Signalling through either the p38 or ERK mitogen-activated protein (MAP) kinase pathway is obligatory for phorbol ester and T cell receptor complex (TCR-CD3)-stimulated phosphorylation of initiation factor (eIF) 4E in Jurkat T cells

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Abstract Initiation factor (eIF) 4E plays a key role in the regulation of translation. Its activity is modulated both by phosphorylation and by its association with an inhibitory protein, 4E-BP1, which precludes its interaction with eIF4G. Although increased eIF4E phosphorylation has been correlated with the activation of protein synthesis in T cells, the kinase(s) and/or phosphatase(s) involved have not been characterised. There is evidence for phosphorylation of eIF4E mediated by both protein kinase C-dependent and -independent signalling pathways. In these studies, I show that activation of protein kinase C with phorbol ester, stimulation via the T cell receptor complex with the monoclonal antibody OKT3 and cellular stresses increase the phosphorylation of eIF4E in Jurkat T cells. In contrast to published data, inhibition of either the ERK MAP kinase or p38 MAP kinase signalling pathways does not affect the PMA- or OKT3-stimulated increase in eIF4E phosphorylation. However, simultaneous inhibition of both of these pathways with selective inhibitors is required to completely abrogate the enhanced phosphorylation of eIF4E. These data show that in Jurkat cells, protein kinase C modulates the phosphorylation status of eIF4E indirectly via the ERK and/or p38 MAP kinase signalling pathways.

Key words: Initiation factor; eIF4E; Mitogen-activated protein kinase

1. Introduction

The T cell antigen receptor complex (TCR-CD3) mediates signalling pathways that can result in activation, anergy or apoptosis, the outcome depending upon the T cell differentiation state and the nature of the signals transmitted by co-receptors (reviewed in [1,2]). The TCR is coupled to a protein tyrosine kinase (PTK) cascade that links the TCR-CD3 to at least three signalling cascades: the guanine nucleotide binding protein, *ras*, phospholipase Cγ1 (PLCγ1) and the c-*jun* N-terminal kinase/stress activated protein kinase (JNK/SAPK) [1–4]. Activation of *ras* via signalling through the TCR-CD3 complex plays a central role in the regulation of mitogen-activated protein (MAP) kinases (such as ERK1, ERK2) and JNK/SAPK [1–4]. PLCγ1 controls the metabolism of inositol phospholipids, thereby generating inositol polyphosphates that mobilise intracellular calcium and diacylglycerol that stimulates protein kinase C (PKC) [2]. Synergistic interactions between *ras*, PKC, JNK/SAPK and calcium-controlled signalling pathways are necessary for TCR signal transduction [4]. In many cells, activation of MAP kinases serves as an integration point for both PTK and PKC signalling pathways, although the role of the latter in this response has been the subject of much debate [5].

When T cells are stimulated through the TCR-CD3 complex with a monoclonal antibody (OKT3), or with mitogens such as phorbol esters (PMA, phorbol 12-myristate 13-acetate) and calcium ionophores (ionomycin) which mimic this effect, the rate of protein synthesis is greatly increased and activation of intracellular signalling pathways utilising tyrosine kinases and protein kinase C (PKC) results in increased phosphorylation of eIF4E and stimulated initiation factor complex formation [6,7]. Physiological regulation of protein synthesis is almost always exerted at the level of polypeptide chain initiation, mediated in part by phosphorylation events that modulate the availability of initiation factors to participate in the initiation process (reviewed in [8,9]). The cap structure present at the 5′ end of mRNA facilitates mRNA binding to the ribosome, promoted by at least three initiation factors (eIF4A, 4B, 4F) and ATP hydrolysis [8,9]. eIF4F, a cap binding protein complex composed of three subunits [8–10], is believed to unwind secondary structure in the mRNA 5′ untranslated region to facilitate binding to the 40S ribosomal subunit. Although increased levels of eIF4E phosphorylation correlate directly with enhanced rates of translation in a variety of cell types (reviewed in [8]), it is still not clear how phosphorylation of eIF4E modulates its activity. The major phosphorylation site of eIF4E is serine-209 in vitro and in vivo [11–14], and there is evidence for phosphorylation mediated by both protein kinase C-dependent and -independent pathways [8]. Protein kinase C will phosphorylate eIF4E in vitro at the correct site, albeit at low stoichiometry [15–17]. In addition, co-injection of PKC and eIF4E into quiescent NIH 3T3 cells led to a synergistic effect on eIF4E mitogenic activity [18]. Whilst there may be a direct effect of phosphorylation on cap structure recognition in vitro [19], phosphorylation of eIF4E in vivo can also be correlated with enhanced interaction with other components of the eIF4F complex [6,7,20].

