whom correspondence should be addressed.

N-terminal kinase (JNK) were provided by Professor P. Cohen (University of Dundee, Scotland, U.K.) and Santa Cruz Ltd. respectively. The phosphorothioate-modified ODNs were synthesized in-house and cartridge-purified.

Cell culture

Primary cultures of rat aortic smooth muscle cells were established by enzymic dispersion and cultured as outlined previously [11].

Expression and purification of histidine-tagged MAP kinase

Wild-type and inactive *Xenopus* MAP kinase were synthesized as histidine-tagged fusion proteins in the *Escherichia coli* strain BL21(DE3)pLysS following transfection with the pRSETB plasmid containing the cDNA inserts previously defined [13]. The proteins were purified from the bacterial cell lysates using a Ni-nitrilotriacetic acid Sepharose matrix [14].

ODN treatments

Cells which were 60–70% confluent were treated with MAP kinase antisense (5'-GCCGCCGCCGCCCAT-3'), sense (5'-ATGGCGGCGGCGGCGGC-3') or scrambled (5'-CGCGCGCTCGCGCACCC-3') phosphorothioate-modified ODNs [12]. The ODNs were prepared in serum-free Dulbecco's modified Eagle's medium (+100 ng/ml insulin) with DOTAP at a ratio of 1:1 (w/w) and incubated with the cells for 8 h. The cells were then washed twice with serum-free medium to remove the DOTAP/ODN mixture and incubated in Dulbecco's modified Eagle's medium containing 1% (v/v) foetal calf serum and ODNs at the required concentration. After a further 16 h this procedure was repeated and the cells were incubated for a further 48 h.

MAP kinase immunoblotting and activity

Cellular MAP kinase levels were analysed by Western blotting as outlined previously using the MK-12 antibody [10,15]. MAP kinase activity was assayed in whole-cell lysates using a commercial MAP kinase assay kit utilizing the epidermal growth factor (EGF) receptor peptide (KRELVEPLTPAGEAPN-ALLR) as substrate [16].

p90^{s6k} activity

Cells were solubilized in a buffer containing peptidase inhibitors as previously described [12], and then precleared lysates were incubated for 90 min with 1.5 µg/ml of a monoclonal anti-p90^{s6k} antibody. Following this step, lysates were incubated for 90 min with 30 µl of a 10% Pansorbin solution pre-coupled to mouse monoclonal IgG. Immunoprecipitates were washed and then assayed *in vitro* for phosphorylation of the p90^{s6k} peptide pseudosubstrate as outlined previously [12].

Agonist-stimulated MEK activity assay

MEK was immunoprecipitated from stimulated cells lysed in 20 mM Tris/HCl (pH 7.5) containing 137 mM NaCl, 5 mM EDTA, 10 mM NaF, 1 mM Na₄P₂O₇, 1 mM Na₃VO₄, 10 µg/ml aprotinin, 0.1 mM PMSF, 1% Triton X-100 and 10% glycerol using 1 µg/ml of a polyclonal rabbit anti-MEK antibody. MEK activity was assayed by resuspending the immunoprecipitates in 25 mM Hepes buffer (pH 7.4) containing 25 mM β-glycero-

phosphate, 15 mM MgCl₂ and 1 mM dithiothreitol. To this was added 50 µM ATP/5 µCi of [γ-³²P]ATP, 1 µg of wild-type exogenous histidine-tagged MAP kinase and 200 µM EGF receptor peptide substrate, in a total volume of 30 µl, and the reaction was incubated at 30 °C for 15 min. An aliquot of this reaction was subjected to SDS/PAGE (10% gel), and the gel was dried and exposed to autoradiographic film [13]. In addition, EGF receptor peptide phosphorylation was assayed by ion-exchange chromatography.

DNA synthesis

Cells were incubated with ODNs as outlined above, and then incubated in the presence or absence of PDGF (30 ng/ml) for 18 h. The cells were then incubated for a further 6 h with 0.1 µCi/ml [³H]thymidine, and incorporation was measured as outlined previously [16].

