Opposing roles of serine/threonine kinases MEKK1 and LOK in regulating the CD28 responsive element in T-cells

Li TAO*, Scott WADSWORTH*, Jason MERCER†‡, Cynthia MUELLER†, Kirsten LYNN†, John SIEKIERKA* and Avery AUGUST*†‡¹

*R. W. Johnson Pharmaceutical Research Institute, Drug Discovery Research, Raritan, NJ 08869, U.S.A., †Immunology Research Laboratories and Department of Veterinary Science, The Pennsylvania State University, University Park, PA 16802, U.S.A., and ‡Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA 16802, U.S.A.

T-cell activation requires signals from both the T-cell receptor (TcR) and other co-stimulatory molecules such as CD28. TcR- and CD28-mediated signals are integrated during T-cell activation resulting in the expression of cytokine genes such as interleukin-2 (IL-2). An enhancer element (CD28RE) of the IL-2 gene specifically responsive to CD28 signals has been previously identified and characterized. This response element and an adjacent Activated Protein-1 (nuclear factor-interleukin-2B) site together (RE/AP1) were shown to complex with c-rel, AP-1 and other factors. However, details of the signal transduction pathways leading from CD28 to the composite response element remain poorly understood. We present data showing that overexpression of the serine threonine kinase, mitogen-activated protein kinase/extracellular-signal-regulated kinase kinase-1 (MEKK1), but not nuclear factor- κ B inducing kinase, or MAP kinase/ERK kinase-1 (MEK1), can significantly increase the level of CD28RE/AP1-driven luciferase (Luc) reporter gene expres-

sion in Jurkat E6-1 cells. A MEKK1 dominant negative mutant blocked such activation induced by stimulation with Raji B cells and the superantigen staphylococcus enterotoxin E (SEE), as well as via CD3/CD28. Mutations in either site of the RE/AP1 element abolished MEKK1-induced Luc expression. Calcineurin inhibitors, CsA and FK520, or inhibitors of p38 kinase (SB 203580), or MEK1 (PD 098059), did not affect MEKK1-induced reporter activation. These results directly implicate MEKK1 in the CD28 signalling pathway that activates the CD28 response element. Co-expression of the lymphocyte-oriented kinase (LOK) kinase attenuated Raji/SEE-induced IL-2 production in Jurkat cells, as well as MEKK1 and Raji/SEE-induced reporter gene activation. These data suggest that MEKK1 and LOK may have opposing roles in regulating the CD28RE/AP1 element.

Key words: c-Jun, c-rel, interleukin-2, T-cell activation.

INTRODUCTION

Efficient activation of T-cells by multiple extracellular signals initiates a number of events leading to cytokine production and cell proliferation. It is well established that T-cell activation requires signals from both the T-cell receptor (TcR) and other co-stimulatory molecules [1,2]. A major co-stimulatory molecule, CD28, is a 44 kDa glycoprotein expressed on the surface of most mature T-cells [3]. CD28 signalling is activated by binding to B7 molecules on the surface of antigen-presenting cells. CD28 co-stimulation acts in concert with TcR/CD3 signals to enhance T-cell proliferation and interleukin-2 (IL-2) production, although alone it does not show any measurable effect on these events [4,5]. Despite the important role it plays, little is known about the signalling pathway leading from CD28 to the IL-2 promoter. Several molecules have been implicated as immediate downstream signal mediators. Phosphatidylinositol-3-phosphate kinase (PI3K) and growth-factor-receptor-bound protein 2 (Grb2) have been shown to associate with CD28 via a pYMN motif in the cytoplasmic tail [6–9]. ITK, a T-cell-specific tyrosine kinase, also binds to CD28 directly upon activation [10]. CD28 signalling also activates p38 mitogen-activated protein kinase

(MAPK) [11]. Beyond these molecules, several other molecules such as small GTP-binding proteins also have been implicated in CD28 signalling pathways [12,13].

CD28 co-stimulation upregulates IL-2 production, due to both transcriptional activation of the IL-2 gene and post-transcriptional stabilization of IL-2 mRNA [14]. CD28 signals activate a number of transcription factors such as Activated Protein-1 (AP-1), nuclear factor κ B (NF- κ B) and nuclear factor of activated T-cells (NFATs) to bind to their consensus sequences in the IL-2 promoter [15]. A unique CD28 response element (CD28RE) was first identified as an enhancer element located at –164 to –154 bp of the minimal IL-2 promoter [16]. Full CD28 responsiveness requires both the CD28RE and the adjacent nuclear factor-interleukin-2B (NF-IL-2B) AP-1 site, which form the CD28RE/AP1 composite element [17]. One prominent feature of this unique sequence, as tested by a number of criteria such as stimulation by CD3 and CD28 antibodies, has been its specific response profile upon CD28 stimulation in Jurkat cells [17,18]. Activation of this composite element depends on B7 molecules expressed by antigen-presenting cells, and disruption of CD28-B7 engagement eliminates such activity [19]. Upregulation of the composite element is insensitive to the calcineurin

Abbreviations used: AP-1, Activated Protein-1; CD28RE, CD28 responsive element; Cot, cancer Osaka thyroid; EGFP, enhanced green fluorescent protein; ERK, extracellular signal-regulated kinase; Grb2, growth-factor-receptor-bound protein 2; IKK, I κ B kinase; IL-2, interleukin-2; ITK, inducible T-cell kinase; JNK, c-Jun N-terminal kinase; JNKK, JNK kinase; LOK, lymphocyte-oriented kinase; LOKKinGFP, LOK kinase domain fused to GFP; Luc, luciferase; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MEK1, MAP kinase/ERK kinase-1; MEKK1, MEK kinase-1; MKK, MAPK kinase; NFAT, nuclear factor of activated T-cells; NF- κ B, nuclear factor κ B; NF-IL-2B, nuclear factor-interleukin-2B; NIK, NF- κ B inducing kinase; PAK, p21-activated kinase; PI3K, phosphatidylinositol-3-phosphate kinase; P/I, PMA/ionomycin; RE/AP, responsive element/AP; SEE, staphylococcus enterotoxin E; SEK, stress-activated protein kinase/ERK kinase; TcR, T-cell receptor.

