Growth hormone and phorbol esters require specific protein kinase C isoforms to activate mitogen-activated protein kinases in 3T3-F442A cells

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Previous studies have shown that the activation of p44 and p42 mitogen-activated protein (MAP) kinases (ERK1 and ERK2) by growth hormone (GH) and phorbol esters, but not by epidermal growth factor, in 3T3-F442A preadipocytes is dependent on protein kinase C (PKC). In the present study two approaches have been taken to determine the PKC isoform dependence of MAP kinase activation in these cells. By immunoblotting with specific antibodies, the cells were found to express PKC- α , - γ , - δ , - ϵ and - ζ . Treatment of cells with 500 nM PMA for 3 h led to the complete depletion of PKC- δ and the partial depletion of PKC- α but did not significantly affect the expression of the other PKC isoforms. In parallel, such treatment severely attenuated the ability of GH to activate MAP kinase. The degree of this attenuation was not increased by more prolonged PMA pretreatment, indicating that PKC- δ and perhaps PKC- α are important for MAP kinase activation by GH. These experiments further revealed that additional PKC isoforms were required for

the full activation of MAP kinases by acute treatment with PMA. A second approach involved the use of anti-sense oligodeoxynucleotides (ODNs) to deplete the individual PKC isoforms selectively. Each of the ODNs used effectively depleted the relevant isoform to undetectable levels and did not affect the expression of the other PKC isoforms. Pretreatment of cells with PKC- δ anti-sense ODN, but not with anti-sense ODN to the other phorbol ester-sensitive isoforms, severely attenuated the activation of MAP kinases by GH. PKC- δ anti-sense ODN also blocked (by approx. 50%) the activation of MAP kinases by PMA. Furthermore a combination of PKC- δ and - ϵ anti-sense ODNs completely blocked the effect of PMA on MAP kinases. Collectively, these results indicate that the novel PKC- δ and - ϵ isoforms can couple to the MAP kinase pathway in 3T3-F442A cells but that the activation of MAP kinases by GH specifically involves PKC- δ .

INTRODUCTION

The cytokine growth hormone (GH) exerts diverse effects on the growth, differentiation and metabolism of a range of cells (reviewed in [1]). In the preadipocyte 3T3-F442A cell line, GH induces growth arrest [2] and promotes differentiation to the adipocyte phenotype [3]. Although at the intracellular level the molecular mechanisms underlying these actions have not been elucidated it is recognized that changes in protein phosphorylation play an important role. One class of serine/threonine protein kinases known to be activated by GH are the mitogen-activated protein (MAP) kinases [4–6]. The MAP kinases have been shown to phosphorylate various cellular proteins including p90^{ras} [7], phospholipase A₂ [8], the epidermal growth factor (EGF) receptor [9] and certain transcription factors including the ternary complex factor Elk 1 [10] and members of the signal transducer and activator of transcription (STAT) family [11]. Although known to play a pivotal role in mitogenesis [12,13], MAP kinases have also been shown to be both necessary and sufficient for the differentiation of PC12 cells [12] and are at least necessary for differentiation of both 3T3-L1 [14] and 3T3-F442A preadipocytes (S. J. Yarwood, E. Kilgour, E. M. Sale, G. J. Sale and N. G. Anderson, unpublished work). MAP kinases are therefore likely

to assume a crucial role in mediating the cellular responses to GH.

The precise mechanisms by which GH induces the activation of the MAP kinase signalling cascade remains unclear. The cloned GH receptor is a member of the cytokine receptor superfamily [15] and as such possesses no intrinsic tyrosyl kinase activity. However, binding of GH results in activation of the GH receptor-associated tyrosyl kinase JAK-2 [16] and to a smaller extent also of JAK-1 [17]. Although JAK-2 is proposed to be required for the activation of MAP kinases by GH [18,19], the signalling events that link it to MAP kinase activation remain to be established. We have shown previously [4] that protein kinase C (PKC) is involved in the activation of MAP kinase by GH; this has been confirmed by others [20]. PKC is a multigene family of phospholipid-dependent serine/threonine kinases consisting, so far, of 12 members divided into groups on the basis of their structural and biochemical properties. Thus the conventional PKCs (α , β I/II and γ) are regulated by Ca²⁺ ions and diacylglycerol or phorbol esters, and the novel PKCs (δ , ϵ , η and θ) are sensitive to diacylglycerol or phorbol esters but are insensitive to calcium [21,22]. There are also the atypical PKCs (ζ and λ /I), which, although structurally related to the other PKCs, are insensitive to Ca²⁺ ions, diacylglycerol and phorbol esters [21,23],

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; GH, growth hormone; MAP kinase, mitogen-activated protein kinase; MEK, mitogen-activated or extracellular signal-regulated kinase kinase; ODN, oligodeoxynucleotide; PI 3-kinase, phosphoinositide 3-OH kinase; PKC, protein kinase C.