RESULTS

Figure 1 shows the effect of treating rat aortic smooth muscle cells for 72 h with antisense ODN. Approx. 90% depletion of MAP kinase was achieved with a concentration of 5–10 µM antisense ODN. The control scrambled ODN caused no depletion of MAP kinase at the same concentrations, whereas the sense

Figure 1 Depletion of MAP kinase expression and reduction of MAP kinase activity in rat aortic smooth muscle cells following MAP kinase antisense pretreatment

(A) Cells were pretreated with the indicated concentrations (10, 5 and 1 µM) of antisense (AS) ODN, or with 10 µM scrambled (Scr) or sense (S) ODNs, or none (C), as outlined in the Experimental section. Whole-cell lysates were immunoblotted for p42 and p44 MAP kinase. The blot is representative of at least three experiments. (B) Cells were incubated in the presence or absence of 10 µM antisense (AS) ODN, as outlined in the Experimental section, and then further incubated in the presence or absence of All (100 nM) for 5 min or PDGF (30 ng/ml) for 10 min (P). Equal amounts of protein (10–20 µg) were assayed for *in vitro* MAP kinase activity as outlined in the Experimental section. Values are means ± S.D. of duplicate determinations from a single experiment repeated a further three times.

Figure 2 Effect of MAP kinase antisense treatment on the expression of p38 and JNK in rat aortic smooth muscle cells

Cells were incubated with 10 μ M of antisense (AS), sense (S) or scrambled (Scr) ODNs, or none (C), for 72 h, as indicated in the Experimental section. Whole-cell lysates were blotted for the presence of p38 (A) or JNK (B). The arrowheads in (B) indicate the p46 and p54 JNK isoforms. The blots are representative of at least three experiments.

ODN caused a small enhancement of MAP kinase protein expression (Figure 1A). Figure 1(B) shows the effect of specific MAP kinase deletion upon the activation of MAP kinase in vascular smooth muscle cells. Treatment with 10 μ M antisense ODN resulted in complete abolition of both PDGF- and AII-stimulated MAP kinase activity in these cells. In additional experiments it was found that scrambled ODN treatment had no effect upon PDGF- and AII-stimulated MAP kinase activity (results not shown).

Antisense ODN treatment did not affect the expression of either p38 MAP kinase (reactivating kinase) or the p46 and p54 JNKs [17,18] (Figure 2), indicating that the MAP kinase depletion observed was a sequence-specific effect. This specificity was further confirmed when the activation of a putative downstream target of MAP kinase, p90^{src} (Figure 3C), and the immediate upstream activator MEK (Figure 4) were assayed. In untreated cells both AII and PDGF stimulated a time-dependent increase in the activation of p90^{src} (Figure 3A), with kinetics similar to those observed for the activation of MAP kinase. AII stimulated an approx. 3-fold increase in activity, while the response to PDGF was greater, giving an approx. 4-fold increase. Treatment of the cells with 10 μ M antisense ODN did not affect the recovery of p90^{src} in cell immunoprecipitates (Figure 3B), but substantially reduced the activation of p90^{src} in response to either AII or PDGF (Figure 3C).

In contrast, treatment of the cells with MAP kinase antisense ODN had no effect on the recovery of MEK in cellular immunoprecipitates (results not shown) and did not affect maximal activation of MEK stimulated in response to AII or PDGF (% activity compared with untreated control: AII, 84 ± 13 %; PDGF, 92 ± 28 %; $n = 2$; and Figure 4). Simultaneous assays of MAP kinase activity showed that MAP kinase activation had been abolished in these experiments. This strongly suggests that activation of the MAP kinase cascade has not been affected in a non-specific manner by MAP kinase antisense ODN treatment.

In a number of preliminary studies, PDGF stimulated a 6–8-

Figure 3 AII- and PDGF-stimulated p90^{src} activity and recovery following MAP kinase antisense pretreatment

(A) Cells were incubated with AII (100 nM) or PDGF (30 ng/ml) for the times shown and then assayed for p90^{src} activity as described in the Experimental section. (B) and (C) Cells were incubated in the presence (AS) or absence (C) of 10 μ M antisense ODN, as outlined in the Experimental section, and then Western-blotted for p90^{src} content in cellular immunoprecipitates (B) or stimulated with AII or PDGF (P) and assayed for p90^{src} activity in the cellular immunoprecipitates (C). The blot is representative of at least three experiments, and values in (C) are means $\pm$ S.D. of three individual experiments.

