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Requirement of MAP kinase for differentiation of fibroblasts to adipocytes, for insulin activation of p90 S6 kinase and for insulin or serum stimulation of DNA synthesis

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A phosphorothioate-oligonucleotide-based antisense strategy for depleting MAP kinase was developed. The 17mer antisense probe, EAS 1, caused a potent and concentration-dependent decrease in the steady state expression of p42 and p44 MAP kinase in 3T3 L1 fibroblasts and adipocytes with submicromolar concentrations effective. Antisense EAS 1 elicited a dose-dependent inhibition of insulin- and serum-stimulated DNA synthesis. Elimination of p42 MAP kinase by >95% and p44 MAP kinase to levels undetected blocked the ability of serum in 3T3 L1 fibroblasts and insulin in 3T3 L1 adipocytes to stimulate DNA synthesis by 87–95%. The differentiation of 3T3 L1 fibroblasts into adipocytes was prevented by 1 μM antisense EAS 1. The corresponding sense, scrambled or sense plus antisense EAS 1 phosphorothioate oligonucleotides did not deplete the p42 or p44 MAP kinase from either cell type, did not inhibit stimulation of DNA synthesis and did not interfere with differentiation. Two kinases on different MAP kinase activation pathways were not depleted by antisense EAS 1 whereas the ability of insulin to activate p90 S6 kinase was >90% eliminated in 3T3 L1 adipocytes by 4.5 μM antisense EAS 1. In conclusion these results show that MAP kinase is required for insulin and serum stimulation of DNA synthesis, for insulin stimulation of p90 S6 kinase activity and for differentiation of 3T3 L1 cells. Moreover, the development of the antisense probe EAS 1 against a target sequence of p42 MAP kinase that is conserved in p44 MAP kinase and across a range of species provides a molecular tool of general applicability for further dissecting the precise targets and roles of MAP kinase.

Key words: antisense/differentiation/growth factor/insulin/MAP kinase

Introduction

Mitogen activated protein (MAP) kinases are thought to play a key role in the signalling process of growth factors such as EGF, PDGF, NGF and of insulin. Additionally, MAP kinases are activated by cytokines and seven-helix membrane spanning receptors linked to heterotrimeric G-proteins, underscoring the wide range of agonists that activate MAP kinase (for reviews see Blenis, 1993; Nishida and Gotoh, 1993; Blumer and Johnson, 1994). Two highly related isoforms of MAP kinase, termed p42 MAP kinase and p44 MAP kinase, have been cloned. MAP kinases are phosphorylated and activated by combined tyrosine and threonine phosphorylation in the sequence TEY (Payne *et al.*, 1991). An upstream kinase with dual specificity that phosphorylates both the tyrosine and threonine has been isolated and is called MAPK kinase (Nakielny *et al.*, 1992). Recent suggestions are that cells may in fact contain a group of MAPK kinase homologues (Zheng and Guan, 1993). MAPK kinase is activated by serine/threonine phosphorylation (Gomez and Cohen, 1991; Kosako *et al.*, 1992) catalysed by a MAPKK kinase *in vitro*. Although initial studies identified c-Raf 1 as the MAPKK kinase later reports indicate that additional kinases may act in parallel with c-Raf 1 (Dent *et al.*, 1992; Lange-Carter *et al.*, 1993; Posada *et al.*, 1993; Haystead *et al.*, 1994).

A central role for MAP kinase in growth factor and insulin signalling is probable because MAP kinase can phosphorylate a large number of target substrates *in vitro*. These include p90 S6 kinase (Sturgill *et al.*, 1988), MAPKAP kinase-2 (a kinase that phosphorylates glycogen synthase at Ser7; Stokoe *et al.*, 1992), EGF receptor (Takishima *et al.*, 1991), phospholipase A_2 (Lin *et al.*, 1993), tyrosine hydroxylase (Sutherland *et al.*, 1993a), PHAS 1 (Lin *et al.*, 1994), protein tyrosine phosphatase 2C (Paraldi *et al.*, 1994), stathmin (Leighton *et al.*, 1993) and the transcriptional factors c-myc, c-jun, c-fos, $p62^{TCF}$, NF-IL6 and ATF-2 (Nishida and Gotoh, 1993 and references therein). Few if any of the *in vitro* substrates have been definitively shown to be substrates for MAP kinase *in vivo*.

A p90 S6 kinase isoform phosphorylates the regulatory subunit of protein phosphatase 1 (PP1G) *in vitro*, thereby activating the phosphatase (Cohen *et al.*, 1992). Additionally this p90 S6 kinase, together with p70 S6 kinase, phosphorylates and inactivates glycogen synthase kinase-3β *in vitro* (Sutherland *et al.*, 1993b). These pathways may underlie the ability of insulin to activate glycogen synthesis.

To facilitate the establishment of the full cellular role of MAP kinase and defining whether putative substrates identified *in vitro* are *in vivo* targets of the kinase we have developed an antisense strategy of general applicability for depleting MAP kinase from cells.

We have utilized phosphorothioate oligomers which retain the negative charge on the phosphorus backbone and, like unmodified deoxyoligonucleotides, are substrates for RNase H (Bennett *et al.*, 1992). Phosphorothioates also have the advantage over unmodified deoxynucleotides of displaying high nuclease stability. This combined with the use of *N*-[1-2, 3-dioleyloxylpropyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) which greatly enhances oligonucleotide uptake into cells and improves the

subcellular distribution of the oligonucleotide (Bennett *et al.*, 1992; Wagner *et al.*, 1993), provides a simple and effective antisense technique for the reduction of cellular MAP kinase to near undetectable levels. Using this approach direct evidence was obtained that MAP kinase is essential for the differentiation of fibroblasts into adipocytes, that MAP kinase is necessary for insulin and serum stimulation of mitogenesis and that MAP kinase is required for insulin stimulation of p90 S6 kinase activity.

Results

Design of a MAP kinase antisense oligonucleotide and antisense protocols

Successful application of antisense oligomers depends on the correct selection of oligonucleotide sequences to give strong inhibition of translation, high oligonucleotide stability and good uptake into cells. Effective antisense oligonucleotides are typically 15–22 nucleotides long, i.e. long enough to give a sequence that is theoretically unique to the target protein but short enough to be taken up into cells. Sequences containing the initiation codon and/or surrounding translated or untranslated regions have been particularly successful in many cases. In our studies, the antisense target region on MAP kinase was designed to meet very stringent conditions to select for maximum antisense efficacy, i.e. (i) 15–22mer; (ii) high T_m of binding to complementary oligonucleotides; (iii) low secondary structure; (iv) close to the initiation codon; (v) no self complementarity; (vi) a sequence that was effectively unique to MAP kinase when compared with cDNA sequences from all other known proteins. Using specially designed computer programmes (Bloomfield and Giles, 1992) the result was a MAP kinase sequence that showed particular antisense potential. In the mouse p42 MAP kinase cDNA sequence this corresponded to nucleotides 25–41 (Her *et al.*, 1991) of sequence 5′-ATG GCG GCG GCG GCG GC-3′, where the ATG is the initiation codon. The sequence has a theoretical T_m of 75°C and has no predicted secondary structure. The corresponding 17mer antisense probe, 5′-GCC GCC GCC GCC GCC AT-3′, shows no significant duplex formation. The concurring sequence in mouse p44 MAP kinase has not been fully determined but is identical as far as has been elucidated (see Discussion).

Phosphorothioate oligonucleotide analogues were used to increase antisense potency. Phosphorothioate oligonucleotides are particularly useful as they are resistant to nucleases, relatively simple to synthesize, enter cells well and exhibit similar hybrid kinetics to unmodified oligodeoxynucleotides. Two additional steps taken to enhance oligonucleotide efficacy further were the use of serum that had been heat-treated at 55°C for 30 min and the use of DOTMA. Heat treatment of serum inactivates nucleases (Larcher *et al.*, 1992). Use of DOTMA greatly enhances uptake of oligonucleotides into cells (Bennett *et al.*, 1992) and improves the subcellular distribution of the oligonucleotide (Wagner *et al.*, 1993). The phosphorothioate oligonucleotide with the sequence 5′-GCC GCC GCC GCC GCC AT-3′ was synthesized and is hereinafter referred to as antisense EAS 1. Control phosphorothioate oligonucleotides were also synthesized with sequences as follows: 5′-ATG GCG GCG GCG GCG GC-3′ (sense) and 5′-CGC GCG CTC GCG CAC CC-3′ (scrambled). These oligonucleotides were used routinely.