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and PKC μ , which may represent a new PKC subgroup [24]. There is now increasing evidence to suggest that different PKC isoforms serve diverse functional roles, which is consistent with their displaying distinct regulatory properties and tissue distributions [21–29].

Although it is well established that phorbol ester-sensitive isoforms of PKC can mediate activation of MAP kinases by a number of growth factors (see [30]), it is not clear which isoforms are specifically involved *in situ*. The fact that down-regulation of PKCs by chronic exposure of cells to phorbol ester attenuates the activation of MAP kinases by GH [4,20] implicates an involvement of conventional and/or novel PKC isoforms. In this regard we have recently demonstrated that the lipid kinase, phosphoinositide 3-OH kinase (PI 3-kinase), is required for the activation of MAP kinase by GH [31]. PI 3-kinase preferentially phosphorylates PtdIns(4,5) P_2 *in vivo*, generating PtdIns(3,4,5) P_3 [32,33], a candidate second messenger known to activate the δ , ϵ and η [34] and ζ [35] isoforms of PKC *in vitro*. This prompted us to undertake a study aimed at refining our earlier observations [4] by defining the individual PKC isoforms required for MAP kinase activation by GH. By selective depletion of individual PKC subtypes, with anti-sense oligodeoxynucleotides (ODNs), we show that in 3T3-F442A preadipocytes PKC- δ and PKC- ϵ , but not PKC- α or PKC- γ , mediate the activation of MAP kinases by phorbol esters, whereas only PKC- δ is involved in their activation by GH.

MATERIALS AND METHODS

Materials

Purified sheep GH was obtained from the National Hormone and Pituitary Program (Bethesda, MD, U.S.A.). Culture media, calf serum, foetal calf serum, EGF and lipofectin were obtained from Life Technologies (Paisley, U.K.). Enhanced chemiluminescence (ECL) solutions and [γ - 32 P]ATP were obtained from Amersham (Little Chalfont, Bucks., U.K.). PMA and myelin basic protein were from Sigma (Poole, Dorset, U.K.). Phenyl-Sepharose was purchased from Pharmacia.

Antibodies

Anti-phosphotyrosine (clone 4G10) antibody was purchased from TCS (Botolph Claydon, Bucks, U.K.). Monoclonal antibodies to PKC- α , - β I/II, - γ , - δ , - ϵ , - η and - θ were obtained from Affiniti Research (Exeter, Devon, U.K.). A rabbit polyclonal antiserum to PKC- ζ was raised against a synthetic peptide from the rat sequence (amino acid residues 577–592). Rabbit polyclonal antibodies to p44 MAP kinase (ERK1) were raised against a synthetic peptide from the rat sequence (residues 325–345). A monoclonal antibody to p42 MAP kinase (ERK2) was a gift from Professor Ailsa Campbell (Department of Biochemistry, University of Glasgow, Glasgow, U.K.).

Cell culture

3T3-F442A cells (from Dr. Howard Green, Harvard Medical School, Cambridge, MA, U.S.A.) were grown and treated with agents as previously described [4]. NIH-3T3 cells transfected to stably overexpress PKC- α , - β , - γ , - δ , - ϵ , - η and - ζ (a gift from Dr. Joanne Goodnight, N.C.I., Bethesda, MD, U.S.A.) were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM glutamine and 10% foetal calf serum before the preparation of cell lysates for use as positive controls on immunoblots.

Chronic treatment with phorbol esters

In some experiments cells were preincubated with phorbol esters to render them PKC-deficient. In these studies confluent cultures were washed three times with serum-free medium (DMEM containing 2 mM glutamine and 25 mM Hepes) then incubated in this medium for 24 h. PMA (500 nM) was added to the cultures 3, 6 or 24 h before the end of this incubation period. Parallel control cultures received DMSO (0.01%, v/v). At the end of the 24 h incubation period hormones were added for assessment of acute effects on activation of MAP kinases, and immunoreactive PKC levels were evaluated.