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Abbreviations: eIF, eukaryotic initiation factor; m^7GTP, 7-methylguanosine triphosphate; SDS, sodium dodecyl sulphate; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulphonyl fluoride; PVDF, polyvinylidene difluoride; MOPS, (3-[*N*-morpholino] propanesulfonic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether); PAGE, polyacrylamide gel electrophoresis; VSIEF, vertical slab isoelectric focusing; JNK/SAPK, c-*jun* NH_2-terminal kinase/stress-activated protein kinase; MAP, mitogen-activated protein; 4E-BP, eIF4E binding protein

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Additional eIF4E binding proteins (e.g. 4E-BP1 and 4E-BP2) which interact with eIF4E and inhibit cap structure-dependent translation have been identified as downstream signalling targets [21]. Phosphorylation of 4E-BP1 disrupts its interaction with eIF4E, liberating eIF4E to interact with a conserved hydrophobic region of eIF4G. A similar sequence found in 4E-BP1 is involved in binding to eIF4E and competes with eIF4G for eIF4E binding [22]. Current models suggest that the phosphorylation of 4E-BP1 and consequent liberation of eIF4E lead to the up-regulation of translation [8,21], with association of eIF4E with 4E-BP1 preventing the phosphorylation of eIF4E by protein kinase C in vitro [14]. The phosphorylation of 4E-BP1 is inhibited by the immunosuppressant, rapamycin, which prevents the activation of the 70 kDa ribosomal protein S6 kinase ($p70^{S6K}$) signalling pathway and stabilises the interaction between eIF4E and 4E-BP1 (see references in [8,9,22]). However, rapamycin does not prevent the phosphorylation of eIF4E in primary pig T cells [7], *Xenopus* oocytes [20], CHO.T cells in response to insulin [23] or NIH 3T3 cells in response to serum [24].

In order to understand the mechanism of translational activation in T cells, I have been studying the role of the PKC signalling pathway in the enhanced phosphorylation of eIF4E. In Jurkat cells, the enhanced phosphorylation of eIF4E in response to activation of protein kinase C is facilitated by signalling through the classical MAP kinase and p38 MAP kinase pathways, and does not require dissociation of the eIF4E/4E-BP1 complex. Inhibition of either ERK or p38 MAP kinase activity alone is not sufficient to block eIF4E phosphorylation. However, simultaneous inhibition of both ERK and p38 MAP kinase signalling pathways abrogated the PMA- or OKT3-stimulated phosphorylation of eIF4E. These data indicate that PKC does not directly phosphorylate eIF4E in T cells.

2. Materials and methods

2.1. Chemicals and biochemicals

Materials for tissue culture were from Gibco Life Technologies, Immobilon PVDF was from Millipore and m^7GTP-Sepharose was from Pharmacia-LKB. Microcystin and ionomycin were from Calbiochem and unless otherwise stated, PMA and all other chemicals were from Sigma. Antiserum to eIF4E was as described previously [6,7,20] and antiserum specific for ERK, phospho-ERK, p38 and phospho-p38 were from New England Biolabs. Anti-CD3 monoclonal antibody (OKT3) and rapamycin were kind gifts from Dr. J. Kay (Sussex, UK), PD98059 was from Parke-Davis and SB203580 was a gift from SmithKline Beecham, King of Prussia, PA, USA.

2.2. Tissue culture

Human Jurkat T cells were grown in RPMI 1640 with Glutamax supplemented with 10% foetal calf serum (FCS) as described previously [7]. Cell cultures were incubated for 1 h with vehicle alone or rapamycin, PD98059 or SB203580 as described in individual figure legends, prior to activation.

2.3. Preparation of cell extracts

Following treatment, cells were pelleted in a cooled centrifuge, the medium removed and cells resuspended in 500 μl ice-cold buffer A (50 mM MOPS-KOH, pH 7.4, 2.5 mM EGTA, 1 mM EDTA, 40 mM β-glycerophosphate, 1 μM microcystin, 120 mM NaCl, 7 mM 2-mercaptoethanol, 2 mM benzamidine, 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mM GTP, 2 mM Na_3VO_4). Following a wash in the same buffer, cells were resuspended in 0.1 ml buffer A per 10 ml of original culture and lysed by the addition of 0.5% (by vol.) Nonidet P-40 and vortexing. Cell debris was removed by centrifugation in a microfuge for 5 min at 4°C and the resultant supernatant was frozen in liquid N_2.

