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Activation of MEK-1 and SEK-1 by Tpl-2 proto-oncoprotein, a novel MAP kinase kinase kinase

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The Tpl-2 protein serine/threonine kinase was originally identified, in a C-terminally deleted form, as the product of an oncogene associated with the progression of Moloney murine leukemia virus-induced T cell lymphomas in rats. The kinase domain of Tpl-2 is homologous to the *Saccharomyces cerevisiae* gene product, STE11, which encodes a MAP kinase kinase kinase. This suggested that Tpl-2 might have a similar activity. Consistent with this hypothesis, immunoprecipitated Tpl-2 and Tpl-2ΔC (a C-terminally truncated mutant) phosphorylated and activated recombinant fusion proteins of the mammalian MAP kinase kinases, MEK-1 and SEK-1, *in vitro*. Furthermore, transfection of Tpl-2 into COS-1 cells or Jurkat T cells, markedly activated the MAP kinases, ERK-1 and SAP kinase (JNK), which are substrates for MEK-1 and SEK-1, respectively. Tpl-2, therefore, is a MAP kinase kinase which can activate two MAP kinase pathways. After Raf and Mos, Tpl-2 is the third serine/threonine oncoprotein kinase that has been shown to function as a direct activator of MEK-1.

Keywords: ERK/MAP kinase/oncogene/SAP kinase/Tpl-2

Introduction

One of the major signalling pathways by which growth factors induce cell proliferation and differentiation involves the activation of the extracellular-signal regulated kinases (ERKs), ERK-1 and ERK-2 (Marshall, 1994). ERK-1 and ERK-2 are proline direct kinases which phosphorylate serine or threonine residues in the motif P/LXT/SP (Alvarez *et al.*, 1991). Substrates for these kinases include cytosolic phospholipase A (Lin *et al.*, 1993), the kinases p90rsk (Erikson, 1991) and MAPKAP kinase-2 (Stokoe *et al.*, 1992), and a small family of Ets domain transcription factors, which include Elk-1, SAP-1 and SAP-2 (Hill and Treisman, 1995). The phosphorylation of transcription factors by ERKs suggests that these kinases may provide a cytoplasmic link between the growth factor receptors at the cell surface and transcriptional regulation in the nucleus. Consistent with this hypothesis, activated ERKs can enter the nucleus (Lenormand *et al.*, 1993). Furthermore, genetic evidence in *Drosophila melanogaster* strongly suggests that several transcription factors are targets of receptor tyrosine kinase-mediated activation of ERKs (Wassarman *et al.*, 1995).

Mammalian ERKs are activated by phosphorylation on a threonine and a tyrosine residue by the dual specificity protein kinases, MAPK/ERK kinases 1 and 2 (MEK-1/2), which are themselves activated by phosphorylation (Marshall, 1994). Two MEK kinases (MEK K) have been identified, namely the proto-onco-proteins c-Raf (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992) and c-Mos (Nebreda *et al.*, 1993). Raf is activated in response to growth factor stimulation of protein tyrosine kinase receptors (Marshall, 1995). This involves the Ras-dependent translocation of Raf to the plasma membrane (Leevers *et al.*, 1994) where it is then fully activated by another tyrosine-kinase generated signal (Marais *et al.*, 1995).

The Ras/Raf/MEK/ERK pathway is one example of a mitogen-activated protein kinase (MAP kinase, MAP K) pathway (see Figure 8). These comprise a three-component protein kinase cascade of a serine/threonine protein kinase (MAP kinase kinase kinase, MAP KKK) which phosphorylates and activates a dual-specificity protein kinase (MAP kinase kinase, MAP KK), which in turn phosphorylates and activates another serine/threonine protein kinase (MAP K). MAP K cascades have been identified in *Saccharomyces cerevisiae* (Herskowitz, 1995), *Caenorhabditis elegans* (Kayne and Sternberg, 1995) and *D.melanogaster* (Wassarman *et al.*, 1995) which mediate diverse aspects of cellular function. These include the regulation of cell shape, osmotic integrity and pheromone responses in yeast, the regulation of vulval cell development in *C.elegans* and of eye development in *D.melanogaster*.

The second characterized mammalian MAP K pathway is activated in response to 'stressful' stimuli (e.g. heat shock, translational inhibitors and tumour necrosis factor) and also to certain mitogens (e.g. EGF) (Cano and Mahadevan, 1995). Stress-activated protein kinase (SAP K, also known as JNK) is analogous to ERK-1/2 (Derijard *et al.*, 1994; Kyriakis *et al.*, 1994), SEK-1 (also known as JNK kinase and MKK4) is analogous to MEK-1/2 (Sanchez *et al.*, 1994; Derijard *et al.*, 1995; Lin *et al.*, 1995) and MEK K-1 is analogous to Raf (Minden *et al.*, 1994; Yan *et al.*, 1994). Present data suggest that the ERK-1 and SAP K pathways are parallel and independent of one another. Thus, MEK does not activate SAP K and, conversely, SEK-1 does not activate ERK. Similarly, Raf is not able to activate SEK-1 and *in vivo* MEK K-1 is apparently specific for SEK-1 (Minden *et al.*, 1994; Yan *et al.*, 1994) when expressed at low levels, although MEK K-1 will activate MEK-1 *in vitro* (Lange-Carter *et al.*, 1993). Similar to the ERK MAP kinases, the two substrates of SAP K that have been identified are the transcription factors c-Jun (Hibi *et al.*, 1993; Su *et al.*, 1994) and ATF2

(Gupta *et al.*, 1994). Thus the SAP K pathway may also be involved in the transmission of signals from the cell surface to the nucleus.

Recently, a third mammalian MAP K has been identified, p38 or RK, which is homologous to the *S.cerevisiae* HOG1 kinase that is required for yeast cells to grow at high osmolarity (Han *et al.*, 1994; Rouse *et al.*, 1994). The mammalian p38/RK MAP kinase is activated by high osmolarity, lipopolysaccharide and interleukin 1. Two proteins have been reported to act as MAP KKs for p38, namely MKK4 (the human homologue of the murine SEK-1 protein) and MKK3 (Derijard *et al.*, 1995; Lin *et al.*, 1995). The identity of the MAP KKK for the p38 MAP K pathway is not known but does not appear to be MEK K-1.

The Tpl-2 proto-oncogene encodes a protein serine/threonine kinase which is activated by provirus insertion in Moloney murine leukemia virus (MoMuLV)-induced T cell lymphomas during the late stages of oncogenesis (Patriotis *et al.*, 1993). The provirus integrates into the last intron of the Tpl-2 gene which results in the enhanced expression of a truncated Tpl-2 mRNA transcript which encodes a protein that is altered at its C-terminus (Makris *et al.*, 1993). The proto-oncogene Tpl-2 is expressed primarily in the spleen, thymus, liver and lung, and its expression in spleen cells is elevated after concanavalin A stimulation (Patriotis *et al.*, 1993). Tpl-2 may therefore be involved in regulating the transition of resting T cells into the cell cycle. Tpl-2 is homologous in its kinase domain to that of the *S.cerevisiae* gene product, STE11 (Rhodes *et al.*, 1990; Errede and Levin, 1993), which is a MAP KKK involved in the regulation of response to pheromones. This homology suggested that Tpl-2 might also act as a MAP KKK. In this study, this possibility has been investigated by testing whether Tpl-2 can activate the mammalian MAP KKs, MEK-1 and SEK-1, which regulate the ERK and SAP K MAP kinases, respectively.