Treatment of cells with anti-sense ODNs

Phosphorothioate-modified ODNs were purchased from Genosys Biotechnologies (Cambridge, U.K.). The anti-sense sequences used were 5'-CGGGTAAACGTCAGCCAT-3' for PKC- α [36], 5'-GAAGGAGATGCGCTGGAA-3' for PKC- δ [36], 5'-GCCATTGAACACTACCAT-3' for PKC- ϵ [37] and 5'-AGGGCCCAGACCCGCCAT-3' for PKC- γ . The anti-sense sequence for PKC- γ is based on the start codon (ATG) plus the 15 additional downstream bases in the murine PKC sequence [38]. ODNs were dissolved in 10 mM Tris (pH 8.0)/1 mM EDTA and stored at -20°C until required. Before treatment of cells, appropriate dilutions of ODNs in 100 μl of DMEM were combined with 100 μl of 200 $\mu\text{g}/\text{ml}$ lipofectin in DMEM then preincubated at room temperature for 15 min with occasional mixing. 3T3-F442A fibroblasts (typically 70–80% confluent in 30 mm dishes) were washed with 2 ml of DMEM before addition of the ODN/lipofectin mixture along with a further 800 μl of DMEM to give a final ODN concentration of 10 μM , except for the PKC- α anti-sense ODN, which was used at a final concentration of 5 μM . Cells were incubated for 6 h at 37°C then washed twice with 2 ml DMEM before the addition of fresh medium containing 2 mM glutamine, 10% heat-treated calf serum and the appropriate ODN concentration, but no lipofectin. After a further 48 h this medium was replaced with serum-free medium containing ODN; after a further 16–18 h of incubation, cells were washed twice with serum-free medium and incubated for a further 1 h in this medium without ODNs, before acute stimulation with hormones or assessment of immunoreactive PKC levels.

Preparation of rat brain homogenate

Rat brain homogenates were prepared essentially as described by Kuo et al. [39]. Briefly, rat midbrains, which had been snap-frozen in liquid nitrogen, were cryopulverized before homogenization in buffer [25 mM Tris (pH 7.5)/2.5 mM EGTA/250 mM sucrose/2.5 mM MgCl_2 /50 mM 2-mercaptoethanol/1 mM sodium orthovanadate/1 mM PMSF/2 $\mu\text{g}/\text{ml}$ leupeptin/2 $\mu\text{g}/\text{ml}$ aprotinin/2 $\mu\text{g}/\text{ml}$ pepstatin A] with a Polytron homogenizer. Homogenates were centrifuged at 1000 g for 10 min at 4°C and the supernatant was passed through buffer-moistened glass wool. Triton X-100 (1%, v/v) was added to the supernatant, which was then mixed gently for 1 h at 4°C . After centrifugation as before, 0.25 volumes of 5 $\times$ sample buffer was added to the supernatant, followed by boiling. This denatured supernatant was stored at -70°C before immunoblotting.

Western blotting

Cells were scraped into ice-cold lysis buffer [25 mM Hepes (pH 7.5)/2.5 mM EDTA/50 mM NaCl/50 mM NaF/30 mM sodium pyrophosphate/10% (v/v) glycerol/1% (v/v) Triton X-100/1 mM sodium orthovanadate/0.4 mM PMSF/2 $\mu\text{g}/\text{ml}$

leupeptin/2 $\mu\text{g/ml}$ pepstatin A/2 $\mu\text{g/ml}$ aprotinin], clarified by centrifugation (12000 g for 10 min at 4 $^{\circ}\text{C}$) and denatured by adding 0.25 vol. of 5 $\times$ sample buffer and boiling. Lysate proteins were separated by SDS/PAGE on 10% (w/v) gels. For MAP kinase blots 6 M urea was added to the separating gel solution. After transfer to nitrocellulose, blots were probed with antibodies to PKC isoforms, to phosphotyrosine, or to p42 and p44 MAP kinases. Immunoreactive bands were detected with the enhanced chemiluminescence (ECL) system (Amersham). Densitometric analysis of immunoreactive bands was performed with a Molecular Dynamics Personal Densitometer.

MAP kinase assay

MAP kinase activities were determined after partial purification of cell lysates with phenyl-Sepharose [4]. Activities were calculated from assays performed under standard conditions with myelin basic protein (Sigma) as substrate, as described previously [4].