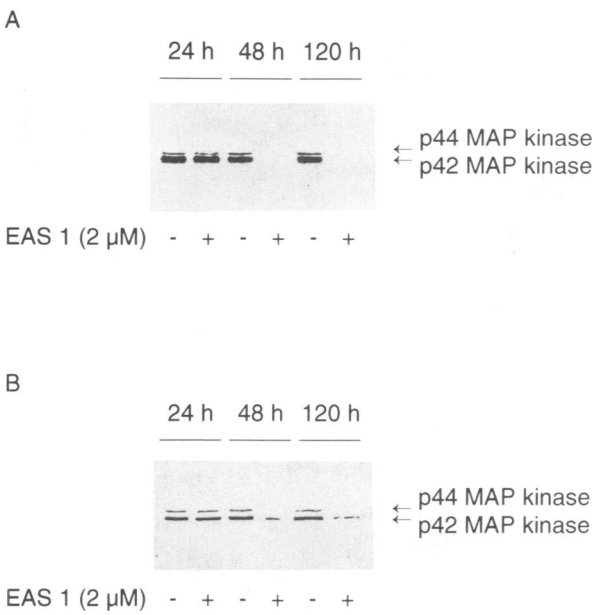

Fig. 1. Time course of inhibition of MAP kinase expression by antisense EAS 1. Cells were incubated with or without antisense EAS 1 for the times indicated with DOTMA present for the first 8 h. MAP kinase expression was determined by Western blotting. Results are typical of three experiments. (**A**) 3T3 L1 fibroblasts. (**B**) 3T3 L1 adipocytes.

Time course and concentration dependence for inhibition of p42 MAP kinase expression by antisense oligonucleotide EAS 1

In the present studies experiments showed that treatment of cells with DOTMA did not affect cellular levels of p42 or p44 MAP kinase in 3T3 L1 cells (Figure 2 and data not shown). Cells were incubated with oligonucleotides in a reduced volume of medium (400 μl in 22 mm dishes) in order to conserve oligonucleotide. This had no effect on the cellular p42 MAP kinase or p44 MAP kinase content or on the signalling responses analysed in the present work (not illustrated). Exposure of 3T3 L1 fibroblasts or 3T3 L1 adipocytes to 2 μM antisense phosphorothioate oligonucleotide EAS 1 caused a time-dependent depletion of p42 and p44 MAP kinase (Figure 1A and B). In both cell types there was a 24 h delay before the very rapid depletion of MAP kinase occurred. A large majority of the MAP kinase protein was lost over 24–48 h. p42 MAP kinase was the major MAP kinase isoform observed in immunoblots of extracts of 3T3 L1 fibroblasts and 3T3 L1 adipocytes (Figure 1 and also Figures 2 and 3). After exposure of cells to 2 μM antisense EAS 1 for 2 days, p42 MAP kinase was depleted by 98% ± 2% (mean ± SEM, n = 3) in the 3T3 L1 fibroblasts and by 85–90% in the 3T3 L1 adipocytes when compared with cells grown under identical conditions and for the same times but in the absence of oligonucleotide. Levels of p42 MAP kinase in 3T3 L1 adipocytes were lowered further to 5% ± 3% (mean ± SEM, n = 4) by using a higher concentration of antisense EAS 1 (4.5 μM) over 3 days; Figure 3. Figure 1 also shows that treatment of cells with 2 μM EAS 1 for 2 or more days resulted in the loss

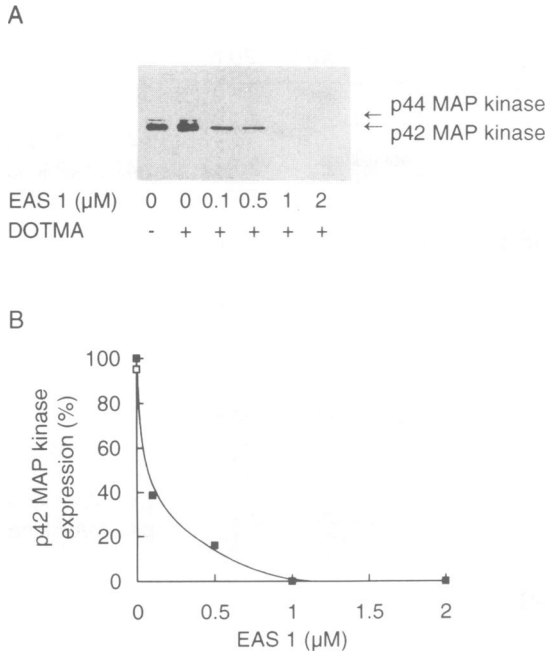

Fig. 2. Antisense EAS 1 causes a concentration-dependent decrease in the steady state expression of p42 and p44 MAP kinase in 3T3 L1 fibroblasts. The cells were exposed to antisense EAS 1 at the concentrations indicated for 48 h with DOTMA present for the first 8 h. (**A**) Western blot. (**B**) Expression of p42 MAP kinase, ■. Results obtained with cells incubated in the absence of both DOTMA and antisense EAS 1 are shown in the far left lane of panel A and the p42 MAP kinase expression is represented by □ in (B). Results are typical of three experiments.

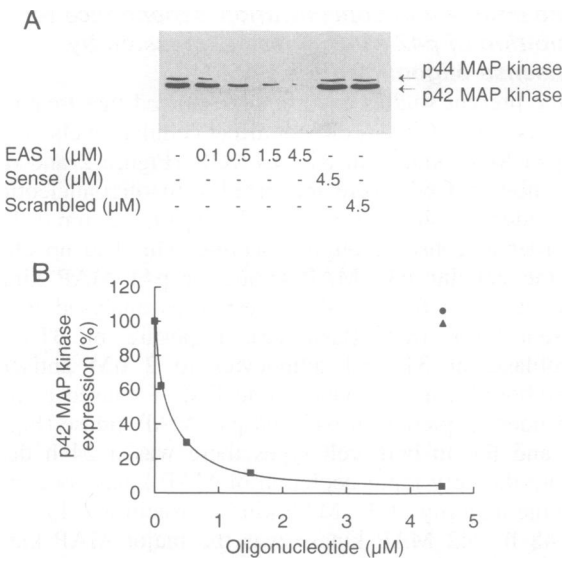

Fig. 3. Antisense EAS 1 causes a concentration-dependent decrease in the steady state expression of p42 and p44 MAP kinase in 3T3 L1 adipocytes. The cells were exposed to antisense EAS 1 (■), sense (●) or scrambled (▲) oligonucleotides at the concentrations indicated for 72 h with DOTMA present for the first 8 h. (**A**) Western blot. (**B**) Expression of p42 MAP kinase. Results are typical of four experiments.

of the p44 MAP kinase to levels undetected in both 3T3 L1 fibroblasts and 3T3 L1 adipocytes.

Figures 2 and 3 show that both p42 MAP kinase and p44 MAP kinase in 3T3 L1 fibroblasts and 3T3 L1 adipocytes were extremely sensitive to depletion by antisense EAS 1 with submicromolar concentrations being effective. Levels of depletion caused by EAS 1 were concentration dependent. In the 3T3 L1 fibroblasts the concentration of EAS 1 required to deplete 50% of p42 MAP kinase ($K_{0.5}$) when compared with cells incubated in the absence of oligonucleotide was ~0.04 μM. In the 3T3 L1 adipocytes the corresponding $K_{0.5}$ for p42 MAP kinase was 0.13 ± 0.04 μM (mean ± SEM, n = 3). The concentrations of EAS 1 required to deplete p42 MAP kinase in 3T3 L1 fibroblasts and 3T3 L1 adipocytes were consistent using two different anti-MAP kinase antibodies (Zymed or PA10) and antisense EAS 1 prepared in four separate syntheses. The somewhat higher $K_{0.5}$ for 3T3 L1 adipocytes relative to 3T3 L1fibroblasts could reflect slight dilution of the oligonucleotide−DOTMA complex in the triglyceride stores of the adipocytes, thereby effectively lowering the active concentration of oligonucleotide in these cells. In analogy with p42 MAP kinase, the p44 MAP kinase isoform was also potently depleted by submicromolar concentrations of EAS 1. Exposure of the cells to EAS 1 concentrations ⩾0.1 μM (3T3 L1 fibroblasts) or 0.5 μM (3T3 L1 adipocytes) reduced p44 MAP kinase to a level no longer detected. Low immunoblot levels of p44 MAP kinase made determination of an accurate $K_{0.5}$ value difficult. Clearly, however, antisense EAS 1 was very effective in extensively depleting the steady state levels of both p42 and p44 MAP kinase with the $K_{0.5}$ value for p44 MAP kinase being at least as low, if not lower, than that for p42 MAP kinase.