¹ To whom correspondence should be addressed (e-mail axa45@psu.edu).

inhibitor FK506, as is CD28-dependent IL-2 production [17]. Sequence analysis of the CD28RE/AP1 element revealed that it contains poor consensus NF- κ B and AP-1 binding sequences [17], although c-rel and p65, members of the NF- κ B family of transcription factors, and AP-1 have been shown to bind to the respective sites [19–22]. Thus it is likely that the upregulation of the CD28RE/AP1 element depends on both NF- κ B and AP-1 transcription factors.

In T-cells, TcR/CD3 signalling alone strongly activates the Raf $\rightarrow$ MEK1 $\rightarrow$ ERK/MAP kinase pathway (see the abbreviations footnote for definitions of individual kinase abbreviations in these pathways) [23]. However, activation of members of the MEKK1 $\rightarrow$ SEK $\rightarrow$ JNK pathway results from the integration of signals from both the TcR and CD28 as shown by the requirement for both TcR/CD3 and CD28 signalling for activation of JNK [24]. A good candidate for regulating the coordination of TcR/CD3 and CD28 signalling is MEKK1, a 196 kDa serine-threonine protein kinase that activates both JNK and ERK signalling pathways [25]. MEKK1 phosphorylates JNK kinases which leads to phosphorylation and activation of JNKs. JNKs phosphorylate and activate c-jun, a component, along with c-fos, of the AP-1 transcription factor [26,27]. Recently, it has been shown that MEKK1 can also phosphorylate and activate IKK α and IKK β , two kinases of the IKK (I κ B kinase) complex regulating the activation of the NF- κ B transcription factors, suggesting that MEKK1 is involved in regulating multiple downstream signalling pathways [28–30]. It has also been shown that MEKK1 in T-cells is activated by coligation of CD3 and CD28 only [31]. Despite the emerging information suggesting that MEKK1 can be a regulator for multiple signalling cascades, its precise role in CD28 signalling is not yet clear.

A number of mammalian serine/threonine kinases related to the *Saccharomyces cerevisiae* STE20 kinase have recently been discovered. Mammalian STE20 kinases may act upstream of the MAPK kinase $\rightarrow$ MAPK kinase $\rightarrow$ MAPK pathway analogous to the STE20 $\rightarrow$ STE11 $\rightarrow$ STE7 $\rightarrow$ FUS3/KSS1 mating signal pathway in yeast [32,33]. It has been demonstrated that p21-activated kinase (PAK), a STE20 family kinase, is involved in CD3/CD28 signalling [13]. Lymphocyte-oriented kinase (LOK) is a STE20 kinase family member whose expression appears to be restricted to lymphocytes [34]. LOK has a kinase domain in its N-terminus, a proline rich region in the middle and a coiled-coil C-terminus region of unknown function [34]. LOK does not activate MEK, MEKK1 or the p38 pathways *in vitro* and the physiological role of LOK in lymphocytes is unclear.

In order to study the function of MEKK1 and LOK in CD28 signalling, we examined their effect on the CD28RE/AP1 composite response element using a luciferase (Luc) reporter construct. Transient transfections of Jurkat E6-1 cells with MEKK1, LOK and other kinases, suggest that MEKK1 is uniquely able to activate the CD28RE/AP1 element in the absence of any other stimulus. This signalling requires activation of both the AP-1 and the NF- κ B transcription factors via both the MEKK1 $\rightarrow$ JNKK/SEK $\rightarrow$ JNK and MEKK1 $\rightarrow$ IKKs $\rightarrow$ I κ B/NF- κ B signalling cascades. Furthermore, the MEKK1 signal resembles that delivered by stimulation with superantigen-pulsed Raji B cells in activating the response element. Thus MEKK1 likely acts as a signal integrator for the TcR/CD3 and CD28 signalling pathways. We also found that LOK can down-regulate Raji/SEE-induced production of IL-2 by Jurkat cells, as well as MEKK1 and Raji/staphylococcus enterotoxin E (SEE)-mediated activation of the CD28RE/AP1 response element, suggesting that LOK may modify TcR/CD28 signals for activation of cytokine transcription.

MATERIALS AND METHODS

Reagents and antibodies

PMA, ionomycin, the p38 MAP kinase inhibitor SB203580, the MEK1 inhibitor PD098059, cyclosporin A, and FK520 were purchased from Calbiochem (La Jolla, CA). SEE was purchased from Toxin Technology (Sarasota, FL). Anti-human CD28 mAb was purchased from Pharmingen (San Diego, CA).

Plasmids

The CD28RE/AP1 wild type and mutant constructs contained three copies of the following oligos in tandem, cloned into the *Xho*I site of the luciferase reporter plasmid pGL3-Promoter vector (Promega, Madison, WI). The orientation and copy number of the oligos were confirmed by sequencing.

RE/AP:

5' tcgagTTTAAAGAAATTCCAAAGAGTCATCag
cAAATTTCTTTAAGGTTTCTCAGTAGTcagct

REm/AP:

5' tcgagTTTAAAGAccTcgaAAAGAGTCATCag
cAAATTTCTggAgctTTTCTCAGTAGTcagct

RE/APm:

5' tcgagTTTAAAGAAATTCCAAATcaCATCag
cAAATTTCTTTAAGGTTTAggtGTAGTcagct

pFC MEKK, pFC MEK1, p53-luciferase reporter and pFA2-cJun DNA constructs were purchased from Stratagene (La Jolla, CA). The MEKK1 dominant negative construct MEKK1/KR was obtained from Dr Shinya Tanaka (Hokkaido University Graduate School of Medicine, Sapporo, Japan). The NF- κ B-luciferase reporter construct was obtained from Dr T. Ouchi (Mount Sinai Medical Center, New York). The IL-2 promoter/luciferase construct was a kind gift of Dr Mike Lenardo (NIH) via Dr Yong Won Choi (University of Pennsylvania, Philadelphia, PA). The I κ B α and NIK constructs were provided by Mr Haiyun Wang (R. W. Johnson Pharmaceutical Research Institute, Raritan, NJ). The LOK kinase domain DNA was obtained from dbest clone #AA459448 [35], with the DNA insert subsequently subcloned into the pEGFP vector (Clontech, Palo Alto, CA) to make LOK kinase domain fused to enhanced green fluorescent protein (EGFP). The full length flag tagged LOK was a kind gift of Dr H. Karasuyama (The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan).