Presentation of results

Except where indicated all results shown are representative of experiments done on at least three separate occasions. Statistical analysis was by Student's *t* test for unpaired samples.

RESULTS

We previously reported that cellular depletion of PKC by chronic treatment of 3T3-F442A preadipocytes with a high concentration (16 μM) of phorbol ester markedly decreased the stimulation of MAP kinases by GH [4]. As shown in Figure 1, immunoblot analysis of cell lysates with a panel of antibodies revealed that, of the PMA-sensitive, conventional and novel PKC isoforms, these

Figure 1 PKC subtypes present in 3T3-F442A preadipocytes

Cell lysates, prepared from NIH-3T3 cells transfected to overexpress the appropriate PKC isoform (lane 1, 50 μg of protein) or confluent 3T3-F442A preadipocytes (lane 2, 50 μg of protein), were run alongside a rat midbrain homogenate (lane 3, 75 μg of protein). Samples were immunoblotted with monoclonal antibodies to PKC- α , PKC- β /I/II, PKC- γ , PKC- δ , PKC- ϵ or a polyclonal antibody to PKC- ζ as described in the Materials and methods section. The positions of the PKC immunoreactive bands are indicated by arrows.

Figure 2 Effect of chronic treatment with phorbol ester on immunoreactive protein kinase levels in 3T3-F442A preadipocytes

Cells were treated with vehicle or PMA (500 nM) for the times indicated before the preparation of cell lysates for assessment of PKC levels by immunoblotting as described in the legend to Figure 1. Immunoblots were subjected to densitometric analysis and the levels of each PKC isoform expressed as a percentage of the level in cells treated with vehicle alone (100%). Results are means $\pm$ S.E.M. for three separate observations.

cells express the α , γ , δ and ϵ subtypes and also the phorbol ester-insensitive, atypical PKC- ζ isoform. The apparent molecular masses of the isoforms were 82 kDa (α), 80 kDa (γ), 78 kDa (δ), 90 kDa (ϵ) and 73 kDa (ζ), in close agreement with values reported in the literature [21–29]. Depending upon the gel conditions, PKC- δ was sometimes detected as a doublet (see Figure 1). We believe that both bands represent PKC- δ for the following reasons. First, immunoreactive levels of both bands are markedly higher in the NIH 3T3 cells overexpressing this isoform compared with non-transfected parental cells. Secondly, where both bands were observed, both were down-regulated with chronic treatment with phorbol ester. Others have observed a doublet representing PKC- δ [39a] and it has been suggested that these might represent different phospho-forms of the enzyme. For the purposes of quantification, the combined densities of both PKC- δ bands were measured. We were unable to detect PKC- β /I/II in the cells, using either the commercially available monoclonal antibody from Affiniti (Figure 1) or two different polyclonal antibodies, one which was raised in-house and another was from Dr. P. Parker (London, U.K.) (results not shown). These experiments were repeated on several occasions and PKC- β was never detectable in 3T3-F442A cells despite loadings of up to 400 μg of lysate protein on the gel and heavy overexposure of the autoradiographs. Using the antibodies available commercially we were also unable to detect either PKC- η or PKC- θ (results not shown).

To identify which of the expressed isoforms of PKC were involved in the activation of MAP kinases two experimental approaches were taken. The first involved chronic pretreatment of cells with the phorbol ester PMA to deplete cells of endogenous PKC. Chronic treatment of cells with 16 μM PMA resulted in the rapid and complete down-regulation of all of the conventional and novel PKC subtypes present in the cells within 3–6 h (results not shown). Thus this treatment failed to provide information on the role of specific PKC isoforms in MAP kinase activation. However, when cells were treated chronically with 500 nM PMA the down-regulation of PKC isoforms followed differential time

Table 1 The effect of chronic treatment with phorbol ester on the stimulation of MAP kinase activity by PMA, GH or EGF

Cells were preincubated for the times indicated with PMA (500 nM) followed by the addition of PMA (10 nM), GH (10 nM) or EGF (10 nM) for a further 10 min. Cells were then lysed and processed for the determination of total MAP kinase activity as described in the Materials and methods section. Data are expressed as fold increases in MAP kinase activity above those in cells chronically pretreated with 500 nM PMA without further treatment and are means $\pm$ S.E.M. for three independent experiments.