Incubation of the cells with 4.5 μM (3T3 L1 adipocytes) or 2 μM (3T3 L1 adipocytes) sense, scrambled or sense plus antisense phosphorothioate oligonucleotides for up to 4 days (3T3 L1 adipocytes) or 2 days (3T3 L1 fibroblasts) did not significantly deplete p42 MAP kinase or p44 MAP kinase indicating that antisense EAS 1 was acting specifically in these cells (e.g. Figures 3 and 5 and data not shown). This was further supported by the observations that the expression of c-Raf 1 and protein kinase C_δ (as determined by immunoblotting), which appear to be upstream activators of the MAP kinase cascade, and the levels of all other major cellular proteins (as determined by densitometric scanning of Coomassie blue-stained polyacrylamide gels) were not significantly affected by the presence of antisense EAS 1 oligonucleotide (0–4.5 μM, 0–5 days; not illustrated).

Antisense EAS 1 depletes MAP kinase whether assayed by kinase activity or Western blotting

To verify that the removal of MAP kinase protein by antisense EAS 1 as determined by Western blotting was associated with a depletion of MAP kinase activity, MAP kinase was assayed in cell extracts with myelin basic protein (MBP) as substrate. For this 3T3 L1 fibroblasts were stimulated with or without EGF and 3T3 L1 adipocytes were stimulated with or without insulin. Figure 4A shows in a representative experiment that treatment of 3T3 L1 fibroblasts with 2 μM antisense EAS1 for 2 days essentially eliminated MAP kinase as determined by Western blotting and depleted EGF-stimulated MBP kinase activity by 92%. Figure 4B shows in a representative experiment that treatment of 3T3 L1 adipocytes with 4.5 μM antisense EAS1 for 3 days removed 96% of MAP kinase as determined by Western blotting and depleted

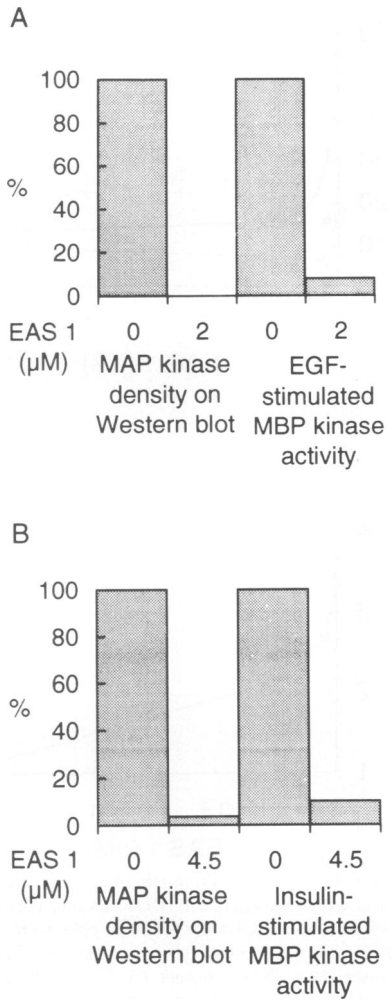

Fig. 4. Antisense EAS 1 depletes MAP kinase whether determined by kinase assay or Western blotting. 3T3 L1 fibroblasts (**A**) or 3T3 L1 adipocytes (**B**) were incubated with DOTMA and the indicated concentrations of antisense EAS 1 for 8 h. The cells were then incubated with the appropriate concentrations of antisense EAS 1 in the presence of 0.25% BSA for 40 h (3T3 L1 fibroblasts) or 64 h (3T3 L1 adipocytes). The cells were incubated for a further 10 min in the presence or absence of 33 nM EGF (3T3 L1 fibroblasts) or in the presence or absence of 80 nM insulin (3T3 L1 adipocytes). The cells were then extracted and aliquots assayed for MBP kinase activity or subjected to Western blotting. The MAP kinase Western blot densities are combined p42 MAP kinase + p44 MAP kinase values. Results are typical of three experiments.

insulin-stimulated MBP kinase activity by 90%. Thus antisense EAS 1 depleted ⩾90% of MAP kinase from 3T3 L1 fibroblasts or 3T3 L1 adipocytes when determined by kinase assay. The extent of depletion measured by kinase assay was slightly less than that obtained by Western blot analysis. This was not unexpected because the MBP assays are likely to be less specific even though inhibitors of major kinases were present in the assays.

Having defined conditions for depleting MAP kinase, we then determined the effects of the depletion on the stimulation of DNA synthesis, cell differentiation and the activation of p90 S6 kinase.

Antisense EAS 1 inhibits insulin- and serum-stimulated DNA synthesis

To test directly whether MAP kinase mediates in the signalling pathway by which growth factors and insulin stimulate DNA synthesis, the effect of depletion of MAP kinase on DNA synthesis in insulin-stimulated 3T3 L1 adipocytes and serum-stimulated 3T3 L1 fibroblasts was determined. 3T3 L1 fibroblasts gain insulin responsiveness following differentiation into adipocytes due to acquirement of insulin receptors (Rubin *et al.*, 1978).

Figure 5A shows that in insulin-stimulated 3T3 L1 adipocytes antisense EAS 1 caused a marked and concentration-dependent loss of the incorporation of [^{3}H]methylthymidine into DNA. Basal levels of [^{3}H]methylthymidine incorporation in the absence of insulin remained relatively constant with increasing antisense EAS 1 apart from a small initial decline. Anti-MAP kinase Western blot analysis of the same cells showed that MAP kinase was also depleted; this is quantified for the p42 MAP kinase isoform in Figure 5C. As p44 MAP kinase accounted for only 5–9% of the total immunoreactive MAP kinase there was insufficient for accurate quantification at all the antisense EAS 1 concentrations used. However, as described above, p44 MAP kinase was at least as sensitive to depletion by antisense EAS 1 as p42 MAP kinase and was not detected at antisense EAS 1 concentrations of ⩾0.5 μM. Antisense EAS 1 at a concentration of 4.5 μM reduced p42 MAP kinase by 95% (Figure 5C) and reduced insulin stimulation of DNA synthesis by 87% (Figure 5B) compared with cells incubated under identical conditions but in the absence of oligonucleotide. There was a good correlation between the level of p42 MAP kinase expression and extent of incorporation of [^{3}H]methylthymidine into DNA in insulin-treated cells as evident from the similar curves in Figure 5A and C. Incubation of 3T3 L1 adipocytes with sense, scrambled or sense + antisense EAS 1 oligonucleotides under identical conditions in the same experiments did not deplete p42 MAP kinase (Figure 5C) or p44 MAP kinase (not illustrated but see Figure 3) and had no effect on insulin-stimulated or basal DNA synthesis (Figure 5A). Further evidence that antisense EAS 1 was acting specifically was provided by using a modified EAS 1 phosphorothioate oligonucleotide sequence (5′-GCC TCC TCC TCC GCC AT-3′) which contained three mismatches. Treatment of 3T3 L1 adipocytes with 4.5 μM of the mismatched oligonucleotide for 72 h did not significantly affect the expression or MAP kinase (the expression was always >80%) or the insulin-stimulated incorporation of [^{3}H]methylthymidine into DNA (not illustrated).

Figure 6 shows that in 3T3 L1 fibroblasts antisense EAS 1 also elicited a marked concentration-dependent reduction in the ability of serum to increase DNA synthesis. Suppression of MAP kinase again appeared to correlate well with the inhibition of [^{3}H]methylthymidine incorporation into DNA (compare the curves in Figures 2B and 6A). In the experiment of Figure 6, antisense EAS 1 at a concentration of 1.5 μM decreased p42 MAP kinase by 98%, depleted p44 MAP kinase to an undetected level and decreased serum stimulation of DNA synthesis by 95%. Incubation of the cells with sense, scrambled or sense + antisense EAS 1 phosphorothioate oligonucleotides up to 1.5 μM did not affect serum-stimulated DNA synthesis (results not shown).