Transfection and luciferase assay

For each transfection, 5×10^6 Jurkat cells were incubated with the indicated DNA, on ice, for 10 min before electroporation. Cells were electroporated using a BTX Electroporator T820 (BTX, San Diego, CA) at 215V, 1 pulse for 65 ms. After a brief incubation on ice, cells were cultured at 37 °C overnight in RPMI 1640 medium (Gibco BRL) supplemented with 10% foetal bovine serum. For PMA/ionomycin/CD28 stimulation, 24 h post-electroporation, cells were washed and incubated with fresh RPMI medium with PMA (20 ng/ml), ionomycin (1 μ M), and anti-CD28 Ab (10 μ g/ml) for an additional 16 h. Before the luciferase assay, cells were pelleted by centrifugation (400 g), washed and resuspended in PBS. Cells were then lysed with the lysis buffer from the Luciferase Reporter Gene Assay Kit (Packard Instrument Company Meriden, CT) and luciferase activity was measured using a Packard microplate luminescent counter. Stable Jurkat cells were generated by electroporation with plasmid containing the LOK kinase domain fused to green fluorescent protein (GFP), or GFP alone, selected with 2 mg/ml G418 and cloned by GFP expression. Several clones of each type were combined to avoid clone to clone variation.

Raji and SEE stimulation

Jurkat cells were transfected with either CD28RE/AP1-Luc reporter DNA alone, or together with MEKK1/KR (a dominant negative kinase-inactive mutant of MEKK1), or LOK kinase GFP by electroporation as described above. Cells were incubated for 20 h at 37 °C before stimulation with the Raji B cell line and SEE. Raji cells (1:1 with Jurkat cells) and SEE (100 ng/ml) were incubated together for 30 min before adding to the Jurkat cells. After 4, 7 and 22 h, aliquots of the cell mixture were collected, washed and assayed by the same luciferase-assay protocol.

RESULTS AND DISCUSSION

MEKK1 activates the CD28RE/AP1 element

CD28 plays an important co-stimulatory role in helping T-cells to achieve maximum activation and IL-2 production. It is unclear whether the signals from CD28 lead to activation of a new array of signalling molecules that synergise with TcR signals, or simply enhance the existing ones generated from TcR/CD3. To evaluate the role of various candidate signalling molecules in CD28 signalling, we measured expression of a CD28RE/AP1-luciferase reporter construct co-transfected into Jurkat cells with MEKK1 or other MAP kinase pathway serine/threonine kinases.

The expression and function of the CD28RE/AP1 reporter gene was confirmed by transiently transfecting the construct into Jurkat cells and subsequently stimulating with PMA and ionomycin, or PMA, ionomycin and CD28 Ab (Figure 1A). As previously reported [17], activation of the CD28RE/AP1 element required both PMA and ionomycin, and a further increase in luciferase activity was seen upon addition of the CD28 antibody. We then tested the effect of MEKK1 on the CD28RE/AP1 response element. We surmised that MEKK1 may be involved because CD28 signalling activates JNK and MEKK1, a direct upstream activator of JNK [28]. Also, MEKK1 has been shown to activate both the AP-1 and the NF- κ B family of transcription factors [26–30]. When MEKK1 was co-transfected with the CD28RE/AP1-Luc reporter DNA into Jurkat cells, a significant, dose-dependent increase in reporter activity was seen (Figure 1B). Control transfection of MEKK1 with a p53 reporter construct in Jurkat cells did not result in activation of the p53 reporter (results not shown). MEKK1/KR, a kinase-inactive MEKK1 mutant, failed to induce any activity under the same transfection conditions. These data suggest that MEKK1 can upregulate the CD28RE/AP1 element in the absence of any other stimulus and that MEKK1 kinase activity is required for this effect. We also examined whether stimulation of the CD28RE/AP1 element by overexpression of MEKK1 was further stimulated by PMA/ionomycin (P/I) and/or CD28 Ab. We transfected lower amounts of MEKK1 plasmid, which by itself caused little increase in CD28RE/AP1 activity, then stimulated with P/I or P/I plus anti-CD28 antibodies. As seen with the response element only, MEKK1 at this low level enhances P/I, and P/I and CD28 induced activation of the CD28RE/AP1 suggesting that MEKK1 signals can mimic the CD28 signal (Figure 1C).

A MEKK1 dominant negative mutant blocks activation of the CD28RE/AP1 by physiological stimulation with Raji/SEE stimulation

If MEKK1 is an important molecule in the CD28 to CD28RE/AP1 signalling pathway, then a kinase-inactive MEKK1 mutant should be able to affect the ability of CD28 to activate the CD28RE/AP1 reporter. Due to the poorly defined and po-

Figure 1 Ectopic expression of MEKK1 can activate the CD28RE/AP1 element in Jurkat E6-1 cells

(A) A representative control experiment showing that the reporter gene was expressed and functional. The CD28RE/AP1-Luc vector that contains three copies of the response element, was transfected into Jurkat E6-1 cells by electroporation. Cells were then stimulated with 20 ng/ml PMA (P), 1 μ M ionomycin (I) and 10 μ g/ml of CD28 mAb (CD28Ab) as indicated 24 h post-transfection. After 8–16 h, cells were lysed and assayed for luciferase activity. (B) Kinase active pFC-MEKK1 (●) or kinase inactive MEKK1/KR (○) were transfected together with the reporter construct. After 20–24 h, cells were lysed and assayed for luciferase expression. (C) Jurkat cells transfected with/without pFC-MEKK1 (0.3 μ g) were stimulated 24 h post transfection with 20 ng/ml PMA (P), 1 μ M ionomycin (I) and 10 μ g/ml of CD28 mAb (CD28Ab) for 16 h before being assayed. Figures represent one of three independent experiments.

tentially non-physiological nature of the P/I signals in this pathway, we used a more physiological stimulation of T-cells, the Raji B cell line and the superantigen SEE as the stimulus to examine the role of MEKK1 in activating the CD28RE/AP1 element. This represents a more physiologically relevant system for activating T-cells than using antibodies CD3 and CD28, or P/I. The kinase-inactive mutant MEKK1/KR construct was co-transfected with the reporter gene into Jurkat cells and 24 h post-transfection, cells were stimulated with Raji/SEE for another 4–22 h. As shown in Figure 2(A), incubation of Jurkat transfectants with Raji/SEE activated the CD28RE/AP1 reporter gene, and the kinase-inactive MEKK1/KR mutant consistently blocked 30–50 % of such activity over several time points while control vectors showed no inhibition (results not shown). This result strongly suggests that MEKK1 is involved in the