	MAP kinase activation (fold)			
	0	3	6	24
PMA	6.74 $\pm$ 0.56	3.98 $\pm$ 0.42	3.25 $\pm$ 0.61	1.37 $\pm$ 0.25
GH	3.45 $\pm$ 0.51	2.01 $\pm$ 0.35	1.59 $\pm$ 0.39	1.79 $\pm$ 0.36
EGF	8.20 $\pm$ 0.76	7.95 $\pm$ 0.57	8.07 $\pm$ 0.66	7.98 $\pm$ 0.65

Figure 3 Effect of chronic treatment with phorbol ester on the activation of p42 MAP kinase by PMA, GH or EGF

3T3-F442A preadipocytes were preincubated for the times indicated with PMA (500 nM) followed by the addition of GH (10 nM), PMA (10 nM) or EGF (10 nM) for a further 10 min. Cell lysates were prepared and immunoblotted with antibodies to p42 MAP kinase as described in the Materials and methods section. The immunoblot showing the relative shifts in MAP kinase mobility under each condition is representative of three separate experiments.

courses (Figure 2). Concurrent examination of the activation of MAP kinases was performed by assay of phosphotransferase activity in partly purified cell extracts [4] (Table 1) and by mobility shift due to their enhanced phosphorylation, which correlates with activation of these enzymes [40] (Figure 3). Preincubation of cells with 500 nM PMA for 3 h was sufficient to cause marked inhibition of the acute activation of MAP kinase by GH (Table 1 and Figure 3). At this time cellular PKC- δ had fallen to undetectable levels and levels of PKC- α had been decreased by $63.5 \pm 12\%$ ($P < 0.001$). In contrast, the γ and ϵ isoforms were markedly less sensitive to chronic PMA treatment, with no significant decrease in their levels occurring after 3 h (Figure 2). No further significant inhibition of the GH effect on MAP kinases was achieved when the PMA treatment was continued for up to 24 h (Table 1 and Figure 3), although cellular levels of PKC- α , γ and ϵ continued to decrease, resulting in undetectable levels of PKC- α , a $60.1 \pm 7\%$ ($P < 0.06$) decrease in PKC- γ and a $53.3 \pm 7\%$ ($P < 0.003$) decrease in PKC- ϵ levels,

Figure 4 Specific down-regulation of PKC isoforms by treatment with anti-sense ODNs

Cells were preincubated with anti-sense ODNs (10 μ M for PKC- γ , δ and ϵ ; 5 μ M for PKC- α) for 72 h before the preparation of lysates as described in the Materials and methods section. Immunoblots show the amounts of each isoform in lysates from NIH-3T3 cells overexpressing the appropriate PKC subtype (ox), control 3T3-F442A cells preincubated with lipofectin alone (c) or 3T3-F442A cells preincubated with the anti-sense ODN to the PKC isoform indicated. The positions of the immunoreactive PKC bands are indicated. The immunoblots shown are representative of experiments performed on at least two occasions for each PKC isoform.

compared with levels in control cells, after 24 h of treatment with PMA (Figure 2). As expected, chronic treatment with PMA had no effect on the cellular levels of atypical PKC- ζ (Figure 2). Collectively these results suggested a role for PKC- δ in the activation of MAP kinases by GH. However, from these results alone, an involvement of other PKC isoforms, particularly PKC- α , could not be excluded. Although the activation of MAP kinases by an acute challenge with PMA was also decreased within 3 h of chronic exposure to PMA, it continued to decline, resulting in the complete inhibition of MAP kinase stimulation after 24 h of chronic treatment with PMA (Figure 3). In contrast with the marked effects of chronic treatment with PMA on the activation of MAP kinases by GH there was no detectable effect on the activation induced by EGF (Table 1 and Figure 3).