Antisense EAS 1 prevents differentiation of fibroblasts into adipocytes

The differentiation of 3T3 L1 fibroblasts into adipocytes provides an excellent system to test the role of MAP

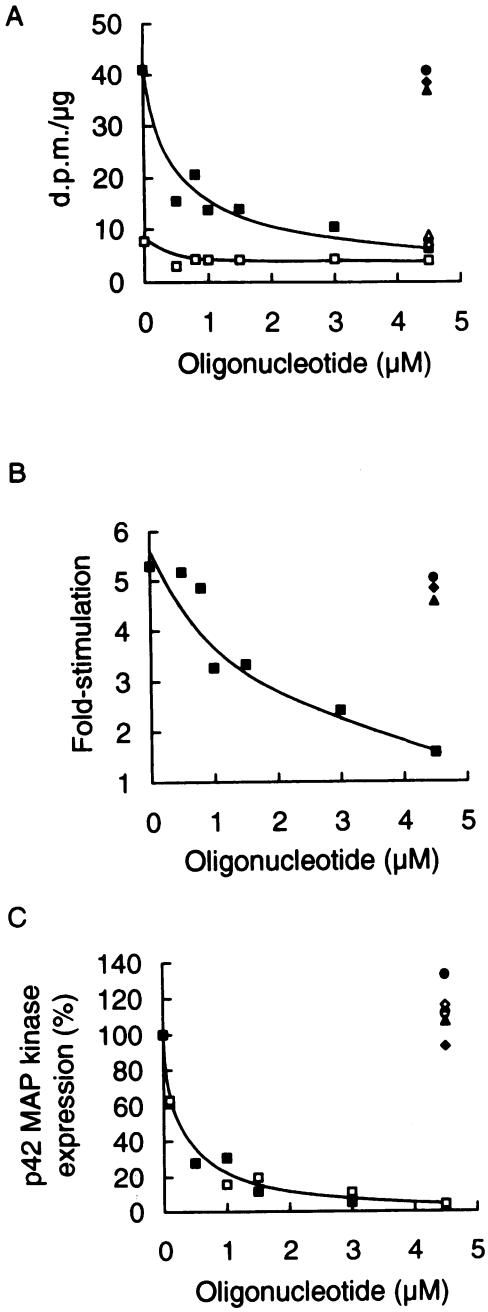

Fig. 5. Antisense EAS 1 inhibits insulin-stimulated DNA synthesis. 3T3 L1 adipocytes were incubated with DOTMA and the indicated concentrations of the oligonucleotide for 8 h. The cells were then incubated with the appropriate concentrations of the oligonucleotides in the presence of 0.25% BSA for 64 h. 16 h before the end of the incubations cells were treated with or without 80 nM insulin. [^{3}H]methylthymidine was added 1 h before the end of the incubations. Cells were extracted and aliquots taken for measurement of incorporation of radioactivity into DNA (**A**) and of p42 MAP kinase expression by Western blotting (**C**). In (A) and (C) values are for antisense EAS 1 (■, □), sense (●, ○), scrambled (▲, △) or sense + antisense EAS 1 (◆, ◇) oligonucleotides for incubations performed in the presence (closed symbols) or absence of insulin (open symbols). In (**B**) the degree of insulin stimulation of [^{3}H]methylthymidine incorporation into DNA is shown for antisense EAS 1 (■), sense (●), scrambled (▲) or sense + antisense EAS 1 (◆) oligonucleotides. Results are representative of three experiments.

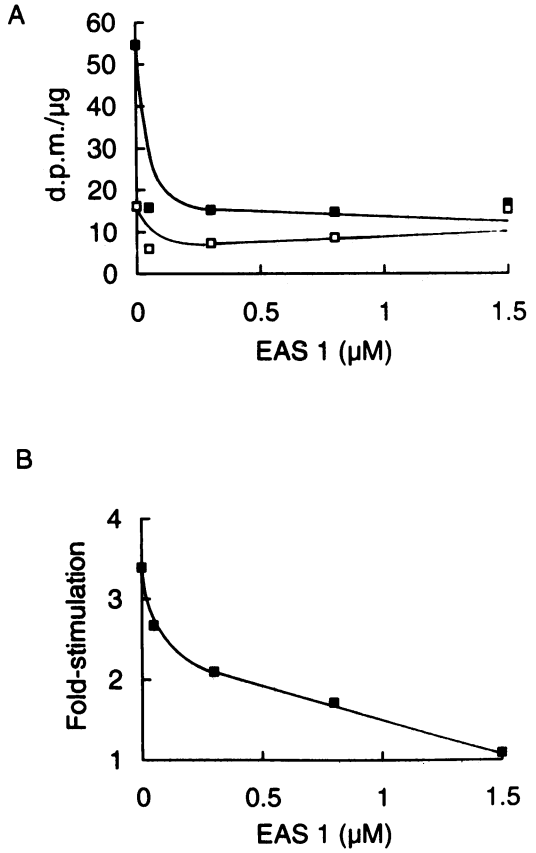

Fig. 6. Antisense EAS 1 inhibits serum-stimulated DNA synthesis. 3T3 L1 fibroblasts were incubated with DOTMA and the indicated concentrations of antisense EAS 1 for 8 h. The cells were then incubated for 40 h with the appropriate concentrations of antisense EAS 1 in the presence (**A**, ■) or absence (A, □) of 10% heat-treated fetal calf serum. [^{3}H]methylthymidine was added 1 h before the end of the incubations and the incorporation into DNA determined (A). (**B**) shows the degree of serum stimulation of [^{3}H]methylthymidine incorporation into DNA. Western blot analysis showed that in this experiment 1.5 μM antisense EAS 1 depleted p42 MAP kinase by 98% and depleted p44 MAP kinase to a level no longer detected. Results are of a typical experiment which was duplicated.

kinase in transducing differentiation. Confluent 3T3 L1 fibroblasts were pretreated for 48 h with antisense oligonucleotide EAS 1 (0–2.5 μM) with DOTMA present for the first 8 h of this period. The differentiating agents (Frost and Lane, 1985) dexamethasone (0.25 μM), methylisobutylxanthine (0.5 mM) and insulin (1 μg/ml) were then added as described in Materials and methods and incubations continued in the presence of antisense EAS 1 (0–2.5 μM). Figure 7 shows results obtained at day 7 after addition of the differentiating agents. In the absence of DOTMA and antisense EAS 1, >98% of cells differentiated into adipocytes (Figure 7A). Treatment of cells with DOTMA alone did not affect the ability of the fibroblasts to differentiate (Figure 7B). Cells treated with 0.05 μM antisense EAS 1 and DOTMA largely differentiated (Figure 7C). Treatment of cells with 0.1 μM antisense EAS 1 in the presence of DOTMA markedly prevented differentiation, with only ~25% of cells displaying the adipocyte phenotype on day 7 after addition of the differentiating agents (Figure 7D). Use of antisense EAS 1 at concentrations of 1 μM and above in the presence of DOTMA resulted in cells exclusively retaining their fibroblast phenotype and no adipocytes were formed (Figure 7E and F). In the experiment of Figure 7 p42 MAP kinase levels measured in the same cells were 86.0%