2.4. Polyacrylamide gel electrophoresis (SDS-PAGE), vertical slab isoelectric focusing (VSIEF) and immunoblotting

One-dimensional polyacrylamide gels and vertical slab isoelectric focusing gels were run as described [7,20]. Proteins transferred to PVDF and eIFE, ERK, phosphorylated ERK, p38 and phosphorylated p38 MAP kinases were detected with specific rabbit anti-peptide antisera as described in the individual figure legends.

2.5. m^7GTP-Sepharose chromatography

For the isolation of eIF4E and associated proteins, cell extracts of equal protein concentration were subjected to m^7GTP-Sepharose chromatography as described [6,7,20]. The beads were washed three times in buffer A and bound protein eluted with either SDS-PAGE or VSIEF sample buffer, as indicated.

2.6. Assay for protein kinase C activity in cell extracts

For assays of protein kinase C activity, incubations (total volume 20 μl) contained 50 mM MOPS pH 7.2, 5 mM $MgCl_2$, 1 mM dithiothreitol, 40 μg/ml leupeptin, 500 nM PKI (peptide inhibitor of protein kinase A), 2 mM $CaCl_2$, 1 mM EDTA, 100 μM [γ-^{32}P]ATP (16 800 cpm/pmol), 37.5 μg/ml phosphatidylserine, 75 nM PMA and 8 μg of cell extract. Reactions were initiated by the addition of 10 μM of the protein kinase C-specific substrate peptide FKKSFKL (Bachem, Switzerland), designed after the known phosphorylation site of the myristoylated alanine-rich C-kinase substrate protein (MARCKS) [25], in the absence or presence of 400 μM of the protein kinase C-specific pseudosubstrate inhibitor peptide, RFARKGALRQKNVHDVKQ [26] and incubated for 20 min at 30°C. Reactions were moved into ice and stopped with the addition of 10 μl 0.5% (by vol.) phosphoric acid containing 5 mM adenosine. Samples (15 μl) were spotted onto P81 ion-exchange paper, which was washed three times in 0.5% phosphoric acid, once in acetone, dried and counted with liquid scintillant

3. Results and discussion

3.1. PMA and OKT3 stimulate the phosphorylation of eIF4E

Despite growing evidence for a role for phosphorylation of eIF4E in translational control in T cells [6–8], the kinase(s) responsible for enhanced eIF4E phosphorylation in vivo and

Table 1
Incubation with PD98059 and SB203580 does not prevent the activation of protein kinase C

Treatment	Phosphorylation of peptide substrate (pmol/min/ng protein)	Fold increase over unstimulated
Unstimulated	1.5	1
PMA	14	9.3
OKT3	7.6	5
PMA+PD98059+SB203580	10.5	7

Jurkat T cells (10 ml culture) were preincubated with vehicle, or 20 μM SB203580 and 50 μM PD98059 for 1 h, prior to incubation with or without 20 nM PMA or OKT3 for 15 min, as indicated. Cell extracts were prepared and assayed for their ability to phosphorylate a PKC-specific peptide [25], in the absence or presence of a pseudosubstrate peptide inhibitor of PKC [26], as described in Section 2. The activity of PKC assayed by incorporation of [^{32}P]phosphate into the MARCKS peptide is expressed as pmol/min/ng protein after subtraction of PKC activity recovered in the presence of the pseudosubstrate inhibitor. Under these assay conditions, the pseudosubstrate inhibitor peptide decreased the activity of recombinant PKCα by 97% (data not shown).