To define further the involvement of specific PKC subtypes in the activation of MAP kinases we next used anti-sense ODNs, designed to down-regulate selectively the expression of each isoform. Immunoblot analysis revealed that preincubation of cells with the indicated concentrations of anti-sense oligonucleotides to PKC- α , γ , δ or ϵ resulted in the depletion of over 90% of the appropriate PKC subtype (Figure 4). Importantly there was no detectable effect of any of the anti-sense treatments on cellular levels of other PKC subtypes (Figure 4). Further analysis of this nature revealed that the anti-sense treatments had no significant effect on the cellular levels of the GH receptor-

Figure 5 Effect of down-regulation of PKC subtypes by anti-sense ODNs on the activation of p42 MAP kinase by phorbol ester, GH or EGF

3T3-F442A cells were preincubated with anti-sense (AS) ODNs to the PKC subtype indicated, as described in the legend to Figure 4, before the addition of PMA (10 nM), GH (10 nM), EGF (10 nM) or vehicle for a further 10 min. Cells were then lysed and immunoblotted with antibodies to p42 MAP kinase as described in the Materials and methods section. The immunoblot shown is representative of three independent experiments.

associated tyrosyl kinase JAK-2 or of the p42 and p44 isoforms of MAP kinases (results not shown).

Cells were pretreated with the individual anti-sense ODNs or with lipofectin alone (control samples) for 70–72 h before the addition of PMA, GH or EGF for 10 min. MAP kinase activation was then assessed by immunoblotting. The addition of lipofectin alone to the cells did not affect the levels of expression of the PKC isoforms nor the activation of MAP kinases by GH, PMA or EGF (results not shown). As shown in Figure 5 (top panel), acute treatment of cells with PMA converted the entire cellular population of p42 MAP kinase protein to the more slowly migrating activated form. Cellular depletion of the α or γ isoforms of PKC had little effect on this activation. However, depletion of either the δ or the ϵ isoform, by preincubating cells with the appropriate anti-sense ODN, decreased the PMA-induced activation of MAP kinases by approx. 50% (Figure 5, top panel, lanes 5 and 6). Consistent with these observations, pretreatment of cells with a combination of anti-sense ODNs to both PKC- δ and - ϵ resulted in the complete inhibition of the acute effect of PMA on the activation of p42 MAP kinase (Figure 5, top panel, lane 7). Identical results were obtained when the acute effect of PMA on activation of the p44 isoform of MAP kinase was examined (results not shown). Thus in these cells the δ and ϵ subtypes of PKC are capable of coupling to the MAP kinase signalling pathway.

As reported previously [31], acute stimulation of cells with GH converted approx. 50% of the cellular population of p42 MAP kinase to the activated form (Figure 5, middle panel). This effect of GH was markedly decreased after the depletion of PKC- δ with anti-sense ODN (Figure 5, middle panel, lane 5). In contrast, depletion of PKC- α , - γ or - ϵ had no effect on the activation of p42 MAP kinase by GH. Essentially identical results were obtained when the activation of p44 MAP kinase was examined (results not shown). This lack of effect of the PKC- ϵ anti-sense

Figure 6 Effect of down-regulation of PKC subtypes by anti-sense ODNs on the tyrosine phosphorylation of MAP kinase induced by GH

3T3-F442A cells were preincubated with anti-sense (AS) ODNs to the PKC subtype indicated, before the addition of GH (10 nM, 10 min). Cell lysates were prepared and immunoblotted with anti-phosphotyrosine antibodies as described in the Materials and methods section. The relative positions of p44 and p42 MAP kinases are indicated by arrows. The immunoblot shown is representative of three independent experiments.

treatment is therefore in marked contrast with the inhibitory effect that it was observed to exert on the activation of MAP kinase by PMA (Figure 5, top panel). These results demonstrate a role for PKC- δ in the activation of MAP kinases by GH and further show that PKC- α , - γ and - ϵ are not involved in this action of GH. The anti-sense treatments failed to affect the EGF-induced activation of both p42 (Figure 5, bottom panel) and p44 (results not shown) MAP kinases, which is consistent with a similar lack of effect of the depletion of cellular PKC by chronic PMA treatment (Table 1 and Figure 3) [4] and of PKC inhibitors (results not shown) on the activation of MAP kinases by EGF in these cells.

To obtain further evidence that PKC- δ is required for the activation of MAP kinases by GH, samples from cells treated with each of the anti-sense ODNs were immunoblotted with anti-phosphotyrosine antibodies. The activated forms of p42 and p44 MAP kinases are readily detectable by anti-phosphotyrosine immunoblotting of crude cell lysates. Figure 6 shows that GH induced the tyrosyl phosphorylation of both p42 and p44 MAP kinase. Prior treatment with anti-sense ODN to PKC- δ markedly decreased the extent of tyrosyl phosphorylation of these proteins. In contrast, the depletion of PKC- α , - γ or - ϵ failed to affect p42 and p44 MAP kinase tyrosyl phosphorylation significantly.