Figure 2 A MEKK1 dominant negative mutant blocks activation of the CD28RE/AP1 reporter or IL-2 promoter reporter induced by Raji/SEE or CD3/CD28

(A) CD28RE/AP1-Luc (○, ●) alone or CD28RE/AP1-Luc plus 4 μ g MEKK1/KR DNA (▽, ▼) were transfected into Jurkat cells as described in Figure 1. After 20–24 h, cells were aliquoted and incubated either with (●, ▼) or without Raji (1:1) and SEE (100 ng/ml) (○, ▽) for an additional 4–22 h before luciferase assay. Figures represent one of three independent experiments. (B) CD28RE/AP1-Luc was transfected into Jurkat T cells alone (white bars) or along with MEKK1/KR (black bars). After 18 h, cells were stimulated with antibodies to CD3 and CD28 for an additional 8 h, then harvested for luciferase assay. (C) IL-2-promoter-Luc was transfected into Jurkat T-cells alone (white bars) or along with MEKK1 (black bars). After 20–24 h, cells were harvested for luciferase assay. (D) IL-2-promoter-Luc was transfected into Jurkat T-cells alone (white bars) or along with MEKK1/KR (black bars). 20–24 h later, cells were stimulated with antibodies to CD3 and CD28 for an additional 8 h, then harvested for luciferase assay. DN, dominant negative.

upregulation of CD28RE/AP1 by a CD28-activating signal for two reasons. Firstly, the Raji B cell/SEE system provides the Jurkat T-cells with two physiological activating signals (antigen/MHC [TcR/CD3] and B7 molecules [CD28]), which have been shown to induce IL-2 production by Jurkat cells [18,36], results not shown). Secondly, activation of the CD28RE/AP1 by Raji/SEE is CD28-specific [18]. To confirm that MEKK1 could regulate the ability of TcR/CD28 signals to activate the IL-2 gene, we tested whether MEKK1/KR could also block CD3/CD28-induced activation of the CD28RE/AP1. We transfected Jurkat T-cells with the CD28RE/AP1-luciferase alone or in the presence of MEKK1/KR. Cell cultures were split 18 h later, when aliquots were either left unstimulated, or stimulated with antibodies against the CD3 and CD28 receptors. Luciferase activity was analysed 8 h later (Figure 2B). As seen in Figure 2(B), the expression of MEKK1/KR inhibited the ability of CD3 and CD28 antibodies to activate the CD28RE/AP1 reporter construct. We also wanted to find out if like the CD28RE/AP1, MEKK1 could regulate the full IL-2 promoter. We therefore transfected Jurkat T-cells with the IL-2-promoter driving a luciferase gene alone, or with MEKK1, and measured luciferase activity 24 h later. Figure 2(C) shows that transfection of MEKK1 alone can cause activation of transcription from the IL-2 promoter. To find out if MEKK1 could affect CD3/CD28-induced activation of this IL-2 promoter reporter, we transfected the IL-2 promoter-luciferase construct into Jurkat cells in the presence or absence of MEKK1/KR and stimulated cells with antibodies to CD3 and CD28. Figure 2(D) demonstrates that

MEKK1/KR also inhibits the ability of CD3/CD28 antibodies to activate the IL-2 promoter. In the latter 3 sets of experiments, P/I induced at least 20-fold induction of the respective reporter plasmids, indicating that these cells were still capable of responding (results not shown). These results on the role of MEKK1 in CD28 signalling are consistent with results showing that MEKK1/KR can inhibit the induction of endogenous IL-2 message by anti-CD3 and CD28 antibodies in Jurkat cells, and also consistent with results from knockout mice lacking molecules downstream of MEKK1, such as c-rel, SEK1 and JNK2, all having significant defects in IL-2 production [31,37–39].

Activation of CD28RE/AP1 by MEKK1 depends on both NF- κ B and JNK pathways

We next examined the downstream pathways that MEKK1 may regulate in order to activate the CD28RE/AP1 reporter. MEKK1 has been well characterized as an upstream activator of the JNKK/SEK $\rightarrow$ JNK pathway, which leads to activation of the AP-1 transcription factor [26,28]. AP-1 has been implicated as the protein factor that recognizes the AP-1 (NF-IL-2B) site of the CD28RE/AP1 element [20]. MEKK1 can also activate the NF- κ B transcription factor via direct phosphorylation of IKK α and IKK β in the IKK complex [30]. Furthermore, c-rel and p65 of the NF- κ B family can bind to the CD28RE site [19]. Since the CD28RE/AP1 element contains sequences similar to both the NF- κ B and the AP-1 consensus binding sites, and requires both sites for TcR/CD28 responsiveness [17], we tested whether

Figure 3 Activation of CD28RE/AP1 by MEKK1 requires both the NF- κ B and AP-1 sites; MEKK1 is the major MAP kinase that can activate the CD28RE/AP1 element

(A) Cells transfected with either NF- κ Bm/AP-1 or NF- κ B/AP-1m reporters, which are unresponsive to PMA/ionomycin/CD28 stimulation, are also not activated by pFC-MEKK1 (3 μ g). (B) Co-expression of I κ B blocks MEKK1-induced CD28RE/AP1 reporter expression. 15 μ g of I κ B α DNA was co-transfected with 5 μ g of pFC-MEKK1 into 15×10^6 Jurkat cells. (C) Expression of a mutant c-jun blocks CD28RE/AP1 expression induced by pFC-MEKK1. 12 μ g of pFA-c-jun was co-transfected into 5×10^6 Jurkat cells with 1 μ g MEKK1. (D) Jurkat cells were transfected with the reporter construct and pFC-MEKK1 (4.5 μ g) for 16 h before treatment with the respective compounds. The compounds were added for 6 h, and cells were lysed and assayed for luciferase activity. Controls are Jurkat cells transfected with CD28RE/AP1-Luc reporter DNA only. Results are expressed as % MEKK1-induced activation. (E) The NIK kinase construct was transfected with either CD28RE/AP1-Luc or NF- κ B-Luc into Jurkat cells. Luciferase assay was carried out 24 h post-transfection. DN, dominant negative.