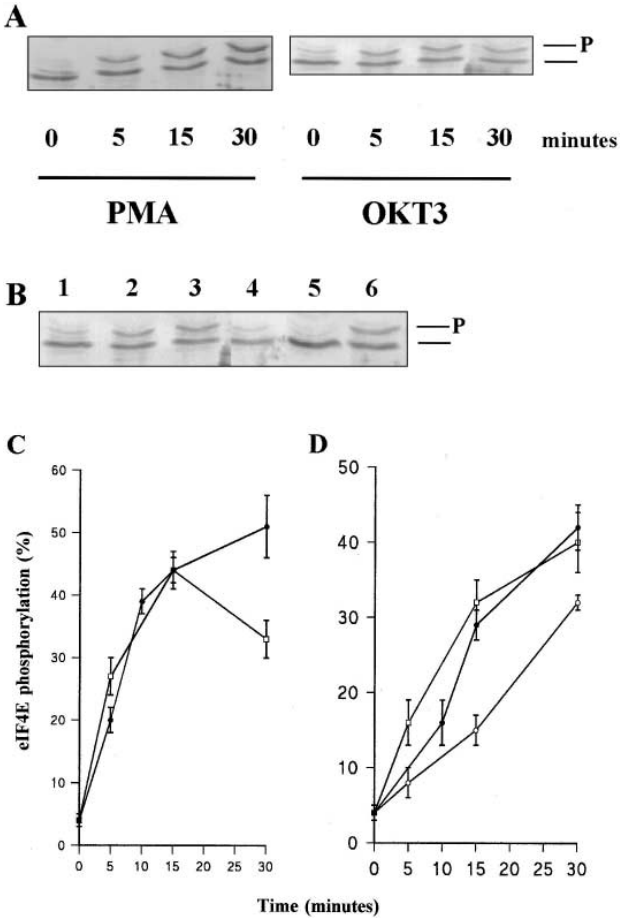

Fig. 1. Phosphorylation of eIF4E is stimulated in response to PMA, OKT3 and cellular stress. A: Jurkat T cells (10 ml culture), grown for 4 days to high density to reduce the background level of eIF4E phosphorylation [7], were incubated in the presence of PMA (20 nM) or OKT3 (5 μg/ml) for the indicated times. Cells were transferred to ice and extracts prepared as described. Total eIF4E was isolated from aliquots (25 μl) of equal protein concentration by m^7GTP-Sepharose chromatography and the phosphorylation status of recovered protein monitored by VSIEF and immunoblotting. The more phosphorylated variant in this gel system is the upper band, as indicated on the right. These data are representative of those obtained in three separate experiments. B: Jurkat T cells (10 ml) were incubated for 15 min without further addition (lane 5), for 5 min in the presence of 5 mM sodium arsenite (lane 1) or 10 μg/ml anisomycin (lane 2), or for 15 min with 20 nM PMA/100 nM ionomycin (lane 3), 0.5M sorbitol (lane 4) or 1 μM okadaic acid (lane 6). Cell extracts were prepared and eIF4E isolated and subjected to VSIEF and immunoblotting as above. These data are representative of those obtained in three separate experiments. C: Jurkat T cells were incubated in the absence or presence of 20 nM PMA (●) or 5 μg/ml OKT3 (□) as described above. At each time point, the percentage of total eIF4E in the phosphorylated form was determined by scanning densitometry. The amount of eIF4E in the phosphorylated form in unstimulated cells at 30 min incubation was 4% (±2%, $n=3$). Presented data are the means and S.D. (bars) of three separate experiments. D: Jurkat T cells were incubated for the indicated times in the absence or presence of 5 mM sodium arsenite (○), 20 nM PMA/100 nM ionomycin (●) or 10 μg/ml anisomycin (□) and the percentage of total eIF4E in the phosphorylated form was determined as above. Presented data are the means and S.D. (bars) of three separate experiments.

the signalling pathways utilised remain elusive. There is, however, evidence for a role of protein kinase C in these processes, as eIF4E is phosphorylated in vivo in response to PMA in reticulocytes, 3T3-L1 cells, T cells and CHO.T cells [6–8,15–17,23], and in vitro by protein kinase C [14–17]. I now report the results of experiments designed to address the question as to whether PKC directly phosphorylates eIF4E in T cells, or whether enhanced phosphorylation of eIF4E is mediated via downstream signalling pathways, particularly those involving members of the MAP kinase family.