fold increase in DNA synthesis, whereas AII, thrombin and endothelin-1, which stimulate a transient activation of MAP kinase [11], were without effect (results not shown). Pretreatment with the control scrambled ODN had no effect upon the stimulation of DNA synthesis in these cells, which remained approx. 6–8-fold throughout (Table 1). Antisense ODN pretreatment reduced basal [³H]thymidine incorporation by approx. 90%, when compared with control, and the fold stimulation of [³H]thymidine incorporation by PDGF treatment was decreased by approx. 60%, with the total incorporation being < 5% of that of the untreated control (Table 1). Sense ODN pretreatment, however, was observed to increase basal [³H]thymidine incorporation by approx. 60% when compared with the untreated control. PDGF-stimulated [³H]thymidine incorporation in sense and scrambled ODN pretreatment controls was decreased to 65% and 68% of the untreated control value respectively, but this reduction was relatively small when compared with the decrease observed on antisense pretreatment (< 5% of the untreated control).

Figure 4 Effect of MAP kinase antisense pretreatment on MEK activity in rat aortic smooth muscle cells

Cells were incubated in the presence (AS) or absence (C) of 10 μ M antisense ODN, as outlined in the Experimental section. Cells were stimulated with either 100 nM AII or 30 ng/ml PDGF (P) for 5 min. Lysates were immunoprecipitated for MEK1 and 2 and then assayed for MEK activity as outlined in the Experimental section. The blot in (A) is representative of at least three experiments. Panel (B) shows the corresponding radioactivity counts incorporated into the EGF receptor peptide substrate by the MEK-activated exogenous wild-type (WT) MAP kinase. This is representative of two MEK activity assays.

Table 1 Effect of MAP kinase antisense pretreatment on PDGF-stimulated DNA synthesis in rat aortic smooth muscle cells

Cells were incubated with 10 μ M antisense, scrambled or sense ODN, as outlined in the Experimental section, or with vehicle (control). Cells were then stimulated or not with 30 ng/ml PDGF for 18 h and incubated with 0.1 μ Ci/ml [3 H]thymidine for 6 h. Incorporation was measured as outlined in the Experimental section. Values are means $\pm$ S.D. of triplicate determinations from a single experiment repeated at least three times.

Treatment	[3 H]Thymidine incorporation (d.p.m.)
Control	8526 $\pm$ 2501
Antisense	963 $\pm$ 448
Sense	13372 $\pm$ 2423
Scrambled	6365 $\pm$ 2372
PDGF	55818 $\pm$ 4507
PDGF + antisense	2378 $\pm$ 209
PDGF + sense	36078 $\pm$ 2951
PDGF + scrambled	37935 $\pm$ 2193

DISCUSSION

This study has used a specific antisense ODN to demonstrate the necessity for MAP kinase activation in vascular smooth muscle DNA synthesis. Previously we and others have shown that growth factors such as PDGF stimulate both the sustained activation of MAP kinase and DNA synthesis in both vascular and airways smooth muscle cells, whereas agents such as AII and bradykinin stimulate a transient activation of MAP kinase and do not stimulate DNA synthesis [11,16]. This implies that

sustained MAP kinase activation may be required in the initiation of mitogenesis. However, since a number of studies have implied a role for other signalling events in the regulation of vascular smooth muscle cell growth, particularly p70 ribosomal S6 kinase [19], it was important to determine whether MAP kinase is a requisite for the growth process.

The conclusions of the present study depend on the assumption that the results observed are due to sequence-specific antisense effects. To confirm the sequence specificity, we utilized two control ODNs (sense and scrambled), the minimum number required to be able to make any mechanistic claims for an antisense effect [20]. In a more extensive study, additional controls such as a variety of mismatch ODNs could have been used to confirm target specificity, and/or another set of ODNs could have been designed to target another region of the MAP kinase RNA molecule. However, the controls used in the present study indicated that MAP kinase depletion was due to a sequence-specific antisense effect, as neither the sense nor the scrambled control ODNs caused MAP kinase depletion (Figure 1A). Target sequence specificity was further confirmed by the observed 'sense' MAP kinase enhancement effect (Figure 1A and Table 1), which has been proposed previously to be specific for sense ODNs targeted to the ATG translation initiation codon of RNA molecules [21].