MEKK1, like a TcR/CD28 signal, is also dependent on both pathways to activate the CD28RE/AP1 element. Consistent with this model, we found that mutations in either the NF- κ B site or the AP-1 site rendered the composite element unresponsive to MEKK1 in the co-transfection assay (Figure 3A). This suggests that both sequences of the composite element indeed are required for the upregulation of CD28RE/AP1 activity induced by MEKK1 or CD28 signalling. Using dominant negative mutants, we then tested individual molecules from either the NF- κ B or the JNK pathway for their involvement in MEKK1-induced reporter

gene expression. Overexpression of I κ B α , which leads to sequestration of NF- κ B transcriptional factors in the cytoplasm, blocked MEKK1-induced CD28RE/AP1 expression (Figure 3B). IKK α K44R, an IKK α mutant which blocks CD28RE/AP1 activation by PMA and CD28 Ab [40], inhibited approx. 40 % of the MEKK1-induced activation (results not shown). Overexpression of a c-jun mutant construct (pFA2-cJun), which contains the JNK activation site but no DNA binding site and competes with the endogenous c-jun for upstream activation signals, also blocked MEKK1-induced CD28RE/AP1 activity (Figure 3C).

Although it is unclear whether there is any co-operativity between the transcription factors in binding to the composite element, as mutation in one site should not affect factors binding to the other, it is clear that activation of this composite element by either TcR/CD28 signals or overexpression of MEKK1 requires co-operation between signals from the NF- κ B and the JNK-AP1 pathways. The inhibition of MEKK1-induced activation of the CD28RE/AP by these factors was not due to apoptosis or cell cycle effects as no differences in these parameters were observed in cells transfected with these reagents (results not shown).

MEKK1 activation of the CD28RE/AP1 element does not require MEK1, p38, NF- κ B inducing kinase (NIK), or calcineurin

At least three MAP kinase pathways, Raf $\rightarrow$ MEK1 $\rightarrow$ ERK, MEKK1 $\rightarrow$ SEK/JNKK $\rightarrow$ SAPK/JNK and MEKK3 $\rightarrow$ MKK3/6 $\rightarrow$ p38, have been characterized in numerous cellular signalling systems. However, only the MEKK1 $\rightarrow$ JNKK/SEK $\rightarrow$ JNK/SAPK pathway has been implicated in CD28 costimulation of IL-2 production. To test the specificity of the MEKK1 induction of the CD28RE/AP1 response element, we compared activation by transfected MEK1 and MEKK1 in Jurkat cells. The MEK1 construct, which activates the ERKs, failed to induce a significant increase in CD28RE/AP1 reporter gene activity (results not shown). Furthermore PD 098059, a known MEK1 inhibitor, had no effect on activation of CD28RE/AP1 reporter activity by MEKK1 (Figure 3D).

Since MEKK1 can, under certain conditions, activate other MAPK pathways [41], we further tested the effect of specific kinase inhibitors on MEKK1-induced CD28RE/AP1 reporter gene expression. The p38 MAPK has been shown to be activated by CD28 signalling [11], but the specific p38 inhibitor SB203580 at 1 μ M (sufficient for the inhibition of p38 in cell) [11], had no significant effect on MEKK1-induced CD28RE/AP1 reporter activity, consistent with its inability to inhibit IL-2 production in response to TcR/CD28 stimulation [11]. Taken together, our findings suggest that ectopic expression of MEKK1 mimics CD28RE/AP1 activation by TcR/CD28, which proceeds in part via downstream activation of JNK (MEKK1 $\rightarrow$ JNKK/SEK $\rightarrow$ JNK/SAPK), rather than via Raf $\rightarrow$ MEK1 $\rightarrow$ ERK or MEKK3 $\rightarrow$ MEK3/6 $\rightarrow$ p38. The data also suggest that MEKK1 is more likely to be the molecule that integrates TcR and CD28 signals, as p38 α is activated by CD28 signalling alone [11], and the MEK1 $\rightarrow$ ERK pathway is strongly activated by TcR/CD3 signalling alone [23].

It has been shown that activation of the CD28RE/AP1 in response to TcR/CD28 stimulation is relatively insensitive to calcineurin inhibitors such as FK506 and CsA, although some other regulatory elements of the IL-2 gene are calcineurin-dependent [17,42]. As shown in Figure 3(E), both inhibitors failed to block the MEKK1-induced reporter gene activation. The results suggest again that activation of CD28RE/AP1 by ectopic expression of MEKK1 mimics the TcR/CD28 activation of CD28RE/AP1 in that it proceeds via a pathway parallel to that of calcineurin, although we can not rule out that calcineurin may act upstream of MEKK1.

NIK is a MAP3 kinase that can activate a NF- κ B reporter gene upon TNF α or IL-1 stimulation when overexpressed [43]. Although NIK has been shown to be a potent activator of both IKK α and IKK β in the IKK complex, it does not appear to activate the JNK pathway [44]. We found that while NIK alone weakly activated a NF- κ B-Luc reporter in Jurkat cells, NIK could not activate the CD28RE/AP1 composite element (Figure 3E), and in fact, inhibited CD28RE/AP1-Luc reporter gene

expression. This may have been due to a feedback inhibition caused by NIK-induced transcription of endogenous I κ B, as observed by others [45].

Our results with NIK differ in detail from Kempiaik et al. [46] who suggested that NIK could weakly activate the CD28RE/AP1. This discrepancy may be due to differences in transfection efficiencies of the NIK expression vectors used, or in the timing of stimulation and analysis of expression. Although overexpression experiments must be interpreted with caution, our results, and those previously reported both suggest that MEKK1 is a much more efficient activator of the CD28RE/AP1 reporter than NIK [46]. Minimal activation by NIK of the CD28RE/AP1 composite element is consistent with our response element mutagenesis data, which suggest that full activation of CD28RE/AP1 requires strong activation of both NF- κ B and AP-1 (Figure 3A). Nel and colleagues have also reported that MEKK1 is involved in TcR/CD28 induction of IL-2 production as the MEKK1/KR mutant can inhibit the production of IL-2 message when Jurkat cells are stimulated with anti-CD3 and CD28 [31]. In addition, they have recently shown that the JNK pathway activated by MEKK1 can activate the CD28 responsive element [46]. Thus MEKK1 may be a highly efficient activator because of its ability to simultaneously activate both the JNKK $\rightarrow$ JNK $\rightarrow$ AP-1 pathway and the IKK $\rightarrow$ I κ B/NF $\rightarrow$ κ B pathway.