To look at the effect of activation of PKC and stimulation of the TCR-CD3 receptor complex on the phosphorylation of eIF4E, Jurkat T cells were incubated with either 20 nM PMA or 5 μg/ml OKT3 (half maximal concentrations of agonist (data not shown)), for various times prior to the preparation of extracts. Total cytoplasmic eIF4E was then isolated by m^7GTP-Sepharose chromatography and bound protein subjected to VSIEF and immunoblot analysis. With this gel system, the upper immunoreactive band represents the more phosphorylated variant of eIF4E [7,14,20,23]. Fig. 1A shows that activation of PKC with 20 nM PMA led to enhanced levels of eIF4E phosphorylation from 3% in control cells to 20% within 5 min, reaching 50% of total eIF4E in the phosphorylated state at 30 min (see Fig. 1C). The response of eIF4E phosphorylation to OKT3 was slightly more rapid (28% at 5 min) and was maximal at 15 min (42%; Fig. 1C). Increased phosphorylation of eIF4E in response to PMA apparently did not require the dissociation of eIF4E from 4E-BP-1 (data not shown), suggesting that association of eIF4E with 4E-BP-1 does not preclude its phosphorylation in vivo. This is contrary to the in vitro studies of Whalen et al. [14], who have shown that association of recombinant 4E-BP1 with eIF4E prevents the phosphorylation of the latter by protein kinase C.

3.2. *Cellular stress and okadaic acid increase the phosphorylation of eIF4E*

In addition to phosphorylation of target proteins that may be attributed directly to it, PKC is also likely to contribute to cellular signalling events through cross-talk with other cascades. These include the activation of the classical (ERK1 and ERK2) MAP kinases, the JNK/SAPK MAP kinase and possibly the p38 MAP kinase family [27]. In fibroblasts, stresses induced by arsenite and sorbitol have been reported to strongly induce p38 MAP kinase but not the classical (ERK1, ERK2) MAP kinases (reviewed in [27] and references therein). To examine the potential role of these MAP kinase signalling pathways in modulating the phosphorylation of eIF4E in T lymphocytes, Jurkat cells were incubated in the absence or presence of 5 mM arsenite, 10 μg/ml anisomycin, 20 nM PMA/100 nM ionomycin, or 0.5 M sorbitol for the times indicated. Extracts were prepared and the phosphorylation status of eIF4E monitored by VSIEF and immunoblotting. Fig. 1B,D shows that arsenite caused an increase in eIF4E phosphorylation from 4% in the control cells (Fig. 1B, lane 5) to 7% in the presence of arsenite at 5 min exposure (lane 1), rising to 31% by 30 min (Fig. 1D). PMA/ionomycin increased the phosphorylation of eIF4E to 42% at 30 min (Fig. 1B, lane 3; Fig. 1D), whilst anisomycin resulted in a more rapid increase in eIF4E phosphorylation at early times of incubation (Fig. 1B, lane 2; Fig. 1D). Under these conditions, sorbitol had little effect on the phosphorylation of eIF4E at early times of incubation (Fig. 1B, lane 4). The phosphatase inhibitor, okadaic acid [28,29] was found to increase eIF4E phosphorylation to a level similar to that caused

by PMA (Fig. 1B, lane 6), suggesting that phosphate turnover is important in modulating the level of eIF4E in the phosphorylated form in these cells.

3.3. The PMA-induced phosphorylation of eIF4E involves both ERK and p38 MAP kinase pathways

Previous studies have shown that treatment of T cells with PMA or activation of the TCR-CD3 complex predominantly results in the activation of the classical MAP kinase, ERK2 [5,30]. In numerous cell types, activation of ERK1 and ERK2 is prevented by the specific upstream (MEK1) kinase inhibitor, PD98059 (reviewed in [31]). In addition, PMA/ionomycin or OKT3 and CD28 cause a transient activation of JNK/SAPK which is required for full T cell activation [4], an effect not observed with PMA alone [30]. Similar activation of JNK/SAPK has been reported for γ-radiation [32] and antioxidant treatment of T cells [33]. Arsenite, bacterial lipopolysaccharide, heat shock and interleukin-1 predominantly activate the p38 MAP kinase pathway [27,31], which itself leads to the activation of a family of downstream kinases [31,34]. Recently, it has been demonstrated that platelet-derived growth factor [35] can activate p38 MAP kinase in airway smooth muscle cells and that T cell proliferation in response to interleukins 2 and 7 requires p38 MAP kinase activation [36]. At least two of the isoforms of the p38 MAP kinase family are selectively inhibited by the compound SB203580 [31,34,37,38]. Although PMA results in a modest activation of some members of this family of kinase [38,39], the physiological relevance of this is not known at this time.