Results

Full-length rat Tpl-2 is >90% identical to the proto-oncogene products of human and murine Cot (Miyoshi *et al.*, 1991; Aoki *et al.*, 1993; Ohara *et al.*, 1993). Protein homology searches (data not shown) revealed that Tpl-2 and Cot kinases are next most closely related to STE11 (Rhodes *et al.*, 1990; Errede and Levin, 1993), a MAP KKK which regulates the response to pheromones in *S.cerevisiae*. Surprisingly, this homology was not noticed when Tpl-2 cDNA was originally cloned (Patriotis *et al.*, 1993). Tpl-2 is also homologous to several other MAP KKKs [murine MEK K-1 (Lange-Carter *et al.*, 1993), *Schizosaccharomyces pombe* BYR2 (Wang *et al.*, 1991) and *S.cerevisiae* BCK1 (Lee and Levin, 1992)]. The homology between Tpl-2 and STE11 is restricted to the kinase domain (32% identical, 54% similar), with the longest stretches of homology encompassing subdomains 4–6b (42% identical, 65% similarity) and subdomains 8 and 9 (45% identical and 62% similar). This similarity raised the possibility that Tpl-2 might function as a MAP KKK.

Activation of ERK-1 by Tpl-2 in transfected COS-1 cells

To investigate whether Tpl-2 could regulate the ERK-1 MAP kinase pathway, COS-1 cells were transiently transfected with expression vectors containing Tpl-2 cDNA or a C-terminally truncated Tpl-2 cDNA (Tpl-2ΔC) and a haemagglutin (HA) epitope-tagged human ERK-1 cDNA. Tpl-2ΔC is deleted at a homologous point to the oncogenic form of human Cot (Miyoshi *et al.*, 1991). After 36 h of culture, ERK-1 was immunoprecipitated from lysed cells via its epitope tag and kinase activity was assayed using myelin basic protein (MBP) as a substrate. Expression of either the wild-type or truncated forms of Tpl-2 resulted in strong activation of the co-transfected ERK-1 (Figure 1a). The level of activation of ERK-1 by Tpl-2ΔC was of the same order as that achieved with a constitutively active Raf mutant, Raf(CAAX) (Leevers *et al.*, 1994). However, in contrast to wild-type Tpl-2, c-Raf-1 overexpression in COS-1 cells had little effect on the activity of ERK-1.

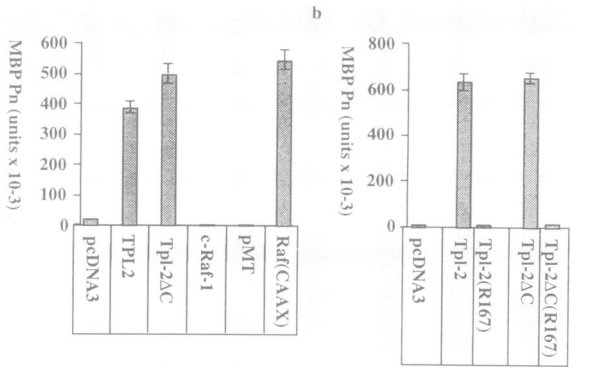

Fig. 1. Activation of ERK-1 by Tpl-2 in transfected COS-1 cells. (**a** and **b**) Lysates were prepared from COS-1 cells 36 h after transfection with plasmids encoding HA epitope-tagged ERK-1 together with the kinases indicated or control empty vectors (pcDNA3 or pMT). Tpl-2ΔC encodes a C-terminally deleted form of Tpl-2 which stops at residue 387, homologous to the human Cot oncogene product. Raf(CAAX) encodes a plasma membrane targeted mutant of c-Raf-1, which is constitutively active. Tpl-2(R167) and Tpl-2ΔC(R167) are kinase-dead mutants of Tpl-2 and Tpl-2ΔC, respectively. ERK-1 was immunoprecipitated from cell lysates and assayed for its ability to phosphorylate MBP. Results shown have been normalized against the amount of ERK-1 in immunoprecipitates and are expressed in arbitrary units. Data are the mean (± standard error) of triplicate assays. Western blot analysis confirmed the expression of the transfected proteins and in (b) that the point mutants Tpl-2(R167) and Tpl-2ΔC(R167) were expressed at comparable levels with their wild-type partners (data not shown).

Kinase-inactive point mutants of Tpl-2 and Tpl-2ΔC were generated by PCR, in which the lysine (residue 167) at the ATP binding site was mutated to arginine. Transfection of COS-1 cells with cDNAs encoding these mutants of Tpl-2, Tpl-2(R167) or Tpl-2ΔC(R167), resulted in no activation of co-transfected HA–ERK-1 (Figure 1b). These data indicate that the activation of ERK-1 by Tpl-2 required its kinase activity.

Activation of ERK-1 by Tpl-2 is independent of Ras and Raf

Although sequence homology suggested that Tpl-2 might directly activate MEK by acting as a MAP KKK, it was formally possible that the activation of ERK-1 by Tpl-2 was mediated via Ras and/or Raf (see Introduction). In this section the possibility was investigated that Ras or Raf might contribute to the activation of ERK-1 by Tpl-2.

To determine whether Ras was required for ERK-1 activation by transfected Tpl-2, COS-1 cells were co-

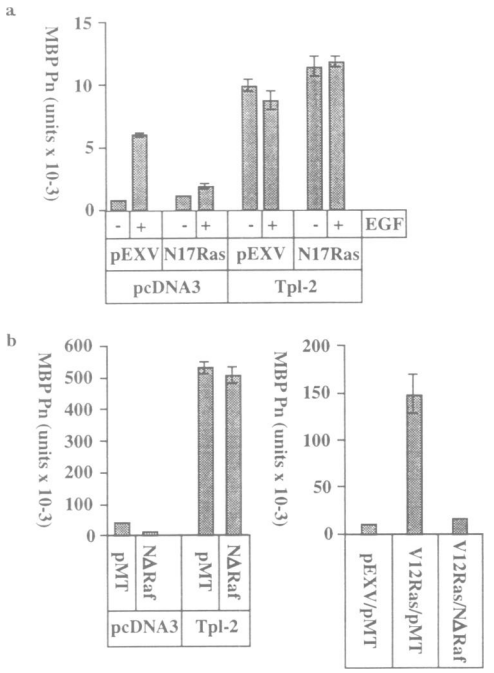

Fig. 2. Effect of dominant-negative mutants of Ras and Raf on the activation of ERK-1 by Tpl-2. (**a**) COS-1 cells were co-transfected with plasmids encoding HA-tagged ERK-1 (3 μg) together with Tpl-2 (1 μg) or the empty vector, pcDNA3 (1 μg) and either the control empty vector, pEXV (4 μg) or pEXV-N17Ras (4 μg). Prior to cell lysis, COS-1 cells were stimulated with EGF for 15 min or left unstimulated. Transfected ERK-1 was purified from cell lysates by immunoprecipitation and assayed for its ability to phosphorylate MBP as in Figure 1. The decreased MBP phosphorylation in this experiment, compared with Figure 1, was due to the dramatically reduced expression of transfected ERK-1 protein. This resulted from the transfection of COS-1 cells with a triple vector combination rather than the double vector transfections of the experiments in Figure 1. Additionally, the pEXV vector was found to decrease the expression of co-transfected proteins from the pcDNA3 vector. (**b**) To test the effect of dominant-negative Raf expression on the activation of ERK-1 by Tpl-2 (left-hand graph), COS-1 cells were co-transfected with plasmids encoding HA–ERK-1 (3 μg), Tpl-2 (1 μg) and NΔRaf or control empty pMT vector (5 μg). To confirm that the NΔRaf construct was active (right-hand graph), COS-1 cells were co-transfected with plasmids encoding HA–ERK-1 (3 μg), V12Ras (1 μg) and either NΔRaf or control empty pMT vector (5 μg). ERK-1 was immunoprecipitated from cell lysates and tested for its ability to phosphorylate MBP as in Figure 1.

transfected with cDNAs encoding a dominant-negative allele of Ras, N17Ras (Feig and Cooper, 1988), together with Tpl-2 and HA–ERK-1 cDNAs. Co-expression of N17Ras did not inhibit the activation of ERK-1 by Tpl-2 (Figure 2a). In contrast, the activation of ERK-1 after EGF stimulation was strongly inhibited by expression of N17Ras, as expected (Howe *et al.*, 1992). Thus, Tpl-2 mediated activation of ERK-1 in transfected COS-1 cells was independent of Ras activity.