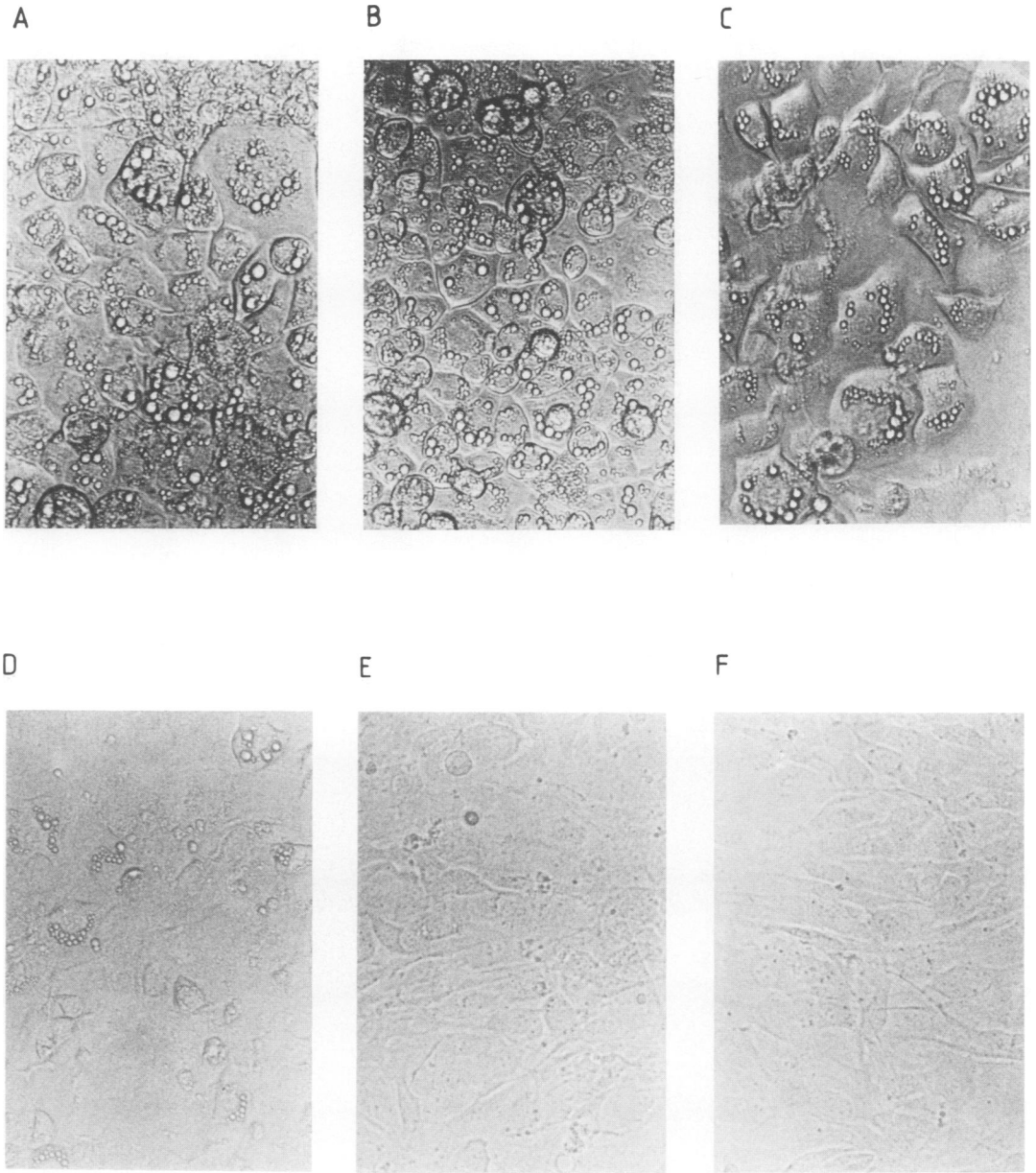

Fig. 7. Antisense EAS 1 causes a concentration-dependent inhibition of differentiation. Confluent 3T3 L1 fibroblasts were incubated for 8 h without (**A**) or with (**B**–**F**) DOTMA in the absence (A and B) or presence of antisense EAS 1 at concentrations of 0.05 μM (C), 0.1 μM (D), 1 μM (E) and 2.5 μM (F). After incubation for a further 40 h with the appropriate concentrations of antisense EAS 1 in the absence of DOTMA the differentiating agents were added in the continued presence of the appropriate concentrations of antisense EAS 1. Photographs were taken on day 7 after adding the differentiating agents.

(no DOTMA, no antisense EAS 1), 100% (DOTMA, no antisense EAS 1), 65.9% (DOTMA, 0.05 μM antisense EAS 1), 12.9% (DOTMA, 0.1 μM antisense EAS 1) 0.0% (DOTMA, 1.0 μM antisense EAS 1) and 0.0% (DOTMA, 2.5 μM antisense EAS 1). p44 MAP kinase levels measured in the same cells were 89.0% (no DOTMA, no antisense EAS 1), 100% (DOTMA, no antisense EAS 1), ~25% (DOTMA, 0.05 μM antisense EAS 1) with the p44 MAP kinase not detected at antisense EAS 1 concentrations of 0.1 μM and above.Thus antisense EAS 1 caused a dose-dependent inhibition of differentiation with loss of differentiation occurring within the same antisense EAS 1 concentration range that depleted p42 MAP kinase and p44 MAP kinase. Treatment of cells with 2 μM sense oligonucleotide (Figure 8B), 2 μM sense oligonucleotide plus 2 μM antisense EAS 1 (Figure 8C) or 2 μM scrambled oligonucleotide (Figure 8D) did not inhibit differentiation nor deplete the cells of p42 or p44 MAP kinase (see legend to Figure 8). Thus the oligonucleotide-mediated inhibition of differentiation was specific for antisense EAS 1 and was not mediated by the control oligonucleotides.

Antisense EAS 1 inhibits insulin activation of p90 S6 kinase

To test directly if MAP kinase mediates in the activation of the p90 S6 kinase by insulin in intact cells the effect of MAP kinase depletion on the insulin stimulation of the p90 S6 kinase activity in 3T3 L1 adipocytes was determined. Figure 9 shows that treatment of 3T3 L1 adipocytes with 4.5 μM antisense EAS 1 for 72 h,

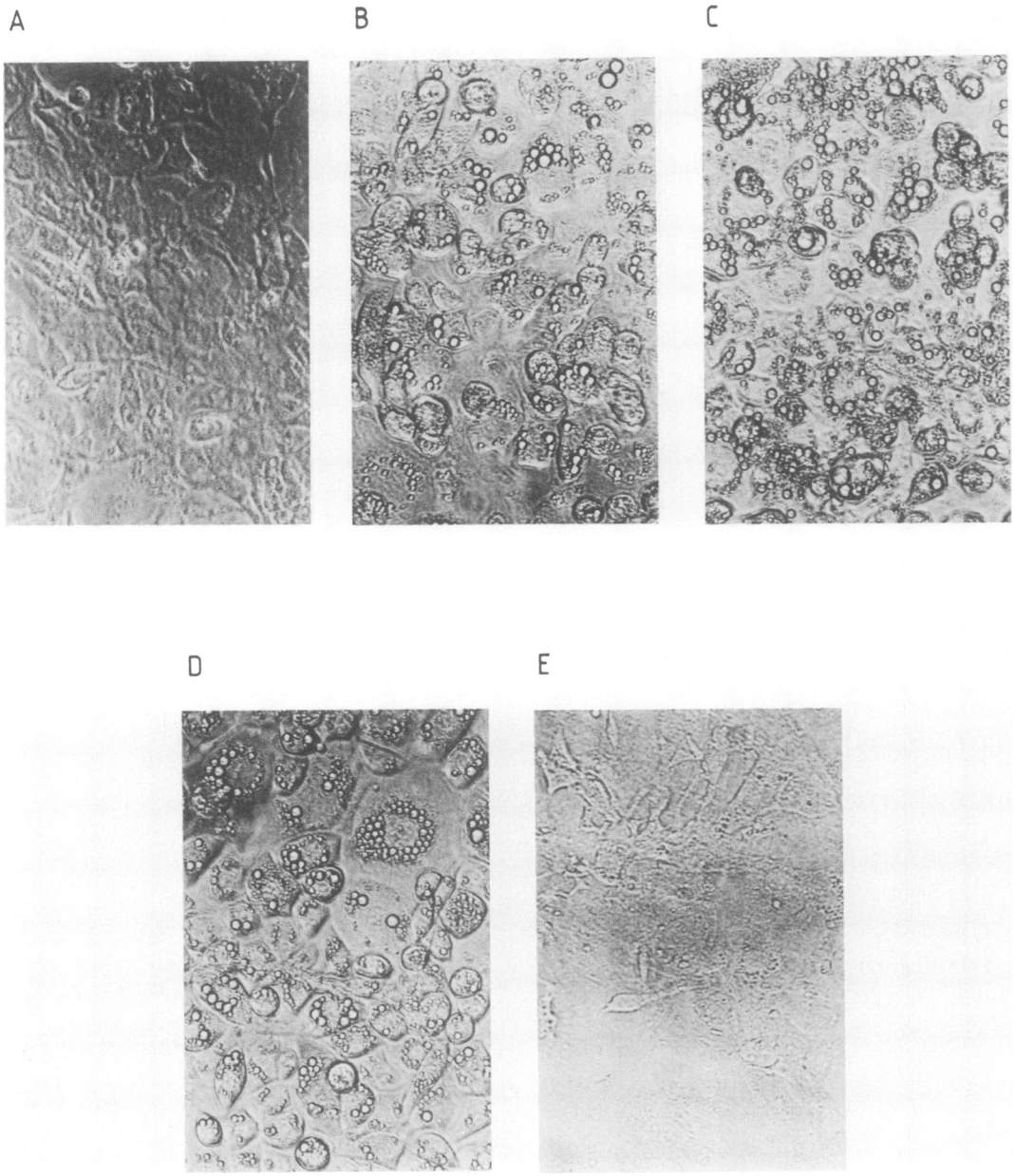

Fig. 8. Antisense EAS 1, but not control oligonucleotides, prevent differentiation. Confluent 3T3 L1 fibroblasts were incubated for 8 h with DOTMA and antisense EAS 1 (**A**), sense phosphorothioate oligonucleotide (**B**), antisense EAS 1 + sense phosphorothioate oligonucleotides (**C**) or scrambled phosphorothioate oligonucleotide (**D**). After incubation for a further 40 h with the appropriate oligonucleotides the differentiating agents were added in the continued presence of the appropriate oligonucleotides. The oligonucleotide concentrations were 2 μM. Photographs were taken at day 7 after adding the differentiating agents. (**E**) In the same experiment 3T3 L1 fibroblasts were incubated as described in (A)−(D) but in the absence of oligonucleotide and differentiating agents, then photographed at the same time as (A)−(D). MAP kinase expression was determined at the end of the experiment by Western blotting. Values for p42 and p44 MAP kinase, respectively, were 3.6 and 0% (antisense EAS 1), 140.6 and 105.6 % (sense), 111.6 and 108.0% (sense plus antisense EAS 1), 77.7 and 100.5% (scrambled) and 117.8 and 110.6% (undifferentiated fibroblasts).

compared with cells incubated in the absence of oligonucleotide, markedly reduced the ability of insulin to increase MAP kinase activity as assayed with MBP and that this was matched by a reduction in the ability of insulin to increase the p90 S6 kinase activity assayed with the same cells.