We also examined the possibility that non-sequence-specific anti-protein effects may have resulted from the ODN treatment protocol. However, although we found that expression of both p42 and p44 MAP kinases was substantially reduced ($> 95\%$) by antisense pretreatment, the expression of the MAP kinase homologues p38 (reactivating kinase) [17] and the p46 and p54 JNKs [18] was not affected (Figure 2). The expression of other proteins such as MEK and p90^{s6k} was also unaffected. In addition, we found that AII- and PDGF-stimulated activation of the upstream MEK was not significantly altered by antisense pretreatment (Figure 4), indicating that the upstream components of the MAP kinase signal transduction cascade, such as protein kinase C [11,22] and p21^{ras} [23], which are activated by either agonist, are not affected in a non-sequence-specific manner. This is an important observation, as ODN non-specific binding to the PDGF receptor has been reported [20].

Both AII and PDGF were found to stimulate p90^{s6k} activity with a kinetic profile similar to that for the activation of MAP kinase [11]. Several studies have implicated MAP kinase as a putative upstream regulator of p90^{s6k} activity within a number of different cell types [24–26]. The results of the present study have shown that the specific antisense ODN depletion of MAP kinase protein (Figure 1A) results in the loss of MAP kinase activity (Figure 1B), and that this in turn is linked directly to the loss of p90^{s6k} activity (Figure 3C), which is not accounted for by p90^{s6k} protein depletion (Figure 3B). These results indicate that p90^{s6k} is an *in vivo* downstream target of MAP kinase in vascular smooth muscle cells, consistent with findings from similar studies using antisense approaches or the novel MEK inhibitor PD 098059 in fibroblasts [12,27,28].

However, it should be noted that PDGF-stimulated p90^{s6k} activity was not completely abolished by antisense pretreatment. This may indicate the presence of a MAP kinase-independent pathway which is involved in the regulation of p90^{s6k}. Alternatively, it is possible that MAP kinase expression, although reduced by $> 95\%$, may not be completely abolished and that the residual MAP kinase activity may allow for a substantial proportion of p90^{s6k} to be activated. This would be consistent with recent findings indicating a substantial degree of amplification within the MAP kinase cascade down to the level of p90^{s6k} activation itself [28].

Preliminary studies did not suggest a role for MAP kinase in the acute regulation of glucose transport in smooth muscle cells, since deoxyglucose uptake was not affected by either antisense ODN or PD 098059 pretreatment (results not shown). This contrasts with findings from other cell types, where MAP kinase has been implicated in the regulation of transporter function [4,5].

Pretreatment of cells with MAP kinase antisense ODNs markedly affected *de novo* DNA synthesis. PDGF-stimulated [³H]thymidine incorporation was significantly diminished by pretreatment with antisense ODN. In addition, basal [³H]-thymidine incorporation was substantially reduced, probably reflecting the relatively high basal activity of MAP kinase in cells in the presence of 1% foetal calf serum. However, the fold stimulation was also substantially decreased (approx. 40% of control), indicating that the observed decrease in the PDGF-stimulated response was not merely due to an effect on basal incorporation. In some instances scrambled and sense ODN pretreatment also reduced PDGF-stimulated [³H]thymidine incorporation. This could be accounted for by non-specific effects of the transfection reagent recorded in preliminary experiments (results not shown). In a recent study we have also shown that PD 098059 is able to decrease PDGF-stimulated DNA synthesis in rat aortic smooth muscle cells; taken together, these studies clearly indicate a role for classical MAP kinase in the regulation of smooth muscle cell division [22].

One additional feature observed was the effect of the sense ODN in stimulating DNA synthesis. This phenomenon has previously been observed in vascular smooth muscle cells, where a 20-mer phosphorothioate-modified ODN directed against the insulin growth factor-1 receptor increased receptor number and basal and serum-stimulated DNA synthesis [20]. The authors of that report suggested that this effect could be due to ODN interaction with endogenous antisense mRNA species, competitive binding to mRNA-binding proteins, facilitation of DNA helix opening or ODN binding to transactivating factors. Their and our results indicate that this phenomenon may be a feature of sense ODNs directed against the ATG translation initiation codon.

Our results clearly confirm for the first time that sustained activation of MAP kinase in vascular smooth muscle cells is essential for DNA synthesis in response to growth factors. Since a number of disease states, such as hypertension and atherosclerosis, involve vascular smooth muscle remodelling, these findings may provide the basis for a future therapeutic approach.

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