The possible role of c-Raf-1 in the activation of ERK-1 by transfected Tpl-2 in COS-1 cells was investigated by co-transfecting a dominant-negative Raf-1 mutant, NΔRaf (Schaap *et al.*, 1993) which encodes the N-terminal regulatory domain which interacts with Ras. Expression of NΔRaf did not inhibit the activation of ERK-1 by Tpl-2 (Figure 2b). In contrast, the activation of ERK-1 by an activated Ras mutant, V12Ras (Howe *et al.*, 1992; Leevers and Marshall, 1992), was strongly inhibited by NΔRaf, confirming that the latter acted in a dominant-negative fashion. Co-expression of a kinase inactive Raf-1 mutant, Raf-1(K373W), also had no inhibitory effect on ERK-1 activation by Tpl-2 (data not shown). These experiments indicated that the activation of ERK-1 by Tpl-2 was independent of c-Raf-1 activity.

Activation of SAP K in cells transfected with Tpl-2

To investigate whether Tpl-2 could activate the MAP K pathway which leads to the activation of SAP K, COS-1 cells were co-transfected with plasmids encoding Tpl-2 or Tpl-2ΔC and an HA epitope-tagged p54β SAP K. After 36 h of culture, the transfected SAP K was immunoprecipitated via its epitope tag and tested for its ability to phosphorylate a glutathione *S*-transferase (GST)–Jun fusion protein *in vitro*. Transfection with either Tpl-2 or Tpl-2ΔC resulted in the strong activation of co-transfected SAP K (Figure 3a). Expression of the constitutively active Raf-1 mutant, Raf(CAAX), in COS-1 cells did not activate SAP K, contrasting its biological activity with that of Tpl-2. The kinase inactive mutants of Tpl-2, Tpl-2(R167) and Tpl-2ΔC(R167), did not activate SAP K (Figure 3b).

Expression of MEK K-1 at low concentrations in HeLa or NIH 3T3 cells specifically activates the SAP K MAP kinase pathway with no effect on ERK-1 (Minden *et al.*, 1994; Yan *et al.*, 1994). However, when expressed at much higher levels, MEK K-1 can activate both the ERK and SAP K MAP kinase pathways (Lange-Carter *et al.*, 1993; Minden *et al.*, 1994). The activation of both the ERK-1 and SAP K MAP kinase pathways by Tpl-2 was also possibly a non-specific effect due to its overexpression. A titration experiment was therefore carried out to determine the specificity of Tpl-2 when expressed at low concentrations in transfected COS-1 cells.

In Figure 3c, it can be seen that ERK-1 and SAP K were activated even when only 4 ng of Tpl-2 plasmid were used for transfection and that both pathways were maximally activated with similar amounts of plasmid. Western blotting confirmed that the level of Tpl-2 protein decreased proportionally as the amount of transfected plasmid was reduced (data not shown).

The effect of expression of Tpl-2 was also tested in transiently transfected Jurkat T cells. Jurkat T cells express relatively low levels of transfected protein compared with COS-1 cells as the plasmid is not autoreplicated. Transient expression of ΔMEK K, a truncated form of MEK K-1 (Lange-Carter *et al.*, 1993), in Jurkat T cells stimulated SAP K without affecting the activity of ERK-1 (Figure 3d). Thus, ΔMEK K was specific for the SAP K pathway in this system. In contrast, Tpl-2 could strongly activate both ERK-1 and SAP K after transfection into Jurkat T cells (Figure 3d). Taken together with the titration experiment (Figure 3c), these data suggested that Tpl-2 could specifically activate both the ERK-1 and SAP K MAP kinase pathways.

To test whether Tpl-2 expression could activate the second 'stress-activated' MAP K pathway which involves the p38/RK MAP kinase, Jurkat T cells were co-transfected with plasmids encoding Tpl-2 and Myc epitope-tagged MPK 2, the *Xenopus* homologue of p38/RK. MPK 2 is >80% identical to mammalian p38/RK (Lee *et al.*, 1994; Rouse *et al.*, 1994). After 48 h of culture, the transfected

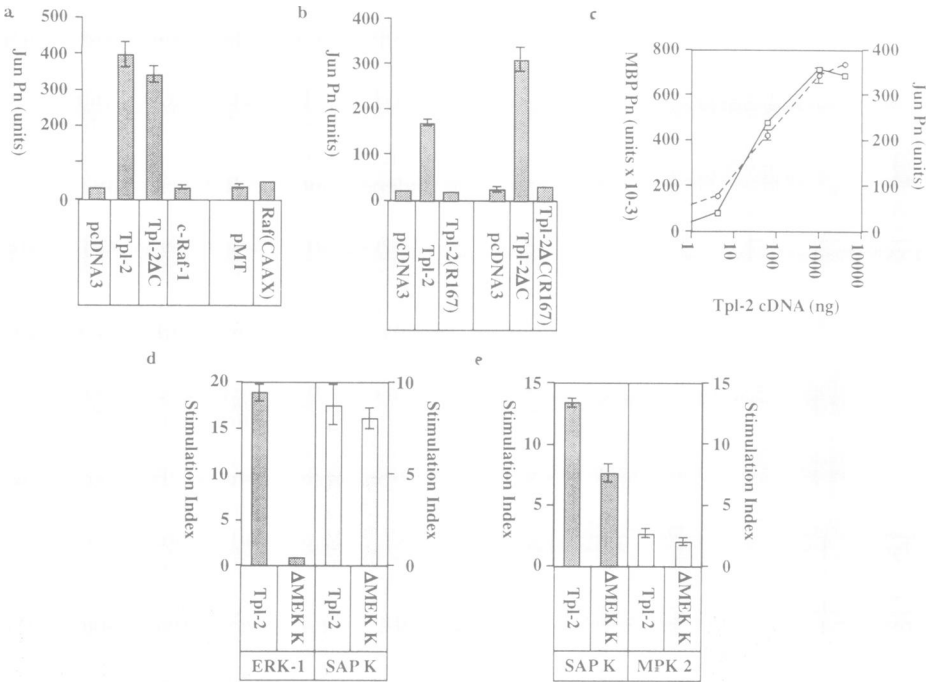

Fig. 3. Activation of p54β SAP K by Tpl-2 in transfected COS-1 cells. (**a–c**) COS-1 cells were transfected with plasmids encoding HA epitope-tagged p54β SAP K together with the kinases indicated or empty vector (pcDNA3 or pMT) and cultured for 36 h. SAP K was immunoprecipitated from cell lysates and assayed for its ability to phosphorylate GST–Jun *in vitro*. GST–Jun phosphorylation data have been normalized against the amount of SAP K in the immunoprecipitates and are expressed in arbitrary units. Results shown are the mean (± standard error) of triplicate assays. In (a) and (b), Western blotting of total cell lysates confirmed the expression of each of the transfected kinases (data not shown). In (c), COS-1 cells were transfected with either HA–ERK-1 or HA–SAP K cDNA together with the indicated amounts of pcDNA3-Tpl-2 cDNA. The total amount of pcDNA3 vector added was adjusted to 4000 ng for each transfection with empty vector. ERK-1 (□) or SAP K (○) were immunoprecipitated from cell lysates and assayed for their ability to phosphorylate MBP or GST–Jun, respectively. (**d**) Jurkat T cells were transiently transfected with HA–ERK-1 or HA–SAP K cDNA together with the kinases indicated or control empty vector. ERK-1 or SAP K activity was assayed as described above. Phosphorylation data are expressed as the mean stimulation index of duplicate assays (± standard error) versus control empty vector. (**e**) Jurkat T cells were transiently transfected with HA–SAP K or Myc–MPK2 cDNA together with the kinases indicated or control empty vector. SAP K and MPK2 activity was determined by assaying phosphorylation of GST–Jun and GST–ATF2(C2), respectively. Phosphorylation data are expressed as the mean stimulation index of duplicate assays (+/– standard error) versus control empty vector.