DISCUSSION

It is now clear that multiple pathways exist to couple receptors with the regulation of the p42 and p44 MAP kinases. Activation of the linear Ras/Raf/mitogen-activated or extracellular signal-regulated kinase kinase (MEK)/MAP kinase cascade can be achieved via the coupling of the Grb2/Sos complex to phosphotyrosyl proteins [13,30,41]. Additionally the MAP kinase signalling cascade is subject to regulatory inputs from the lipid-activated PKC family [22,30,41], the cAMP signalling pathway [30] and sphingolipid-derived signalling pathways [42,43]. Hence the MAP kinases have a key role in the integration of the plethora of different signals to which a cell can be exposed. In this regard there now exists considerable evidence that, in particular, the PKC family of isoforms have an important part in the cross-talk that occurs between different signalling pathways [22,27,44–46] and that the multiple members of this family assume distinct functional roles [21,22,26,29,44,45].

In the present study we have employed two independent approaches to permit an examination of the consequences of cellular depletion of individual subtypes of the phorbol ester-sensitive (conventional and novel) PKC subtypes on the acute activation of MAP kinases by GH. In the first approach, and consistent with the results of others [47], we found that the PKC subtypes expressed in 3T3-F442A preadipocytes display dif-

ferential sensitivities to down-regulation by chronic exposure to phorbol ester. The order of susceptibility to phorbol ester-induced down-regulation was $\text{PKC-}\delta > \text{PKC-}\alpha > \text{PKC-}\gamma > \text{PKC-}\epsilon$. The maximal attenuation of GH-induced activation of MAP kinases, in response to chronic treatment with phorbol ester, occurred at times when more than 99% of the cellular $\text{PKC-}\delta$ was depleted, implying a requirement for $\text{PKC-}\delta$ in this process. However, the partial depletion of other PKC isoforms at these times meant that we could not rule out the participation of other isoforms. Interestingly, a residual activation of MAP kinase by GH was observed after 24 h of pretreatment with PMA (Table 1). That this pretreatment was sufficient to block completely the activation of MAP kinase by acute phorbol ester stimulation (Table 1) strongly suggests that all of the phorbol ester-sensitive PKCs, capable of coupling to the MAP kinase pathway, were depleted at this time point. We therefore conclude that the residual GH effect is attributable to a mechanism that operates independently of the conventional or novel PKCs.

A further mechanism, which could account for the finding that chronic PMA pretreatment abrogates the activation of MAP kinase by GH, is a PMA-induced desensitization of the GH receptor. However, under identical conditions, chronic PMA treatment induced only a minor attenuation of GH-induced receptor tyrosyl phosphorylation, which is unlikely to account for the decrease in MAP kinase activation by GH (N. G. Anderson, unpublished work).

To define the PKC-isoform dependence of MAP kinase activation more precisely, a second approach, involving the use of anti-sense ODNs, was used to achieve a more specific depletion of the PKC subtypes. Our results showed that we could achieve the complete depletion (assessed by Western blotting) of PKCs by using sequence-specific anti-sense ODNs designed to hybridize with the isoform of interest. Importantly, none of the anti-sense treatments significantly affected the expression of the other PKC isoforms. Using this approach we demonstrated that, in response to acute treatment with PMA, only the δ and ϵ isoforms of PKC are capable of coupling to the MAP kinase pathway. Of these two isoforms, only $\text{PKC-}\delta$ depletion blocked the activation of MAP kinases by GH. Furthermore treatment with the corresponding sense ODN, which failed to deplete $\text{PKC-}\delta$, had no effect on MAP kinase activation by GH (results not shown). Importantly, in these cells depletion of PKC isoforms by anti-sense treatment had no effect on the activation of MAP kinases by EGF, in agreement with previous reports showing no requirement for PKC in the activation of MAP kinases by EGF [4]. Hence, at least in 3T3-F442A cells, there exists a marked selectivity of certain PKC isoforms to elicit the activation of MAP kinases. The activation of MAP kinases by agents that signal via PKC involves the novel class of PKC isoforms (δ and ϵ) and not the conventional α and γ PKC subtypes.