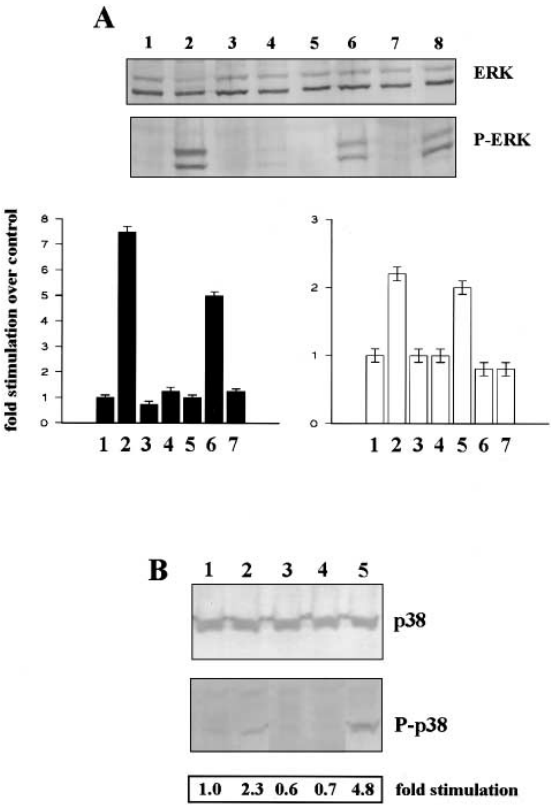

Fig. 2. PMA and OKT3 enhance the phosphorylation of p38 MAP kinase. A: Jurkat T cells (10 ml culture) were preincubated for 1 h with vehicle alone (lane 1), 50 μM PD98059 alone (lane 5), 20 μM SB203580 alone (lanes 3, 6) or 50 μM PD98059 and 20 μM SB203580 (lanes 4, 7), prior to the addition of vehicle (lane 1), 20 nM PMA (lanes 2, 5–7) or 1 μM okadaic acid (lane 8) for 15 min. Extracts were prepared and aliquots (2 μl for ERK; 4 μl for p38 MAP kinase) of equal protein concentration were subjected to SDS-PAGE. ERK1 and ERK2 in the non-phosphorylated form (ERK) or phosphorylated on tyrosine 204 (P-ERK) were visualised by immunoblotting using specific antiserum (upper panel). The level of ERK in the phosphorylated (and hence activated form) was quantified as described above and expressed as fold increase over that present in non-stimulated cells (lower left, filled bars). In a similar manner, p38 and p38 phosphorylated on threonine-180/tyrosine-182 were visualised by SDS-PAGE and immunoblotting (data not shown) and the results are quantified in the lower right panel (open bars). Presented data are the means and S.D. (bars) of three separate experiments. In both cases, these phosphorylation events correspond to catalytic activation of ERK and p38 MAP kinases [27]. B: Jurkat T cells (10 ml culture) were preincubated for 1 h with vehicle alone (lane 1), or 20 μM SB203580 (lanes 3, 4), prior to the addition of vehicle (lane 1), 5 μg/ml OKT3 (lanes 2, 4) or 10 μg/ml anisomycin (lane 5) for 10 min. Extracts were prepared and aliquots (4 μl) of equal protein concentration were subjected to SDS-PAGE and total p38 (upper panel) and phosphorylated p38 (lower panel) visualised as in A. These data are representative of those obtained in four separate experiments.

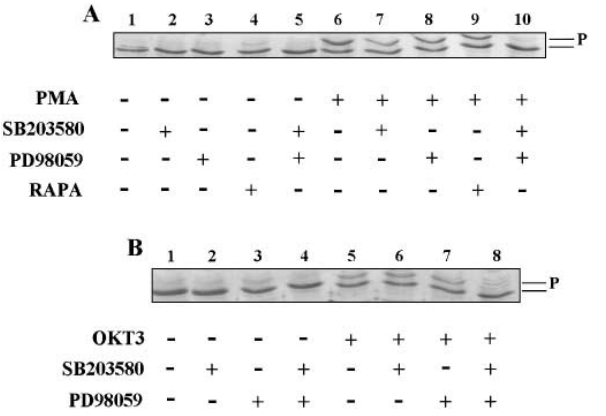

Fig. 3. Protein kinase C does not directly phosphorylate eIF4E in Jurkat T cells. A: Jurkat T cells (10 ml culture) were preincubated with vehicle (−), 20 μM SB203580, 50 μM PD98059 or 20 nM rapamycin for 1 h, prior to incubation with or without 20 nM PMA for 30 min, as indicated. Cell extracts were prepared and aliquots of equal protein concentration (25 μl) were subjected to m^7GTP-Sepharose chromatography to isolate total eIF4E. The recovered protein was subjected to VSIEF and eIF4E visualised by immunoblotting; the more phosphorylated variant (P) is indicated on the right. These data are representative of those obtained in three separate experiments. B: Jurkat cells (10 ml culture) were preincubated in the absence or presence of inhibitors, as described above, and incubated in the absence or presence of 5 μg/ml OKT3 for 15 min prior to preparation of extracts. eIF4E was isolated and visualised as described in A. These data are representative of those obtained in three separate experiments.