MPK 2 was immunoprecipitated via its epitope tag and tested for its ability to phosphorylate a GST–ATF2(C2) fusion protein (Livingstone *et al.*, 1995) *in vitro*. Tpl-2 and ΔMEK K expression resulted in only modest activation of MPK 2 (Figure 3e). In contrast, both Tpl-2 and ΔMEK K clearly activated co-transfected SAP K in the same experiment. These data suggested that Tpl-2 was a poor activator of the p38/RK MAP kinase pathway when expressed at low levels, of comparable efficacy with ΔMEK K, which is not considered to be an activator of this pathway (Derijard *et al.*, 1995; Lin *et al.*, 1995). Expression of much higher concentrations of Tpl-2 in COS-1 cells did result in the activation (5- to 10-fold) of co-transfected MPK 2 (data not shown). This was probably a non-specific effect due to its over-expression.

In vitro phosphorylation of MEK-1 by Tpl-2

As shown in Figure 2, Tpl-2 activated ERK-1 independently of Ras and Raf. The homology of Tpl-2 with the *S.cerevisiae* STE11 MAP KKK suggested that this activation could have resulted from its direct phosphorylation and activation of MEK, which subsequently activated ERK-1. To investigate whether MEK was a substrate for Tpl-2, wild-type Tpl-2 or Tpl-2ΔC were immunoprecipitated from transfected COS-1 cells and tested for their abilities to phosphorylate recombinant kinase-inactive MEK-1 fused to GST, GST–MEK(A207), *in vitro*.

Immunoprecipitated Tpl-2, Tpl-2ΔC and Raf(CAAX), but not control immunoprecipitates, phosphorylated GST–MEK(A207) (Figure 4a). The level of labelling of GST–MEK(A207) obtained with Tpl-2ΔC was considerably greater than that for Tpl-2, reflecting the lower amounts of Tpl-2 in immunoprecipitates relative to Tpl-2ΔC. The level of expression of Tpl-2ΔC in transfected COS-1 cells, determined by Western blotting of total cell lysates, was consistently much higher (10- to 30-fold) than that observed for Tpl-2 (data not shown). By quantifying GST–MEK(A207) phosphorylation relative to the amount of immunoprecipitated Tpl-2, the specific activity ratio of Tpl-2ΔC relative to Tpl-2 (TplΔC/Tpl-2) in the phosphorylation of GST–MEK(A207) was calculated to be 1.25 (mean value of 5 separate experiments, ± 0.1 standard error). The activity of the truncated form of Tpl-2 was therefore similar to that of the wild-type form. Immunoprecipitated Tpl-2(R167) or Tpl-2ΔC(R167) did not label GST–MEK(A207) demonstrating that the kinase activity of Tpl-2 was required for Tpl-2 immunoprecipitates to phosphorylate GST–MEK(A207) (Figure 4b).

Phosphopeptide sequencing of MEK and analysis of various point mutants has indicated that phosphorylation

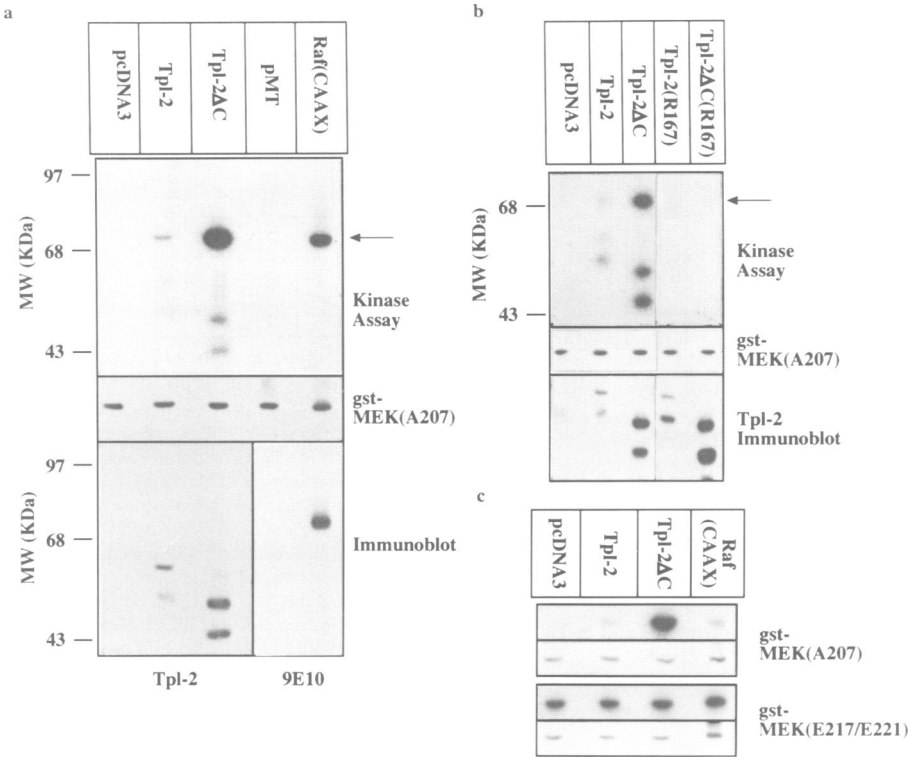

Fig. 4. *In vitro* phosphorylation of MEK-1 by Tpl-2. COS-1 cells were transfected with vectors encoding the kinases indicated or control empty vectors (pcDNA3 and pMT). In (**a**) and (**b**), the transfected kinases were immunoprecipitated from cell lysates and then assayed for their ability to phosphorylate kinase-inactive GST–MEK-1(A207) *in vitro*. Labelled proteins were resolved by SDS–PAGE, transferred onto a PVDF membrane and autoradiographed. The position of the labelled GST fusion protein is indicated with an arrow, as revealed by immunoblotting with an anti-GST mAb (top panel). Immunoblotting confirmed that similar amounts of fusion protein were added to each reaction (middle panel). Both Tpl-2 and Tpl-2ΔC resolved as doublets after SDS–PAGE. This was probably due to alternative initiation of translation, as described for Cot (Aoki *et al.*, 1993). The amount of Tpl-2 and Raf(CAAX) in immunoprecipitates was determined by Western blotting (lower panel). Control experiments determined that Tpl-2 and Tpl-2ΔC did not phosphorylate GST (data not shown). In (**c**), immunoprecipitated kinases were tested for their ability to phosphorylate either GST–MEK(A207) or GST–MEK(E217/E221), as indicated. The upper panels show the ^{32}P-labelled fusion proteins and the lower panels the immunoblots with anti-GST mAb.

of either of two serines, at residues 217 and 221, is sufficient for its activation by Raf (Alessi *et al.*, 1994; Zheng and Guan, 1994). As a first step to investigate whether Tpl-2 also phosphorylated these sites, Tpl-2 immunoprecipitates were tested for their ability to phosphorylate GST–MEK(E217/E221), in which Ser217 and Ser221 were mutated to glutamate, which cannot be phosphorylated (Alessi *et al.*, 1994). GST–MEK(E217/E221) is constitutively active and autophosphorylates resulting in background phosphorylation (Figure 4c). Tpl-2ΔC immunoprecipitates did not increase the phosphorylation of GST–MEK(E217/E221), although GST–MEK-(A207) was strongly phosphorylated (Figure 4c). Similarly, Tpl-2 and Raf(CAAX) immunoprecipitates did not increase the phosphorylation of GST–MEK(E217/E221), although small changes may not have been detected due to the background phosphorylation of GST–MEK(E217/E221). These data therefore suggest that major sites of phosphorylation on MEK by Tpl-2ΔC, and presumably also Tpl-2, correspond to Ser217 and Ser221.