To our knowledge, a direct involvement of $\text{PKC-}\delta$ in the regulation of the MAP kinase pathway has not previously been demonstrated. However, this PKC isoform induces gene expression through the TPA response element [28], a response that is known to involve the MAP kinase pathway [10,12,13]. The activation of transcription factors by MAP kinases [10,13,31] is likely to be an important part of the mechanism by which GH promotes the changes in gene expression associated with growth arrest and the early stages of differentiation. MAP kinases are known to be both necessary and sufficient for the cessation of cell growth and differentiation of PC12 cells [12] and are required for the differentiation of 3T3 preadipocytes [14], and S. J. Yarwood, E. Kilgour, E. M. Sale, G. J. Sale and N. G. Anderson, unpublished work). We noted that the depletion of $\text{PKC-}\delta$ by anti-sense ODN increased the rate of growth of 3T3-F442A pre-

adipocytes (S. J. MacKenzie, M. D. Houslay, N. G. Anderson and E. Kilgour, unpublished work), which is consistent with the decrease in growth rate of NIH-3T3 cells observed after over-expression of this PKC isoform [28]. PKC might therefore play a role in mediating the GH-induced growth arrest in 3T3 preadipocytes [2], although this could be attributable to the activation of signalling pathways in addition to the MAP kinase cascade. Hence the role of $\text{PKC-}\delta$ in the propagation of other downstream GH signalling events warrants further investigation.

The question of where PKC feeds into the MAP kinase cascade remains to be resolved. Although Raf-1 can be phosphorylated by $\text{PKC-}\alpha$, $-\beta$ and $-\gamma$ [48,49] it is a poor substrate for $\text{PKC-}\delta$ [48] and it has been reported that phosphorylation of Raf-1 by $\text{PKC-}\alpha$ does not stimulate its ability to phosphorylate MEK [49]. $\text{PKC-}\delta$ lies upstream of Ras, at least with respect to activation of transcription via the TPA response element [28]. The activation of Ras by $\text{PKC-}\delta$ could be achieved via the stimulation of a guanine nucleotide exchange activity or the inhibition of a GTPase-activating factor. The mechanism by which $\text{PKC-}\epsilon$ mediates the activation of MAP kinases is unclear but in PC 12 cells it attenuates their deactivation, suggesting that it might inhibit a MAP kinase phosphatase [50]. Further studies are required to determine whether $\text{PKC-}\epsilon$ induces the phosphorylation and activation of Raf.

We have now demonstrated that $\text{PKC-}\delta$ (the present study) and PI 3-kinase [31] are both required for the full activation of MAP kinases by GH; this, together with the inability of the PI 3-kinase inhibitor wortmannin to affect PMA-induced activation of MAP kinases in 3T3-F442A cells [31], suggests that PI 3-kinase might lie upstream of $\text{PKC-}\delta$ in the GH signalling pathway leading to MAP kinase activation. In this regard $\text{PtdIns}(3,4,5)\text{P}_3$, the product of PI 3-kinase activity, has been shown to activate $\text{PKC-}\delta$ and other PKC isoforms *in vitro* [34,35]. Supporting this idea, a very recent study [51] demonstrated that $\text{PKC-}\delta$ and PI 3-kinase co-associate in cells stimulated with granulocyte/macrophage colony-stimulating factor, another member of the cytokine superfamily.

Neither the down-regulation of PKC isoforms by chronic treatment of cells with phorbol ester (Figure 3) or by anti-sense treatment (Figure 5) nor the inhibition of PI 3-kinase by wortmannin [31] completely blocked MAP kinase activation by GH, suggesting that PKC- and PI 3-kinase-independent mechanisms also exist for MAP kinase activation by GH. Others have reported that GH induces the tyrosine phosphorylation of Shc and its association with Grb2 [52]. It is possible that this provides an additional route for GH to activate MAP kinases, although no information is available about its relative contribution to such a pathway.

In conclusion we have shown that the δ and ϵ isoforms of PKC can mediate activation of the MAP kinase pathway in 3T3-F442A preadipocytes but that only $\text{PKC-}\delta$ is required for the activation of MAP kinases by GH. The mechanism by which $\text{PKC-}\delta$ supports the activation of MAP kinases by GH is at present unknown and is the subject of continuing studies. In addition to its role in promoting the differentiation of preadipocytes, the modulation of adipocyte metabolism and the induction of IGF-1 expression by GH have received particular attention [1]. Because evidence implicates PKC in these responses [53,54], our findings might therefore have relevance in disease states such as diabetes and obesity in which GH is implicated [1] and that involve selective changes in the expression of PKC isoforms [55,56].

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