Discussion

We have developed an effective and simple antisense technique for the specific depletion of p42 and p44 MAP kinase from 3T3 L1 fibroblasts and 3T3 L1 adipocytes. The protocol involves using a phosphorothioate oligonucleotide analogue called EAS 1. The p42 MAP kinase isoform represented the major (>90%) MAP kinase band detected in immunoblots of both cell types. Treatment of cells with 2 μM antisense EAS 1 for 2 days caused a 98% ± 2% (mean ± SEM, n = 3) reduction of the p42 MAP kinase in 3T3 L1 fibroblasts (Figures 1A and 2). Similarly a 95% ± 3% (mean ± SEM, n = 4) reduction of the p42 MAP kinase in 3T3 L1 adipocytes was achieved by incubation of the cells with 4.5 μM antisense EAS 1 for 3 days (Figures 3 and 5C). Under these EAS 1 incubation conditions p44 MAP kinase was reduced to

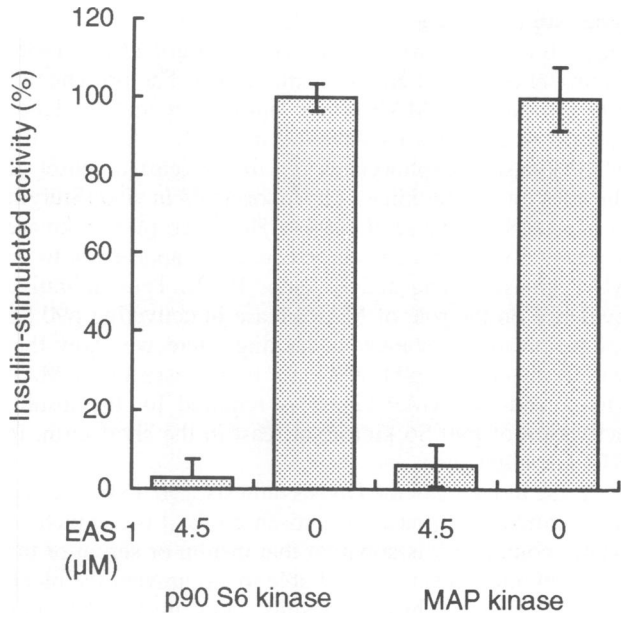

Fig. 9. Antisense EAS 1 inhibits the ability of insulin to activate p90 S6 kinase. 3T3 L1 adipocytes were incubated in the presence or absence of 4.5 μM antisense EAS 1 for 72 h with DOTMA present for the first 8 h and then stimulated for 10 min with or without 100 nM insulin as described in Materials and methods. The cells were extracted and aliquots assayed for p90 S6 kinase and MAP kinase activities. Values are means ± SEM.

undetected levels in both cell types. Cells depleted of p42 and p44 MAP kinase were viable and exhibited apparently normal cellular phenotypes. Treatment of either 3T3 L1 fibroblasts or 3T3 L1 adipocytes with the corresponding sense phosphorothioate oligonucleotide or antisense EAS 1 together with the sense phosphorothioate oligonucleotide or a phosphorothioate oligonucleotide containing the EAS 1 sequence scrambled, at concentrations up to 4.5 μM (3T3 L1 adipocytes) or 2 μM (3T3 L1 fibroblasts), did not deplete the p42 MAP kinase or the p44 MAP kinase.

The extracellular concentration of antisense EAS 1 required to deplete p42 MAP kinase by 50% compared with the amount of p42 MAP kinase in cells incubated in the absence of oligonucleotide ($K_{0.5}$) was ~0.04 μM for 3T3 L1 fibroblasts and 0.13 ± 0.04 μM (mean ± SEM, n = 3) for 3T3 L1 adipocytes. The $K_{0.5}$ values were confirmed using two different anti-MAP kinase antibodies. The p44 MAP kinase isoform represented 5–9% of the MAP kinase on Western blots. Because of the low immunoblot levels of p44 MAP kinase, determination of accurate $K_{0.5}$ values for p44 MAP kinase was difficult and was not undertaken. However, it is clear from Figures 2 and 3 that p44 MAP kinase was at least as sensitive to depletion by antisense EAS 1 as p42 MAP kinase in both 3T3 L1 fibroblasts and 3T3 L1 adipocytes. Thus antisense EAS 1 was a potent inhibitor of both p42 and p44 MAP kinase expression in these cells. The removal of MAP kinase protein as assessed by Western blotting was confirmed by assays of MAP kinase activity (Figure 4). Phosphorothioate antisense oligonucleotides or their analogues have been used in depleting cellular proteins, e.g. Ras (Monia *et al.*, 1992), interleukin 1 receptors (Burch and Mahan, 1991), intracellular adhesion molecule 1 (Chiang *et al.*, 1991; Bennett *et al.*, 1992) and SV40 large T antigen (Wagner *et al.*, 1993). Where measured, $K_{0.5}$ values for the most effective antisense oligonucleotides are in the region of 0.05–3 μM. Unmodified oligonucleotides typically require higher concentrations (~15 μM extracellular concentration) for effect (e.g. Tortora *et al.*, 1991). The high antisense potency of EAS 1 was aided by the following: first, selection of a GC-rich oligonucleotide with a high T_m; secondly, use of a nuclease stable phosphorothioate oligonucleotide analogue; thirdly, use of heat-treated serum to inactivate nucleases; and fourthly, use of DOTMA which enhances oligonucleotide uptake into cells up to 18-fold (Bennett *et al.*, 1992) and diminishes accumulation in endosomes (Wagner *et al.*, 1993). The fact that p42 MAP kinase and p44 MAP kinase were suppressed by antisense EAS 1 even after day 5 in experiments where oligonucleotide-containing medium was not replaced after day 2 indicates that the stability of antisense EAS 1 in the culture medium is high.

Depletion of p42 and p44 MAP kinase by antisense EAS 1 in both 3T3 L1 fibroblasts and 3T3 L1 adipocytes became most evident after 24 h (Figure 1). This delay probably reflects the prevention of turnover of MAP kinase through EAS 1 inhibiting the synthesis of new MAP kinase. This explanation is consistent with the reported half-life of p42 MAP kinase, and also of p44 MAP kinase, being ~24 h (Pagès *et al.*, 1993).

Five points of evidence were obtained that the action of antisense EAS 1 in depleting MAP kinase was specific. First, the expression of MAP kinase was not significantly affected by the sense, sense + antisense, scrambled, or the mismatched (three mismatches) phosphorothioate oligonucleotides. Secondly, the expression of two kinases, c-Raf 1 and protein kinase C, which appear to be on different upstream activation pathways for MAP kinase, was not significantly affected by antisense EAS 1. Thirdly, antisense EAS 1 did not affect the expression levels of any of the major Coomassie blue-stained proteins bands on SDS gels. Fourthly, antisense EAS 1 did not appear to interfere with general cell function. In the presence of antisense EAS 1, cells were viable and retained normal cell morphology. Moreover, treatment of 3T3 L1 adipocytes with antisense EAS 1 at concentrations of up to 6 μM for 3 days had no effect on the ability of insulin to stimulate glucose transport (E.M.Sale and G.J.Sale, in preparation) showing that there was no general impairment of cell function. Finally, the blockage of the signalling responses and the depletion of MAP kinase showed similar sensitivity to antisense EAS 1.

The oligonucleotide sequence of p42 MAP kinase in the antisense EAS 1 probe target region is conserved in mouse, rat and human (Boulton *et al.*, 1991; Her *et al.*, 1991; Owaki *et al.*, 1992) indicating that antisense EAS 1 should be generally applicable in depleting p42 MAP kinase from cells of a wide range of species. Fewer sequences containing the initiation codon are known for p44 MAP kinase than for p42 MAP kinase. Nevertheless the sequence is wholly conserved in rat and human p44 MAP kinase cDNA (Marquardt and Stabel, 1992; Charest *et al.*, 1993) and is reportedly conserved for at least eight nucleotides in mouse p44 MAP kinase cDNA (Tanner and Mueckler, 1993; the mouse sequence lacked 9 bp inclusive of the initiation codon). The potent ability of antisense EAS 1 to deplete mouse p44 MAP kinase suggests that

the remaining 9 bp are also conserved. Thus the antisense probe EAS 1 is also expected to be effective in depleting p44 MAP kinase from cells of a range of species.