Activation of MEK-1 by Tpl-2 in vitro

A coupled assay was used to test directly whether the *in vitro* phosphorylation of MEK-1 by Tpl-2 resulted in its activation. Recombinant GST–MEK-1 was phosphorylated by the immunoprecipitated kinases indicated in the presence of recombinant, histidine-tagged ERK-1 (His–ERK-1), which was subsequently assayed for its ability to phosphorylate MBP. Tpl-2ΔC and Raf(CAAX) immunoprecipitates activated MBP phosphorylation by His–ERK-1 to similar levels (Figure 5a). Wild-type Tpl-2 also strongly activated GST–MEK-1, although to a lesser degree than Tpl-2ΔC. A titration of Tpl-2 and Tpl-2ΔC in the coupled assay revealed that the Tpl-2ΔC immunoprecipitates saturated the coupled assay at a concentration comparable with that shown in Figure 5a (data not shown). Comparison of immunoprecipitates with approximately the same amount of Tpl-2 and Tpl-2ΔC indicated that their MEK K activity was similar. The kinase inactive mutants of Tpl-2, Tpl-2(R167) and Tpl-2ΔC(R167), did not stimulate MBP phosphorylation in the coupled assay (Figure 5b).

In contrast to wild-type Tpl-2, immunoprecipitated c-Raf-1 did not appreciably activate GST–MEK-1 (Figure 5a). The *in vitro* MEK K activity of Tpl-2 and c-Raf-1, therefore, correlated well with their ability to activate ERK-1 *in vivo* (see Figure 1a). The MEK K activity of c-Raf-1 was substantially activated after co-transfection of COS-1 cells with a plasmid encoding V12Ras (Figure 6a). In contrast, the constitutive MEK K activity of Tpl-2 was not increased by co-expression with V12Ras (Figure 6a).

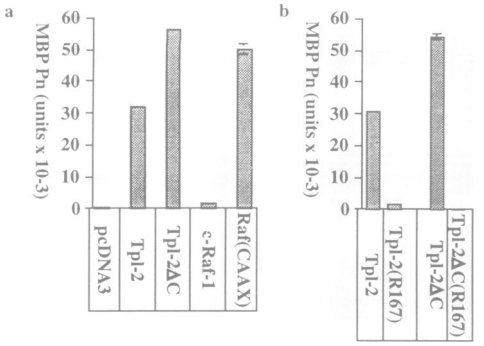

Fig. 5. (**a**) Activation of MEK-1 by Tpl-2 *in vitro*. COS-1 cells were transfected with vectors encoding the indicated kinases or a control empty vector (pcDNA3). After cell lysis, the kinases were immunoprecipitated and then assayed for their ability to activate GST–MEK-1 protein which in turn activated recombinant His–ERK-1 protein, as measured by the phosphorylation of MBP. Control assays in which GST–MEK-1 or His–ERK-1 were omitted indicated that no MEK or ERK-1 activity co-immunoprecipitated with Tpl-2 or with Raf (data not shown). Results are the mean (± standard error) of duplicate MBP phosphorylation assays of activated His–ERK-1. In (**b**), the data have been normalized against the amount of Tpl-2 or Tpl-2ΔC detected in immunoprecipitates analysed by Western blotting to allow comparison between the kinase dead mutants of Tpl-2 and their active counterparts.

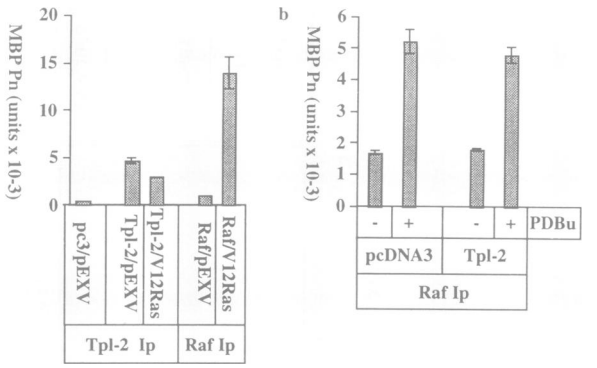

Fig. 6. Effect of V12Ras and Tpl-2 expression on the MEK K activity of c-Raf-1. COS-1 cells were transfected with the vector combinations indicated. In (**a**) Tpl-2 or c-Raf-1 was then immunoprecipitated and assayed for its ability to activate GST–MEK-1 *in vitro* in a coupled assay as in Figure 5. MBP phosphorylation data has been normalized against the amount of Tpl-2 or c-Raf-1 immunoprecipitated to allow comparison between the V12Ras and control pEXV transfections. The decreased activity of the Tpl-2 immunoprecipitates compared with Figure 5 was due to its lower expression in the COS-1 cells as a result of co-transfection with the pEXV vector. In (**b**), COS-1 cells were stimulated with PDBu for 15 min or left unstimulated. After cell lysis, c-Raf-1 was immunoprecipitated and its MEK K activity assessed in a coupled assay. Results are the mean of duplicate assays (± standard error) and are expressed in arbitrary units. The data have been normalized against the amount of c-Raf-1 detected in the immunoprecipitates by Western blotting. Co-expression of Tpl-2 was confirmed by Western blotting of total cell lysates (data not shown).

Expression of Tpl-2 does not activate c-Raf-1 in COS-1 cells

Western blotting did not detect any co-immunoprecipitating endogenous c-Raf-1 in Tpl-2 immunoprecipitates (data not shown). However, due to the high sensitivity of the coupled assay, this did not rule out the contribution of very low levels of c-Raf-1 to the MEK K activity of Tpl-2 immunoprecipitates. c-Raf-1 is inactive without stimulation (e.g. by co-transfection with V12Ras; Figure 6a). Therefore, for co-immunoprecipitating endogenous c-Raf-1 to contribute to the MEK K activity of Tpl-2 immunoprecipitates, it would have to become activated by the expression of Tpl-2 in the COS-1 cells. To test this possibility, c-Raf-1 was co-expressed with Tpl-2 in COS-1 cells. The MEK K activity of immunoprecipitated c-Raf-1 was then tested in a coupled assay. No difference in activity was detected between control and Tpl-2 transfected cells (Figure 6b). In contrast, stimulation of the transfected COS-1 cells with phorbol-12,13-dibutyrate (PDBu) did increase the activity of the c-Raf-1 immunoprecipitates, as expected (Howe *et al.*, 1992). Thus, expression of Tpl-2 in COS-1 cells did not activate the MEK K activity of c-Raf-1.