In order to determine whether PKC was directly or indirectly stimulating the phosphorylation of eIF4E in Jurkat T cells, the selective inhibitors PD98059, SB203580 and the phosphatase inhibitor okadaic acid [28,29] were employed. Activation of ERK1, ERK2 and p38 kinases (Fig. 2A) was monitored by SDS-PAGE and immunoblotting using tyrosine phosphorylation-specific antisera to these proteins. A parallel immunoblot with an antiserum which recognises total ERK or p38 MAP kinase protein was included as a control to show similar loading of cell lysates. Fig. 2A shows that, relative to unstimulated cells (lane 1), treatment with PMA (lane 2), or okadaic acid (lane 8) resulted in a large increase (7.5-fold) in the tyrosine phosphorylation (and hence activation) of ERK1 and ERK2 kinases [34,36,39]. In addition, PMA (lane 2) led

to a small (2.2-fold), but reproducible activation of p38 MAP kinase (quantified in Fig. 2A, right panel). With PMA, the activation of ERK kinase was severely inhibited by preincubation of cells with the MEK1 inhibitor, PD98059 (lane 5), and, in agreement with published data [31,34,38] ERK kinase activation was less sensitive to SB203580 (lane 6). Addition of either SB203580 (lane 3) or SB203580 and PD98059 (lane 4) in the absence of PMA had little effect on ERK or p38 MAP kinase activity. Addition of both PD98059 and SB203580 to cells prior to activation with PMA inhibited the activation of ERK and p38 MAP kinases (lane 7). Similar results were obtained when ERK kinase activity was monitored using immunocomplex assays with a synthetic peptide as substrate (data not shown). Fig. 2B shows the effect of stimulation via the TCR-CD3 complex on the activation of p38 MAP kinase, monitored by SDS-PAGE and immunoblotting using phosphorylation-specific antiserum. As with PMA (Fig. 2A), relative to unstimulated cells (Fig. 2B, lane 1), treatment with OKT3 (lane 2) resulted in over a 2-fold increase in phosphorylation (and hence activation) of p38 MAP kinase. Under similar conditions anisomycin (lane 5) induced a 4.8-fold increase in p38 MAP kinase activation. The selectivity of SB203580 was confirmed by the finding that it prevented the basal level of activation of p38MAP kinase (lane 3), activation observed in the presence of OKT3 (lane 4), and the p38-mediated phosphorylation of MAPKAP-K2 and HSP27 ([31], data not shown).

To look at the effect of inhibition of these MAP kinase signalling pathways on the ability of PMA to stimulate the phosphorylation of eIF4E, cells were preincubated with or without inhibitor prior to addition of PMA for 30 min. Cell extracts were prepared, eIF4E isolated by m^7GTP-Sepharose chromatography and the phosphorylation status of recovered eIF4E monitored by VSIEF. Fig. 3A shows that as expected, the phosphorylation of eIF4E was enhanced in response to PMA (compare lanes 1 and 6). This PMA-increased phosphorylation of eIF4E was unaffected by pretreatment with SB203580, PD98059 or rapamycin alone (lanes 7–9). The lack of effect of rapamycin on the phosphorylation of eIF4E is consistent with studies utilising primary pig T cells [7], *Xenopus* oocytes [20], CHO.T cells in response to insulin [23] or NIH 3T3 cells in response to serum [24,40]. However, the PMA-stimulated phosphorylation of eIF4E was completely abrogated by the simultaneous inhibition of the ERK and p38 MAP kinase signalling pathways (lane 10). The inhibitors alone did not affect the phosphorylation status of eIF4E (Fig. 3A, lanes 2–5) and only preincubation with rapamycin affected the association of eIF4E with its inhibitory partner, 4E-BP1 (data not shown).