Although our results support the notion that MEKK1 is a key molecule that upregulates the CD28RE/AP1 element, it may not be the only factor. As our data show, the CD28RE/AP1 element can be activated by PMA and ionomycin alone, in the absence of CD28 antibody (Figure 1A). Also, MEKK1 can enhance PMA- and ionomycin-induced reporter gene expression in MEKK1-transfected Jurkat cells (Figure 1C). These results suggest that activation of the element can be controlled by multiple pathways including signals from protein kinase C and Ca²⁺. The fact that the MEKK1 dominant negative mutant displayed only a partial blockade of CD28RE/AP1 activation upon B-cell/SEE stimulation suggests that there are multiple pathways leading from CD28 to the CD28RE/AP1 element, a consensus supported by various published results [31,40,46,48]. Mice deficient in SEK1, a direct substrate of MEKK1, retain some ability to make IL-2 upon CD28 co-stimulation, albeit at much reduced levels [37]. A report by Barz et al. [47] suggests that CD28-mediated IL-2 production may require at least three signals: JNK activation, recruitment of PI3K, and a third unknown signal. Molecules that directly regulate the CD28RE/AP1 response element may be activated by a number of upstream signals, just as MEKK1 is able to activate multiple downstream kinases. Most recently, it has been shown that another MAP3 kinase, cancer Osaka thyroid (Cot) kinase, can activate the CD28RE/AP1 in Jurkat cells [48], presumably by activating both the JNK and NF- κ B pathways. Indeed, we have recently found that Cot kinase can enhance MEKK1-mediated CD28RE/AP1 activity, as can an isoform of protein kinase C, PKC- θ (results not shown).

LOK down-regulates MEKK1-mediated CD28RE/AP1 activity and endogenous interleukin-2 production

In searching for molecules that may regulate MEKK1-induced CD28RE/AP1 activation, we tested a number of kinases including the recently discovered lymphocyte-specific LOK kinase [34]. LOK is a STE20-like kinase expressed exclusively in T- and B-lymphocytes [34]. The tissue specificity of this kinase implies its biological function in T-cell signalling, development, or differentiation. The N-terminal kinase domain of LOK shares similarity with other STE20 kinase family members such as haematopoietic

Figure 4 Leukocyte-oriented kinase (LOK) down-regulates MEKK1-mediated or Raji/SEE-induced CD28RE/AP1 reporter activity and endogenous interleukin-2 production

(A) Jurkat cells were transfected with the CD28RE/AP1-Luc reporter gene alone or with pFC-MEKK1 (2.5 μ g), LOKK (10 μ g) or both MEKK1 plus LOKK or full length LOK. LOK alone had no effect on the CD28RE/AP1 (results not shown). Luciferase activity was assayed 24 h post-transfection. Results are expressed as % MEKK1-induced activation. (B) Jurkat cells transfected with the reporter alone or co-transfected with LOK kinase vector were incubated with Raji (1:1) and SEE (100 ng/ml) 24 h post-transfection. Luciferase activity was assayed at 7 h post-stimulation. Figures represent three independent experiments. (C) LOK down-regulates antigen-induced IL-2 production. Jurkat cells stably expressing LOKkinGFP or GFP alone were stimulated with Raji cells & SEE or PMA/Ionomycin for 18 h, supernatants harvested and tested for IL-2 by specific ELISA.

progenitor kinase 1 ('HPK1') and germinal centre kinase ('GCK'). Although LOK is a novel serine/threonine kinase that can autophosphorylate and phosphorylate substrates such as myelin basic protein and histone IIA protein, published data show that wild-type LOK is unable to activate all three known MAP kinase cascades in transfection assays [34]. When we co-transfected MEKK1, and an expression vector containing the kinase domain and the proline rich region of the human LOK gene into Jurkat cells, we found that the presence of either the LOK kinase domain and the proline rich region or the full length LOK significantly decreased MEKK1-induced CD28RE/AP1 reporter activity (Figure 4A). LOK kinase domain or full

length LOK had no effect on CD28RE/AP1 reporter activity alone (results not shown). Further experiments demonstrated that the proline rich region of LOK was not required for this effect (results not shown). To further demonstrate that LOK interferes with the activation of CD28RE/AP1 by CD28 signalling, we used the Raji/SEE system to stimulate LOK-transfected Jurkat cells. The LOK transfectants showed a 30–50% reduction in luciferase activity when stimulated by Raji/SEE as compared to control cells transfected with a β -Gal vector (Figure 4B). As seen with the MEKK1 kinase-inactive mutant, LOK also decreased MEKK1-mediated activation of both AP1 and NF- κ B reporter gene expression independently (L. Tao and A. August, unpublished data). To determine if LOK could regulate the endogenous IL-2 locus, we generated stable lines of Jurkat T-cells expressing a fusion of the kinase domain and proline rich region of LOK to green fluorescent protein (GFP) to make LOKkinGFP. Cells were stimulated with SEE and Raji cells and the production of endogenous IL-2 measured. We also stimulated them with the pharmacological activators PMA and ionomycin, which can bypass the immediate events in T-cell activation. Figure 4C shows that cells that stably express LOKkinGFP secreted less than 60% of the IL-2 compared to GFP transfected cells. By contrast, there was no significant difference in IL-2 production when the cells were stimulated with PMA and ionomycin to bypass the early events in T-cell activation, indicating that LOK may act on early events in T-cell activation (Figure 4C).

Based on these results, we propose that LOK negatively regulates the MEKK1 pathway, either by direct or indirect interaction with MEKK1. Although it has been shown that STE20 kinases tend to activate the JNK/SAPK pathway [49, 50], results from a recent publication suggest that JIK, a novel STE20 kinase family member, inhibits epidermal growth factor-mediated activation of JNK/SAPK [51]. It is likely that some mammalian STE20 kinases upstream of MEKK1 may actually have dual regulatory functions. Further characterization of LOK and its role in regulating T-cell activation pathways leading to the CD28RE/AP1 element are underway.

We thank Dr Crafford Harris for critically reading the manuscript. We also thank other members of the Immunosuppression Team of the R. W. Johnson Pharmaceutical Research Institute for their technical assistance and Dr Hajime Karasuyama for the kind gift of the flag tagged full length LOK.