The MEK K activity of Tpl-2 immunoprecipitates was stable to washing in 0.5 M LiCl and Tpl-2 was the only labelled protein detected in *in vitro* kinase reactions of anti-Tpl-2 immunoprecipitates (data not shown). Taken together with the inability of kinase inactive mutants of Tpl-2 to activate MEK, these data strongly suggest that the MEK K activity associated with anti-Tpl-2 immunoprecipitates was due to Tpl-2 itself, rather than a co-immunoprecipitating protein kinase.

Phosphorylation and activation of SEK-1 by Tpl-2 in vitro

The stimulation of the SAP K pathway by transfected Tpl-2 in COS-1 cells suggested that Tpl-2 might be able to activate MAP KK SEK-1, in addition to MEK-1. This possibility was investigated in a similar fashion to the experiments with MEK. Tpl-2 or Tpl-2ΔC were immunoprecipitated from transfected COS-1 cells and assayed for their phosphorylation of a recombinant GST fusion protein of kinase inactive SEK-1, GST–SEK-1(KR). Immunoprecipitated Tpl-2ΔC phosphorylated GST–SEK(KR) *in vitro* to a similar degree to immunoprecipitated ΔMEK K, a known activator of SEK-1 (Minden *et al.*, 1994; Yan *et al.*, 1994). In contrast, Raf(CAAX) did not phosphorylate the recombinant GST–SEK-1(KR) protein (Figure 7a). This was consistent with the inability of Raf(CAAX) to activate SAP K *in vivo* (Figure 3a). Wild-type Tpl-2 phosphorylated GST–SEK-1(KR) to a lesser extent than Tpl-2ΔC, which again reflected the lower amounts of protein in the immunoprecipitates. Quantification of the level of GST–SEK-1(KR) phosphorylation relative to the amount of immunoprecipitated Tpl-2 or Tpl-2ΔC kinase detected by Western blotting indicated that the specific activity of Tpl-2ΔC was essentially the same as Tpl-2 (Tpl-2ΔC/Tpl-2 mean specific activity ratio from 3 experiments = 1.02 ± 0.1 standard error). The kinase inactive mutants of Tpl-2, Tpl-2(R167) and Tpl-2ΔC(R167), did not phosphorylate GST–SEK-1(KR) (Figure 7b). Tpl-2 immunoprecipitates did not phosphorylate a GST–SEK mutant in which the two residues equivalent to the sites of activation in MEK were mutated so that they were no longer able to be phosphorylated (Yan *et al.*, 1994; data not shown).

To determine directly whether Tpl-2 could activate SEK-1, GST–SEK-1 fusion protein was first phosphorylated by immunoprecipitated Tpl-2 or Tpl-2ΔC and then assayed for its ability to phosphorylate a kinase inactive GST fusion protein of SAP K, GST–SAP K(KA), in a coupled assay. Tpl-2ΔC activated GST–SEK-1 (5.3-fold;

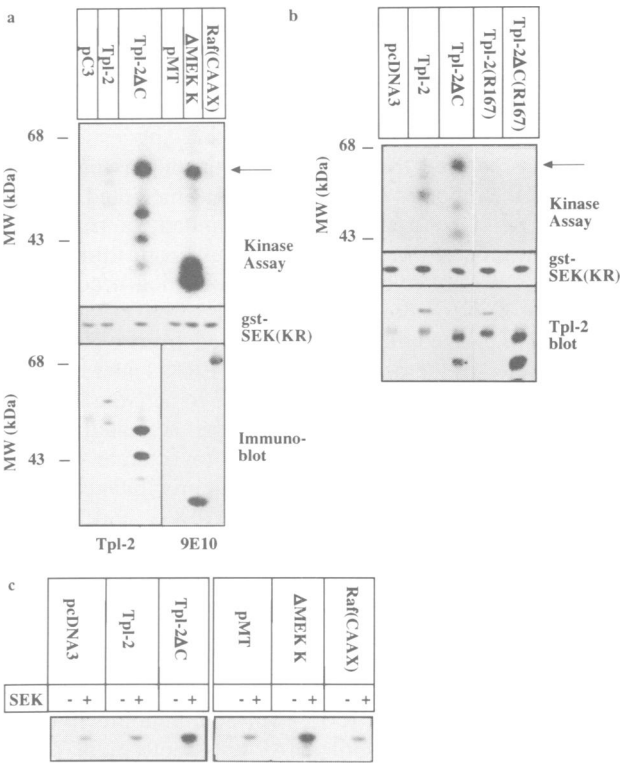

Fig. 7. Phosphorylation and activation of SEK-1 by Tpl-2 *in vitro*. COS-1 cells were transfected with the vectors indicated and the kinases were purified from cell lysates by immunoprecipitation. (**a** and **b**) Immunoprecipitated kinases were tested for their ability to phosphorylate a kinase inactive, GST fusion protein of SEK-1, GST–SEK(KR), *in vitro*. The position of the labelled GST–SEK(KR) in the top panel is shown with an arrow. The middle panel confirms that the amount of fusion protein added to each reaction was comparable as detected by immunoblotting. The lower panel shows the amount of Tpl-2, ΔMEK K and Raf(CAAX) in the immunoprecipitates as determined by Western blotting with the appropriate antibodies. (**c**) Immunoprecipitated kinases were assayed for their ability to activate recombinant SEK-1 fusion protein, GST–SEK-1, to phosphorylate recombinant, kinase inactive SAP K, GST–SAP K(KA), in a coupled assay. Labelled protein was resolved by SDS–PAGE, transferred onto a PVDF membrane and autoradiographed. The panels show the labelled GST–SAP K(KA) protein for each immunoprecipitate which were incubated in the presence or absence of GST–SEK-1.

Figure 7c). The stimulation index obtained was low due to the high basal activity of GST–SEK-1. Thus even in control pcDNA3 immunoprecipitates, the addition of GST–SEK-1 increased the phosphorylation of GST–SAP K(KA) by 5.8-fold. However, the level of activation obtained with immunoprecipitated ΔMEK K, a known activator of SEK-1 (Minden *et al.*, 1994; Yan *et al.*, 1994), was of the same order as that achieved with Tpl-2ΔC in these assays (2.5-fold). The kinase inactive mutant of Tpl-2ΔC, Tpl-2ΔC(R167), did not activate GST–SEK-1 (data not shown). A small, but reproducible, increase in GST–SAP K(KA) phosphorylation was also detected after incubation of GST–SEK-1 with wild-type Tpl-2 (1.5-fold; Figure 7c). However, the assay was insufficiently sensitive to obtain a reliable estimate of the relative specific activity of Tpl-2ΔC compared with Tpl-2. Raf(CAAX) did not activate GST–SEK-1, consistent with its failure to phosphorylate GST–SEK-1 *in vitro* (Figure 7c).

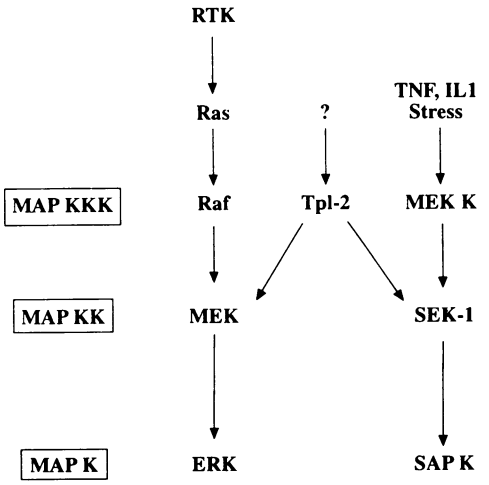

Fig. 8. Summary diagram of MAP K cascades leading to the activation of ERK and SAP K.