As stimulation of the TCR-CD3 complex also resulted in the enhanced phosphorylation of eIF4E, I have analysed the effect of inhibition of the ERK and p38 MAP kinase pathways in this response. As shown in Fig. 3B, increased phosphorylation of eIF4E in response to OKT3 (compare lanes 1 and 5) was unaffected by preincubation of cells with SB203580 or PD98059 alone (lanes 6 and 7). However, the OKT3-stimulated increase in phosphorylation of eIF4E was completely abrogated when signalling through both the ERK and p38 MAP kinases was inhibited (compare lanes 5 and 8). As shown with PMA (Fig. 2A), preincubation of cells with these inhibitors prior to stimulation with OKT3 prevented the activation of ERK and p38 MAP kinases (Fig. 2B).

These data suggest that PKC is indirectly involved in the phosphorylation of eIF4E. Whilst unlikely to affect the activity of PKC alone [31,34,38], an alternative explanation is that SB203580 and PD98059 together inhibit PKC activity in the cell. To discount this possibility, PKC activity was assayed in cell extracts using a PKC-specific peptide substrate designed after the known phosphorylation site of the MARCKS [25]. To further address the specificity of this substrate peptide for PKC, reactions were carried out in the presence of a PKC-specific pseudosubstrate inhibitor peptide, which prevents both autophosphorylation and substrate phosphorylation by PKC in vivo [26]. Table 1 shows that treatment of Jurkat cells with PMA for 15 min resulted in a 9.3-fold increase in PKC-specific phosphorylation of the peptide substrate, whilst OKT3 induced a 5-fold activation. Preincubation of cells with PD98059 and SB203580 for 1 h prior to stimulation with PMA resulted in activation of protein kinase C to 75% of the level observed in the absence of inhibitors. These data suggest that the combination of PD98059 and SB203580 did not prevent the activation of total PKC in these cells, although specific effects on individual isotypes of PKC cannot be discounted.

These data indicate that activation of protein kinase C results in increased levels of eIF4E phosphorylation, which occurs indirectly via the ERK and p38 MAP kinase signalling pathways. Similar results have been obtained upon PMA stimulation of primary human T cells (M. Bushell and S. Morley, unpublished data) and *Xenopus* kidney cells in culture (C. Fraser and S. Morley, unpublished data). Recently, PMA has been shown to activate p38 MAP kinase in a cytokine-dependent T cell line [36], transfected COS-7 [37] and HeLa cells [39] to a level similar to that described for Jurkat cells (Fig. 2A). In all cases, this may be mediated by common kinase(s) and/or phosphatase(s) which integrate multiple extracellular signals necessary for the activation of ternary complex factors, c-*fos* transcription [31,41] and the enhanced phosphorylation of eIF4E [31,40]. Such would be consistent with the recent report that Mnk1/Mnk2 (MAP kinase-interacting kinases 1 and 2), which are downstream of multiple MAP kinase pathways, mediate the insulin-induced and stress-induced phosphorylation of eIF4E [42,43], with Mnk1 able to phosphorylate eIF4E at the correct site in vitro [42]. Whilst phorbol ester increased the activity of Mnk1 expressed in 293 cells [42], this was prevented by either PD98059 or SB203580 alone. In HeLa cells, phorbol ester-stimulated activity of expressed Mnk1 was largely prevented by inhibition of the ERK MAP kinase pathway alone [43]. However, in Jurkat cells stimulated with either PMA, inhibition of both the ERK and p38 MAP kinase signalling pathways was required to abrogate eIF4E phosphorylation in vivo (Fig. 3). These data suggest that PMA activates both the ERK and the p38 MAP kinase pathways in T cells, and that signalling via either is sufficient for the physiological phosphorylation of eIF4E.

At this time, the potential role of Mnk1 in this response is unclear, as eIF4E kinase(s) activity, monitored with recombinant eIF4E protein, was only increased by 1.2-fold with PMA (data not shown). Consequently, this has made any possible biochemical identification of putative eIF4E kinase(s) difficult. The finding that okadaic acid alone induced the phosphorylation of eIF4E to a level similar to PMA (Fig. 1B) suggests that regulation may be directly at the level of phosphatase(s).

Indeed, it has recently been shown that arsenite, acting via the p38 MAP kinase pathway, stimulates AP-1 transcription factor activity by inhibiting a constitutive phosphatase [44]. Alternatively, as okadaic acid has been shown to activate both ERK [27–29] (Fig. 2A) and p38 MAP kinase (data not shown), these data may also reflect effects at the level of Mnk1/Mnk2. The identity of putative eIF4E kinase(s) and phosphatases(s) downstream of the ERK and p38 MAP kinases, and the relative importance of such signalling pathways in T cells is currently under investigation.

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