Growth factors such as EGF and PDGF play important roles in the stimulation of DNA synthesis and cell division. Insulin also regulates normal cell growth and development and induces DNA synthesis in a variety of cell types. Using the antisense strategy we were able to assess in the intact cell the role that MAP kinase plays in signalling the stimulation of DNA synthesis by insulin and by serum. Antisense EAS 1 caused a concentration-dependent inhibition of both insulin-stimulated DNA synthesis in 3T3 L1 adipocytes and serum-stimulated DNA synthesis in 3T3 L1 fibroblasts. In 3T3 L1 fibroblasts treatment with 1.5 μM antisense EAS 1 for 2 days depleted p42 MAP kinase by 98%, depleted p44 MAP kinase to levels no longer detected and inhibited serum stimulation of DNA synthesis by 95% (Figure 6). Similarly, treatment of 3T3 L1 adipocytes with 4.5 μM antisense EAS 1 (the highest concentration tested) for 3 days reduced p42 MAP kinase by 95%, reduced p44 MAP kinase to undetected levels and caused an 87% inhibition of the stimulation of DNA synthesis by insulin (Figure 5). By varying the concentration of antisense EAS 1 we demonstrated a close correlation between [^{3}H]methylthymidine incorporation into DNA in insulin-stimulated 3T3 L1 adipocytes or serum-stimulated 3T3 L1 fibroblasts and the p42 MAP kinase expression. The oligonucleotide-mediated inhibition of DNA synthesis was specific for antisense EAS 1 and was not elicited by treatment of 3T3 L1 fibroblasts or 3T3 L1 adipocytes with the control sense, scrambled or sense plus antisense oligonucleotides at concentrations of up to 1.5 μM (3T3 L1 fibroblasts) or 4.5 μM (3T3 L1 adipocytes). Additionally, the mismatched oligonucleotide which contained three mismatches did not significantly inhibit insulin stimulation of DNA synthesis in 3T3 L1 adipocytes. It is concluded that serum stimulation of DNA synthesis in 3T3 L1 fibroblasts and insulin stimulation of DNA synthesis in 3T3 L1 adipocytes require MAP kinase.

3T3 L1 fibroblasts provide an excellent model to investigate the role of MAP kinase in differentiation. Incubation of these cells with differentiating agents induces the adipocyte phenotype in which the cells accumulate triacylglycerol (Rubin *et al.*, 1978). Treatment of 3T3 L1 fibroblasts with concentrations of antisense EAS 1 of $\geqslant$1 μM completely inhibited differentiation induced by incubation with dexamethasone + isobutylxanthine + insulin (Figures 7E, F and 8A). The action of antisense EAS 1 was specific and was not mimicked by the control sense, scrambled or sense plus antisense phosphorothioate oligonucleotides at concentrations of up to 2 μM (Figure 8B–D). Although the precise mechanism of differentiation is unknown, it is concluded that MAP kinase is required for signalling the differentiation response in 3T3 L1 cells. Additionally, expression of transfected *raf* oncogene in 3T3 L1 adipocytes partially induces differentiation (Porras *et al.*, 1994) and Cowley *et al.* (1994) using MAPK kinase 1 mutants have obtained evidence for the involvement of MAPK kinase 1 in NGF-induced PC12 differentiation.

The observations that MAP kinase phosphorylates and reactivates dephosphorylated p90 S6 kinase *in vitro* and that the activities of MAP kinase and p90 S6 kinase are coordinately regulated in response to growth factors and other stimulii has led to the idea that p90 S6 kinase is a target for MAP kinase in the cell (Sturgill *et al.*, 1988; Chung *et al.*, 1991; Kahan *et al.*, 1992). The presence of p90 S6 kinase in MAP kinase immunoprecipitates lends support to this idea (Scimeca *et al.*, 1992). Additionally, MAP kinase phosphorylates *in vitro* at least a subset of the sites on p90 S6 kinase phosphorylated *in vivo* (Sturgill *et al.*, 1988; Grove *et al.*, 1993). However, p90 S6 kinase kinase activities have been detected that appear not to be MAP kinase (Wang and Erikson, 1992). Thus definitive evidence on the role of MAP kinase in activating p90 S6 kinase *in vivo* is somewhat lacking. Here we show that within intact cells p90 S6 kinase is downstream of MAP kinase and that MAP kinase is required for the insulin activation of p90 S6 kinase, at least in the short term, in 3T3 L1 adipocytes.

As the blocking of the DNA synthesis, p90 S6 kinase and differentiation responses by antisense EAS 1 is complete or nearly complete it is apparent that insulin or serum or the differentiating agents are not able to circumvent the block at MAP kinase. Thus the results show in 3T3 L1 cells that the MAP kinase signalling pathway(s) is necessary for transducing these responses.

As the strategy for optimal depletion of p42 and p44 MAP kinase by EAS 1 involved addition of EAS 1 to cells that were nearly confluent, the effects of the MAP kinase depletion on cell growth were not determined in the present work. Pagès *et al.* (1993) have shown that expression of p44 MAP kinase-deficient mutants or the entire p44 MAP kinase antisense RNA in CCL 39 lung fibroblasts inhibits cell growth.

In conclusion, the results of the present work provide a direct demonstration that MAP kinase is absolutely required for differentiation to occur and also provide a direct demonstration that the ability of insulin or serum to induce DNA synthesis has an absolute requirement for MAP kinase. The ability of insulin to activate p90 S6 kinase was also shown to require MAP kinase. Moreover, the development of the antisense probe, EAS 1, against a broadly conserved target sequence of MAP kinases provides a molecular tool that should be generally applicable to a range of cell types for dissecting precise targets and biochemical roles of MAP kinase.

Materials and methods

Cells

3T3 L1 fibroblasts were the kind gift of Dr G.Gould, University of Glasgow, Scotland and were used to prepare 3T3 L1 adipocytes. For preparation of 3T3 L1 adipocytes, 3T3 L1 fibroblasts at passage 6–9 and 2 days post-confluent were differentiated as described in Frost and Lane (1985) and used between days 8 and 15 after initiating differentiation. For fibroblast studies, 3T3 L1 fibroblasts were from Applied Microbiology and Research, Porton Down, Salisbury, UK. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, 1 g/l glucose, Gibco) containing 10% myoclone fetal calf serum (Gibco) at 37°C in the presence of 5% CO_2. The medium was replaced every 48 h. Where heat-treated serum was used the serum was heated at 55°C for 30 min.

Oligonucleotides

Phosphorothioate oligonucleotides were synthesized on an automated DNA synthesizer (Applied Biosystems 391) replacing the standard iodination bottle with tetraethylthiuram disulfide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation cycle wait step was increased to 900 s. After cleavage and deblocking in concentrated ammonium hydroxide at 55°C for 18 h the phosphorothioate oligo-

nucleotides were purified on OP cartridges (Applied Biosystems), dried down and resuspended in sterile water to give a 20–40× concentrated stock solution. Phosphorothioate oligonucleotides prepared by this method typically contain <1% phosphodiester linkages (Chiang *et al.*, 1991).

Oligonucleotide treatment of cells

Cells (typically 80% confluent in 22 mm dishes) were carefully washed three times with 2 ml DMEM (no additions). Appropriate dilutions of oligonucleotide in 100 µl of DMEM (no serum) were preincubated at room temperature for 15 min with 100 µl of DMEM (no serum) containing 40 µg/ml DOTMA (lipofectin, Gibco). This mixture was added to the cells together with a further 200 µl of DMEM (no serum). The final concentrations of oligonucleotides are as indicated. Cells were incubated for 8 h at 37°C in the presence of 5% CO_2. After this time the medium containing DOTMA was removed and the incubation continued for the times indicated in the figures using fresh medium containing appropriate oligonucleotide concentrations in the presence of 10% heat-treated fetal calf serum or 0.25% bovine serum albumin (BSA) in experiments where the effects of insulin or EGF were studied. The medium was replaced every 2–3 days with new medium containing appropriate concentrations of oligonucleotides and other additions.