Discussion

In this study, the possibility was investigated that Tpl-2 protein serine/threonine kinase might function as a MAP KKK. The results indicate that Tpl-2 can phosphorylate and activate both MEK-1 and SEK-1 *in vitro*. This results in the activation of the ERK-1 and SAP K MAP kinase pathways, respectively, when Tpl-2 is expressed at low levels in either COS-1 cells or Jurkat T cells. Tpl-2 is, therefore, a novel MAP KKK which can activate two MAP K pathways (summarized schematically in Figure 8). Tpl-2 expression resulted in minimal activation of co-transfected MPK2 (p38/RK) in Jurkat T cells, suggesting that Tpl-2 does not act as a MAP KKK for this MAP K pathway.

Constitutively activated forms of MEK, generated by site-directed mutation, induce mitogenesis and transformation in fibroblasts (Cowley *et al.*, 1994; Mansour *et al.*, 1994). Tpl-2 was initially identified as an oncogene (Patriotis *et al.*, 1993) and the transforming activity of this gene product probably results from its ability to phosphorylate and activate MEK. Two other serine/threonine onco-protein kinases, Raf and Mos, also function as MAP KKKs for MEK (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992; Nebreda *et al.*, 1993; Okazaki and Sagata, 1995), suggesting that the ability to activate MEK is a common mechanism for cell transformation by these unrelated kinases. In contrast to Tpl-2, MEK K-1 is not able to activate the ERK-1 pathway unless it is expressed at very high levels and cannot transform cells (Minden *et al.*, 1994; Yan *et al.*, 1994). Indeed, the activation of the SAP K pathway by MEK K-1, in the absence of ERK-1 activation, inhibits cell growth in transfected 3T3 cells (Yan *et al.*, 1994). The ERK-1 and SAP K pathways therefore appear to mediate opposite effects on cell growth when activated separately. Furthermore, the stimuli that trigger ERK and SAP K are largely discrete (Cano and Mahadevan, 1995) suggesting that these pathways are independent and parallel. However, it is evident that certain receptors which promote cell proliferation (EGF receptor, transforming G protein-coupled receptors) will stimulate both MAP K pathways simultaneously (Kyriakis *et al.*, 1994; Coso *et al.*, 1995;

Derijard *et al.*, 1995). It is possible that the stimulation of both ERK and SAP K by such receptors may be mediated by the Tpl-2 MAP KKK, in tissues expressing this kinase.

The deletion of the C-terminus appears to be an important event in the oncogenic activation of Tpl-2 (Patriotis *et al.*, 1993; Makris *et al.*, 1993) and therefore the C-terminus may comprise a regulatory domain of the protein. However, both Tpl-2 and the C-terminally truncated form, Tpl-2ΔC, activated ERK-1 and SAP K when transiently transfected into either COS-1 or Jurkat T cells. Furthermore, the C-terminal deletion had little effect on the specific activity of Tpl-2 towards MEK and SEK. This suggests that wild-type Tpl-2 was constitutively active after transient expression in COS-1 or Jurkat T cells and it is possible that a regulatory factor for Tpl-2 was limiting or absent in these cells. This may have masked the putative activating effect of the C-terminal deletion on its MAP KKK activity.

Tpl-2 is >90% identical to the human proto-oncogene product, Cot, at the amino acid level and may be the rat homologue of the human protein. Cot was isolated, in a C-terminally truncated form, by virtue of its ability to transform SHOK cells (Miyoshi *et al.*, 1991) and also in its full length form as an oncogene which could transform 3T3 cells (Chan *et al.*, 1993; termed *est* in this study). Its high degree of similarity to Tpl-2 suggests that Cot may also function as a MAP KKK. Consistent with this hypothesis, ERK-1 is activated in 293 cells transiently transfected with Cot (Troppmair *et al.*, 1994).

Patriotis *et al.* (1994) recently reported that a C-terminally truncated form of Tpl-2 activates ERK-1 when transiently expressed in COS-1 cells, similar to the data in Figure 1a. This group suggested that the truncated Tpl-2 activates ERK-1 in a Ras- and Raf-dependent fashion and proposed a model in which the truncated Tpl-2 forms a multimolecular complex with Ras and Raf which regulates the activation of MEK. However, several experiments in the present study indicate that the ability of Tpl-2 and Tpl-2ΔC to activate ERK was the consequence of their direct phosphorylation and activation of MEK and was independent of both Ras and Raf.

First, immunoprecipitated Tpl-2 and Tpl-2ΔC phosphorylated and activated recombinant MEK-1 fusion protein *in vitro* (Figures 4 and 5). This activity did not appear to result from endogenous Raf co-immunoprecipitating with Tpl-2, as determined by Western blotting (unpublished data). Furthermore, the MEK K activity of immunoprecipitated c-Raf-1 was not altered by co-expression with Tpl-2 in COS-1 cells (Figure 6b), which also argues against c-Raf-1 mediating the activation of ERK-1 by Tpl-2.

Secondly, contrary to the data of Patriotis *et al.* (1994), the present study indicates that a dominant-negative mutant of Ras did not block the activation of ERK-1 by Tpl-2 (Figure 2a), suggesting that Ras activity was not required for ERK-1 activation by Tpl-2. Furthermore, co-expression of a dominant-negative mutant of Raf-1, NΔRaf, did not block the activation of ERK-1 by Tpl-2 (Figure 2b). Thus, Raf activity was also not required for Tpl-2 activation of ERK-1. In contrast to these data, Patriotis *et al.* (1994) found that the Raf dominant-negative mutant, Raf-1(S621A), did inhibit the activation of ERK-1 by truncated Tpl-2. It is possible that the use of distinct Raf mutants may account for the contrasting effects on Tpl-2 activity. However, the ability of Raf(S621A) to block ERK-1 activation by Tpl-2 may simply result from competition for the same substrate MEK, rather that suggesting that c-Raf-1 is required for Tpl-2 activation of this pathway. Similarly, the reported inhibition of v-Raf-mediated activation of ERK-1 by a kinase-inactive mutant of truncated Tpl-2 (Patriotis *et al.*, 1994) may result from titration of the common substrate MEK and does not necessarily imply that Tpl-2 is downstream of Raf.

Finally, Tpl-2 could be distinguished from c-Raf-1 in its specificity in the activation of the ERK-1 and SAP K MAP kinase pathways. Tpl-2 was able to activate both of these MAP K pathways, whereas the activated Raf-1 mutant, Raf(CAAX), was only able to activate ERK-1 (Figures 1a and 3a). Moreover, immunoprecipitated Tpl-2 phosphorylated and activated both MEK-1 and SEK-1 *in vitro*, whereas Raf(CAAX) was only able to phosphorylate and activate MEK-1 (Figures 4, 5 and 7). Tpl-2 was also distinguished functionally from MEK K-1 in transfected Jurkat T cells, in which it activated both ERK-1 and SAP K, whereas ΔMEK K only activated SAP K (Figure 3d). Furthermore, Western blotting analysis failed to detect any MEK K-1 in Tpl-2 immunoprecipitates (unpublished data). Thus, the MAP KKK activity of Tpl-2 immunoprecipitates did not result from co-precipitation of MEK K-1.

Concanavalin A induces the expression of Tpl-2 mRNA in splenic T cells within 1 h of stimulation (Patriotis *et al.*, 1993) suggesting that Tpl-2 may regulate the transition of resting lymphocytes into the cell cycle. The present study suggests that the ERK-1 and SAP K MAP kinase pathways in activated T cells may be regulated by Tpl-2. However, the induced expression of Tpl-2 after TcR cross-linking, which occurs relatively slowly, implies that it may play a role in regulating MAP K pathways in T cells that have already entered the G_1 phase of the cell cycle, rather than in resting G_0 T cells. In future experiments, it will be important to identify the signals that regulate the MAP KKK activity of endogenous Tpl-2 to determine its precise role in the activation of T cells.