REFERENCES

- Chambers, C. and Allison, J. (1997) Co-stimulation in T-cell responses. *Curr. Opin. Immunol.* **9**, 396–404
- Fraser, J., Straus, D. and Weiss, A. (1993) Signal transduction events leading to T-cell lymphokine gene expression. *Immunol. Today* **14**, 357–362
- Lenschow, D., Walunas, T. and Bluestone, J. (1996) CD28/B7 system of T-cell co-stimulation. *Annu. Rev. Immunol.* **14**, 233–258
- Schwartz, R. (1992) Co-stimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. *Cell* **71**, 1065–1068
- Harding, F., McArthur, J., Gross, J., Raulet, D. and Allison, J. (1992) CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature (London)* **356**, 607–609
- Prasad, K., Cai, Y., Raab, M., Duckworth, B., Cantley, L., Shoelson, S. and Rudd, C. (1994) T-cell antigen CD28 interacts with the lipid kinase phosphatidylinositol 3-kinase by a cytoplasmic Tyr(P)-Met-Xaa-Met motif. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2834–2838
- August, A. and Dupont, B. (1994) CD28 of T lymphocytes associates with phosphatidylinositol 3-kinase. *Int. Immunol.* **6**, 769–774
- Truitt, K., Hicks, C. and Imboden, J. (1994) Stimulation of CD28 triggers an association between CD28 and phosphatidylinositol 3-kinase in Jurkat T-cells. *J. Exp. Med.* **179**, 1071–1076
- Schneider, H., Cai, Y., Prasad, K., Shoelson, S. and Rudd, C. (1995) T-cell antigen CD28 binds to the GRB-2/SOS complex, regulators of p21ras. *Eur. J. Immunol.* **25**, 1044–1050