In the differentiation experiments 3T3 L1 fibroblasts were treated for 8 h with or without oligonucleotide and/or DOTMA, as above, then incubated in medium containing 10% heat-treated fetal calf serum in the presence or absence of oligonucleotide for 40 h. The differentiating agents dexamethasone (0.25 µM), methylisobutylxanthine (0.5 mM) and insulin (1 µg/ml) were then added (Frost and Lane, 1985) in fresh medium containing 10% heat-treated fetal calf serum in the presence or absence of oligonucleotide. After 48 h the medium was replaced with medium containing insulin (1 µg/ml) and 10% heat-treated fetal calf serum with or without oligonucleotide. After a further 48 h the medium was replaced at 2 day intervals with medium containing 10% heat-treated calf serum in the presence or absence of oligonucleotide.

Assay of DNA synthesis

One hour before the end of the oligonucleotide incubation, 1 µCi/well of [^{3}H]methylthymidine (1 mCi/ml, ICN Radiochemicals) was added and the incubation continued at 37°C. Cells were then rapidly washed twice with 2 ml ice-cold PBS and extracted by scraping into 62.5 mM Tris−HCl (pH 7.5) containing 0.1% SDS (3T3 L1 fibroblasts) or 62.5 mM Tris−HCl (pH 7.5) containing 1% SDS (3T3 L1 adipocytes). Appropriate duplicate aliquots were precipitated with 750 µl of ice-cold 10% TCA. After 30 min at 0°C the precipitates were diluted to 5 ml with further ice-cold TCA and collected on G/C filters (Whatman). Filters were washed extensively with ice-cold 10% TCA, dried and counted in Optiphase Hi-Safe 3 scintillant (Fisons Chemicals). Further aliquots were removed for determination of protein concentration and for analysis of MAP kinase by Western blotting. DNA synthesis was calculated as d.p.m. incorporated/µg total cellular protein.

Western blotting

Samples (typically 20 µg total protein) were separated by SDS−PAGE (10% resolving gel) using a Bio-Rad minigel apparatus (Tappia *et al.*, 1993). Proteins were transferred to PVDF membranes (ICN) using a Hoefer semi-dry transfer apparatus (typically 30 mA/gel, 40 min) with 20% methanol, 25 mM Tris base and 192 mM glycine, as the transfer buffer. Membranes were blocked for 1–2 h at room temperature in TBS-T buffer (20 mM Tris−HCl, 137 mM NaCl, 0.1% Tween-20, pH 7.5) containing 10% BSA. Membranes were incubated at room temperature for 1 h in TBS-T containing 10% BSA and antibody (0.1 µg/ml Zymed anti-MAP kinase antibody, 4–6 µg/ml of PA10 anti-MAP kinase antibody, 1 µg/ml anti-Raf 1 antibody or 1 µg/ml of anti-protein kinase C_δ antibody) and washed three times, 10 min each time, with 10 ml TBS-T buffer containing 10% BSA. Membranes were then incubated for 60 min in TBS-T buffer containing 10% BSA with a 1:2000 dilution of sheep anti-rabbit antibody or a 1:5000 dilution of sheep anti-mouse antibody coupled to horseradish peroxidase, as appropriate, at room temperature followed by two 30 min washes with TBS-T buffer. Immunoreactive bands were visualized using an ECL kit (Amersham International) and Hyperfilm-MP (Amersham International). Quantification was then achieved by densitometric scanning. p42 MAP kinase and p44 MAP kinase expression was determined relative to total cellular protein and is calculated as a percentage of that present in cells incubated under identical conditions with DOTMA but in the absence of oligonucleotides. Under the conditions used there was a linear relationship between band density and the amount of MAP kinase. This is illustrated for p42 MAP kinase in Figure 10. To maintain the linear relationship care was taken with the length of exposure so as to avoid underexposure or overexposure of the immunoblots.

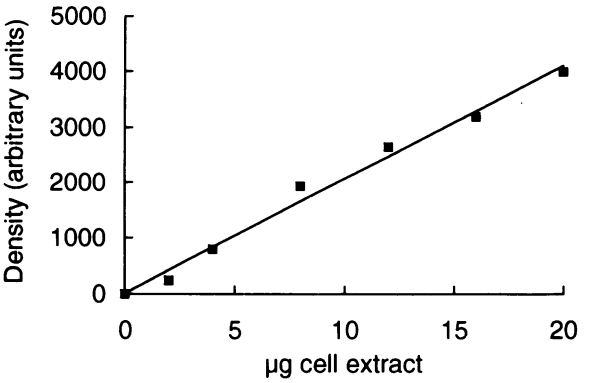

Fig. 10. Western blot quantitation of p42 MAP kinase expression is linear. The indicated amounts of whole cell extract from 3T3 L1 adipocytes were subjected to Western blot analysis with anti-MAP kinase antibody and scanned densitometrically.

Kinase assays

After incubation, cells (in 22 mm dishes) were washed three times with 2 ml of ice-cold 10 mM Tris−HCl, pH 7.4, containing 137 mM NaCl and scraped into 300 µl of ice-cold lysis buffer (20 mM Tris−HCl, pH 7.4, 10 mM β-glycerophosphate, 10 mM NaF, 1 mM Na_3VO_4, 2 mM EDTA, 2 mM EGTA, 0.1% NP-40, 1 mM benzamidine, 1 mM DTT, 10 µg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride). The cells were homogenized by repeated expulsion through a 25 gauge syringe needle, centrifuged at 10 000 *g* for 15 min at 4°C and the supernatant filtered through a 0.22 µm filter (Millipore). Aliquots were taken for kinase assay and protein assay. MAP kinase was assayed using MBP as substrate (Ahn *et al.*, 1990). For this, 1 µg of cell extract in a final volume of 25 µl of 20 mM HEPES, pH 7.4, containing 10 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 5 µM protein kinase A inhibitor peptide (Sigma), 10 µM calmidizolium (Sigma), 0.1 mM Na_3VO_4 and 0.33 mg/ml MBP, was incubated at 30°C for 15 min with 0.1 mM [γ-^{32}P]ATP (5 c.p.m./fmol). The reaction was terminated by the addition of 6.25 µl of 312.5 mM Tris−HCl, pH 7.4, containing 10% SDS, 76 mg/ml dithiothreitol, 0.1% (w/v) bromophenol blue and 20% (w/v) sucrose and boiling for 5 min. MBP was separated by SDS/PAGE with a 15% resoving gel (Smith and Sale, 1988). The MBP band was excised and counted in Optiphase Hi-Safe 3 scintillant. In some experiments the incorporation of ^{32}P into phosphothreonine was determined by phosphoamino acid analysis (Smith *et al.*, 1988); this showed that all the ligand-stimulated ^{32}P incorporation into MBP was into phosphothreonine. p90 S6 kinase was assayed by a standard immunocomplex assay (Terada *et al.*, 1993). For this, cell extract containing 35 µg of protein was incubated at 0°C for 18 h with 2.7 µg of rabbit anti-p90 S6 kinase antibody or 2.7 µg of rabbit IgG for controls. The immune complex was adsorbed to protein A−Sepharose for 1 h and washed twice with lysis buffer (see above) and once with p90 S6 kinase assay buffer (20 mM HEPES, pH 7.4, containing 10 mM $MgCl_2$, 0.4 µM protein kinase A inhibitor peptide and 0.4 mM DTT). Following the final wash the pellet was resuspended in 40 µl of the p90 S6 kinase assay buffer containing 0.1 mM [γ-^{32}P]ATP (3 c.p.m./fmol) and 200 µM S6 peptide, RRRLSSLRA (Upstate Biotechnology Incorporated). After incubation for 20 min at 30°C the reaction was terminated by the addition of 10 µl of stopping buffer (164 mM HCl, 1 mM ATP and 1% BSA). Following centrifugation, 20 µl aliquots of the supernatant were added in duplicate to phosphocellulose paper squares. The squares were washed six times in 1.5 l of 175 mM phosphoric acid (10 min each wash), dried and counted in Optiphase Hi-Safe 3 scintillant.

Antibodies

Horseradish peroxidase conjugated sheep anti-rabbit and sheep anti-mouse antibodies were from Sera Lab. Two anti-MAP kinase antipeptide antibodies were used. A mouse anti-MAP kinase monoclonal antibody from Zymed Immunochemicals was used routinely. A rabbit anti-MAP kinase antipeptide antibody, PA10, raised against the sequence ELDDLPKERC which represents residues 339–347 of the p44 MAP kinase sequence of Boulton *et al.* (1990) but with an added C-terminal

cysteine to enable coupling was used where stated (King *et al.*, 1991). Anti-p90 S6 kinase and anti-Raf 1 antibodies were from Upstate Biotechnology Incorporated. Anti-protein kinase C_δ antibody was from Transduction Laboratories.

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