- 10 August, A., Gibson, S., Kawakami, Y., Kawakami, T., Mills, G. and Dupont, B. (1994) CD28 is associated with and induces the immediate tyrosine phosphorylation and activation of the Tec family kinase ITK/EMT in the human Jurkat leukemic T-cell line. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9347–9351
- 11 Schafer, P., Wadsworth, S., Wang, L. and Siekierka, J. (1999) p38 alpha mitogen-activated protein kinase is activated by CD28-mediated signaling and is required for IL-4 production by human CD4+CD45RO+ T-cells and Th2 effector cells. *J. Immunol.* **162**, 7110–7119
- 12 Kaga, S., Ragg, S., Rogers, K. and Ochi, A. (1998) Stimulation of CD28 with B7-B2 promotes focal adhesion-like cell contacts where Rho family small G proteins accumulate in T cells. *J. Immunol.* **160**, 24–27
- 13 Kaga, S., Ragg, S., Rogers, K. and Ochi, A. (1998) Activation of p21-CDC42/Rac-activated kinases by CD28 signaling: p21-activated kinase (PAK) and MEK kinase 1 (MEKK1) may mediate the interplay between CD3 and CD28 signals. *J. Immunol.* **160**, 4182–4189
- 14 Umlauf, S., Beverly, B., Kang, S., Brorson, K., Tran, A. and Schwartz, R. (1993) Molecular regulation of the IL-2 gene: rheostatic control of the immune system. *Immunol. Rev.* **133**, 177–197
- 15 Jain, J., Loh, C. and Rao, A. (1995) Transcriptional regulation of the IL-2 gene. *Curr. Opin. Immunol.* **7**, 333–342
- 16 Fraser, J., Irving, B., Crabtree, G. and Weiss, A. (1991) Regulation of interleukin-2 gene enhancer activity by the T-cell accessory molecule CD28. *Science* **251**, 313–316
- 17 Shapiro, V., Truitt, K., Imboden, J. and Weiss, A. (1997) CD28 mediates transcriptional upregulation of the interleukin-2 (IL-2) promoter through a composite element containing the CD28RE and NF-IL-2B AP-1 sites. *Mol. Cell. Biol.* **17**, 4051–4058
- 18 Shapiro, V., Mollenauer, M. and Weiss, A. (1998) Nuclear factor of activated T-cells and AP-1 are insufficient for IL-2 promoter activation: requirement for CD28 up-regulation of RE/AP. *J. Immunol.* **161**, 6455–6458
- 19 Ghosh, P., Tan, T., Rice, N., Sica, A. and Young, H. (1993) The interleukin 2 CD28-responsive complex contains at least three members of the NF kappa B family: c-Rel, p50, and p65. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1696–1700
- 20 McGuire, K. and Iacobelli, M. (1997) Involvement of Rel, Fos, and Jun proteins in binding activity to the IL-2 promoter CD28 response element/AP-1 sequence in human T cells. *J. Immunol.* **159**, 1319–1327
- 21 Lai, J., Horvath, G., Subleski, J., Bruder, J., Ghosh, P. and Tan, T. (1995) RelA is a potent transcriptional activator of the CD28 response element within the interleukin 2 promoter. *Mol. Cell. Biol.* **15**, 4260–4271
- 22 Butscher, W., Powers, C., Olive, M., Vinson, C. and Gardner, K. (1998) Coordinate transactivation of the interleukin-2 CD28 response element by c-Rel and ATF-1/CREB2. *J. Biol. Chem.* **273**, 552–560
- 23 Weiss, A. and Littman, D. (1994) Signal transduction by lymphocyte antigen receptors. *Cell* **76**, 263–274
- 24 Su, B., Jacinto, E., Hibi, M., Kallunki, M., Karin, M. and Ben-Neriah, Y. (1994) JNK is involved in signal integration during costimulation of T lymphocytes. *Cell* **77**, 727–736
- 25 Schlesinger, T., Fanger, G., Yujiri, T. and Johnson, G. (1998) The TAO of MEKK. *Front. Biosci.* **3**, D1181–D1186
- 26 Whitmarsh, A. and Davis, R. (1996) Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J. Mol. Med.* **74**, 589–607
- 27 Karin, M., Liu, Z. and Zandi, E. (1997) AP-1 function and regulation. *Curr. Opin. Cell. Biol.* **9**, 240–246
- 28 Zandi, E., Rothwarf, D., Delhase, M., Hayakawa, M. and Karin, M. (1997) The I kappa B kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for I kappa B phosphorylation and NF-kappa B activation. *Cell* **91**, 243–252
- 29 Lee, F., Hagler, J., Chen, Z. and Maniatis, T. (1997) Activation of the I kappa B alpha kinase complex by MEKK1, a kinase of the JNK pathway. *Cell* **88**, 213–222
- 30 Lee, F., Peters, R., Dang, L. and Maniatis, T. (1998) MEKK1 activates both I kappa B kinase alpha and I kappa B kinase beta. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 9319–9324
- 31 Faris, M., Kokot, N., Lee, L. and Nel, A. (1996) Regulation of interleukin-2 transcription by inducible stable expression of dominant negative and dominant active mitogen-activated protein kinase kinase kinase in Jurkat T cells. Evidence for the importance of Ras in a pathway that is controlled by dual receptor stimulation. *J. Biol. Chem.* **271**, 27366–27373
- 32 Leberer, E., Dignard, D., Marcus, D., Thomas, D. and Whiteway, M. (1992) The protein kinase homologue Ste20p is required to link the yeast pheromone response G-protein beta gamma subunits to downstream signalling components. *EMBO J.* **11**, 4815–4824
- 33 Ramer, S. and Davis, R. (1993) A dominant truncation allele identifies a gene, STE20, that encodes a putative protein kinase necessary for mating in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 452–456
- 34 Kuramochi, S., Moriguchi, T., Kuida, K., Endo, J., Semba, K., Nishida, E. and Karasuyama, H. (1997) LOK is a novel mouse STE20-like protein kinase that is expressed predominantly in lymphocytes. *J. Biol. Chem.* **272**, 22679–22684
- 35 Boguski, M., Lowe, T. and Tolstoshev, C. (1993) dbEST—database for “expressed sequence tags”. *Nat. Genet.* **4**, 332–333
- 36 Fraser, J., Newton, M. and Weiss, A. (1992) CD28 and T-cell antigen receptor signal transduction coordinately regulate interleukin 2 gene expression in response to superantigen stimulation. *J. Exp. Med.* **175**, 1131–1134
- 37 Nishina, H., Bachmann, M., Oliveira-dos-Santos, A., Kozieradzki, I., Fischer, K., Odermatt, B., Wakeham, A., Shahinian, A., Takimoto, H., Bernstein, A., Mak, T., Woodgett, J., Ohashi, P. and Penninger, J. (1997) Impaired CD28-mediated interleukin 2 production and proliferation in stress kinase SAPK/ERK1 kinase (SEK1)/mitogen-activated protein kinase kinase 4 (MKK4)-deficient T lymphocytes. *J. Exp. Med.* **186**, 941–953
- 38 Kontgen, F., Grumont, R., Strasser, A., Metcalf, D., Li, R., Tarlinton, D. and Gerondakis, S. (1995) Mice lacking the c-rel proto-oncogene exhibit defects in lymphocyte proliferation, humoral immunity, and interleukin-2 expression. *Genes Dev.* **9**, 1965–1977
- 39 Sabapathy, K., Hu, Y., Kallunki, T., Schreiber, M., David, J., Jochum, W., Wagner, E. and Karin, M. (1999) JNK2 is required for efficient T-cell activation and apoptosis but not for normal lymphocyte development. *Curr. Biol.* **9**, 116–125
- 40 Harhaj, E. and Sun, S. (1998) I kappa B kinases serve as a target of CD28 signaling. *J. Biol. Chem.* **273**, 25185–25190
- 41 Yujiri, T., Sather, S., Fanger, G. and Johnson, G. (1998) Role of MEKK1 in cell survival and activation of JNK and ERK pathways defined by targeted gene disruption. *Science* **282**, 1911–1914
- 42 June, C., Bluestone, J., Nadler, L. and Thompson, C. (1994) The B7 and CD28 receptor families. *Immunol. Today* **15**, 321–331
- 43 Malinin, N., Boldin, M., Kovalenko, A. and Wallach, D. (1997) MAP3K-related kinase involved in NF-kappaB induction by TNF, CD95 and IL-1. *Nature (London)* **385**, 540–544
- 44 Song, H., Regnier, C., Goeddel, D. and Rothe, M. (1997) Tumor necrosis factor (TNF)-mediated kinase cascades: bifurcation of nuclear factor-kappaB and c-jun N-terminal kinase (JNK/SAPK) pathways at TNF receptor-associated factor 2. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 9792–9796
- 45 Scott, M., Fujita, T., Liou, H., Nolan, G. and Baltimore, D. (1993) The p65 subunit of NF-kappa B regulates I kappa B by two distinct mechanisms. *Genes Dev.* **7**, 1266–1276
- 46 Kempf, S., Hiura, T. and Nel, A. (1999) The Jun kinase cascade is responsible for activating the CD28 response element of the IL-2 promoter: proof of cross-talk with the I kappa B kinase cascade. *J. Immunol.* **162**, 3176–3187
- 47 Barz, C., Nagel, T., Truitt, K. and Imboden, J. (1998) Mutational analysis of CD28-mediated costimulation of Jun-N-terminal kinase and IL-2 production. *J. Immunol.* **161**, 5366–5372
- 48 Lin, X., Cunningham, Jr, E. T., Mu, Y., Geleziunas, R. and Greene, W. (1999) The proto-oncogene Cot kinase participates in CD3/CD28 induction of NF-kappaB acting through the NF-kappaB-inducing kinase and I kappa B kinases. *Immunity* **10**, 271–280
- 49 Diener, K., Wang, X., Chen, C., Meyer, C., Keesler, G., Zukowski, M., Tan, T. and Yao, Z. (1997) Activation of the c-Jun N-terminal kinase pathway by a novel protein kinase related to human germinal center kinase. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 9687–9692
- 50 Hu, M., Qiu, W., Wang, X., Meyer, C. and Tan, T. (1996) Human HPK1, a novel human hematopoietic progenitor kinase that activates the JNK/SAPK kinase cascade. *Genes Dev.* **10**, 2251–2264
- 51 Tassi, E., Biesova, Z., Fiore, P. D., Gutkind, J. and Wong, W. (1999) Human JIK, a novel member of the STE20 kinase family that inhibits JNK and is negatively regulated by epidermal growth factor. *J. Biol. Chem.* **274**, 33287–33295

Received 10 December 2001/4 January 2002; accepted 28 January 2002