Materials and methods

Cell lines

COS-1 cells were obtained from the European Collection of Animal Cell Cultures and were maintained in DMEM supplemented with 5% FCS, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (50 U/ml). For experiments, COS-1 cells were cultured until near confluence and were split 1:4. Cells were then harvested for transfection after a further 2 days in culture. EGF and PDBu were both purchased from Sigma and were used at 50 ng/ml and 100 ng/ml, respectively, to stimulate cells. The J6 sub-line of the Jurkat T leukemic cell line (Robb *et al.*, 1981) was passaged in RPMI 1640 medium containing 5% FCS, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (50 U/ml). Cells were maintained in a rapid growth phase prior to transfection.

Antibodies

Immunoprecipitation and Western blotting of HA epitope-tagged ERK-1 and SAP K was carried out using the 12CA5 monoclonal antibody (mAb; Field *et al.*, 1988). The rabbit anti-Tpl-2 antibodies, Tsp1 and Tsp3, were raised against synthetic peptides encoding residues 51–65 and 381–400 of rat Tpl-2, respectively. Tsp1 was used for immunoprecipitation and Tsp3 for immunoblotting of Tpl-2. A rabbit antiserum raised to the C-terminal 12 amino acids of c-Raf-1 was used for

immunoprecipitation and Western blotting of c-Raf-1. The 9E10 mAb (from G.Evan, ICRF, London; Evan *et al.*, 1985) was used for immunoprecipitation and immunoblotting of Raf(CAAX) and ΔMEK K. An anti-GST mAb (from S.Dillworth, RPMS, London) was used for immunoblotting of GST fusion proteins.

DNA constructs for cell transfection

Haemagglutin epitope-tagged ERK-1 (from Tom Geppert, Univesity of Texas SouthWestern Medical center, USA; Robbins *et al.*, 1993) was subcloned into the pcDNA3 expression vector (InVitrogen). The HA epitope-tagged p54β SAP K (from Jim Woodgett, Ontario Cancer Institute, Canada; Yan *et al.*, 1994) was in the pMT expression vector. Myc epitope-tagged MPK 2 (from Angel Nebreda, ICRF, London and Alan Hall, University College, London) was subcloned into the pcDNA3 vector.

PCR was used to remove the 5′ and 3′ untranslated regions of Tpl-2 cDNA (from Peter Tsichlis, Fox Chase Cancer Center, Philadelphia, USA; Patriotis *et al.*, 1993), which was subcloned into the pcDNA3 expression vector. Tpl-2ΔC encodes a C-terminally deleted form of Tpl-2 which terminates at residue 387, homologous to the human Cot oncogene (Miyoshi *et al.*, 1991). This was generated by PCR from pcDNA3-Tpl-2 using the primers: CATGGATGTCTATCTCCCC and AAGTCTAGCTCGAGTCACCGTGGCTGGTCC. The product was digested with *Bgl*II and *Xho*I and this was then ligated with the *Bgl*II fragment of Tpl-2. Kinase-inactive forms of Tpl-2 and Tpl-2ΔC were generated by mutating K167 to R167 using sequential PCR with the mutagenic primers GAATGGCATG*C*A*G*ACTGATCCCTG and CAGG-GATCAGT*CTG*CATGCCATTC using the appropriate flanking primers. All PCR products were verified by DNA sequencing. The c-Raf-1 cDNA was subcloned into the pcDNA3 vector for COS-1 cell expression experiments. The Raf(CAAX) construct, which has a Myc tag at its N-terminus, was in the pMT expression vector and the V12Ras (Val12 Ha-Ras) cDNA was subcloned into the pEXV vector. Both have been described previously (Cowley *et al.*, 1994; Leevers *et al.*, 1994). The ΔMEK K construct (from Chris Marshall and Alan Ashworth, Chester Beatty Laboratories, London), encodes a truncated form of murine MEK K (residues 367–672; Lange-Carter *et al.*, 1993) and was subcloned into the pMT expression vector.

ERK-1 assay

COS-1 cells (2×10^6) were transfected with 3 μg of each of the vectors indicated by electroporation (Bio-Rad Gene Pulser; 250 V, 125 μF) and cultured for 36 h. In some experiments the amounts of plasmid used for transfection were altered, as indicated in the figure legends. Eighteen hours after transfection, cells were washed free of serum and cultured for the remaining period in DMEM plus 1 mg/ml BSA. The cells were lysed with 1 ml of buffer A (1% Triton X-100, 25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM Na_3VO_4, 10 mM $Na_4P_2O_7$ and 1 μg/ml each of aprotinin and leupeptin). Lysates were centrifuged (13 000 *g* for 10 min) and the supernatant incubated with 12CA5 antibody coupled covalently to protein A–Sepharose (Pharmacia) with dimethyl pimelimidate (Schneider *et al.*, 1982) to immunoprecipitate the transfected HA epitope-tagged ERK-1 kinase. Immunoprecipitates were then washed four times with buffer A and once in buffer B (50 mM HEPES, pH 7.2, 100 mM NaCl, 1 mM DTT, 6 mM $MgCl_2$, 1 mM $MnCl_2$). The kinase reaction was carried out in 50 μl of buffer B plus 5 μM ATP, 2.5 μCi [γ-^{32}P]ATP and 0.25 ng/ml MBP, incubated at room temperature for 30 min with continuous gentle agitation. MBP phosphorylation was measured by binding to p81 paper (Whatman) and Cerenkov counting. The MBP phosphorylation (MBP Pn) data, which are expressed in arbitrary units, have been normalized against the amount of ERK-1 in the immunoprecipitates detected by Western blotting and laser densitometry.

SAP K and MPK2 assays

The conditions for transfection and lysis of COS-1 cells for SAP K assays were as for the ERK-1 assay. Transfected HA epitope-tagged p54β SAP K was immunoprecipitated from cell lysates with 12CA5 antibody as for ERK-1. Immunoprecipitated SAP K bound to washed beads was then resuspended in 50 μl of buffer B plus 10 μM ATP, 5 μCi [γ-^{32}P]ATP and 5 μg of GST–Jun. The beads were then incubated with gentle agitation for 30 min at room temperature. The reaction was terminated by addition of two times reducing SDS–PAGE sample buffer and boiling. The labelled proteins were resolved by SDS–PAGE and transferred onto a PVDF membrane and the level of GST–Jun phosphorylation (Jun Pn) was determined by phosphorimager analysis. Jun phosphorylation data, which are expressed in arbitrary units, have been normalized against the amount of SAP K detected in the immunoprecipitates by Western blotting and laser densitometry.

Transfected Myc epitope-tagged MPK2 was immunoprecipitated from Jurkat cell lysates with 9E10 antibody, as described below. After washing, the immunoprecipitated MPK2 bound to beads was resuspended in 50 μl of buffer B plus 10 μM ATP, 5 μCi [γ-^{32}P]ATP and 5 μg of GST–ATF2(C2) (encoding an N-terminal fragment of ATF-2; Livingstone *et al.*, 1995). The kinase reaction and quantitation of GST–ATF2(C2) labelling was carried out as for SAP K phosphorylation of GST–Jun.

Transient transfection of Jurkat T cells

Transfection of Jurkat T cells was carried out by electroporation essentially as described by Izquierdo *et al.* (1994). Briefly, cells were harvested by centrifugation and washed once in serum-free RPMI 1640 medium. Cells (2×10^7) were then resuspended in 250 μl of RPMI medium, transferred into a Bio-Rad gene pulser cuvette and 10 μg of the appropriate plasmid were added. Cells were pulsed at 960 μF and 270 V (Gene pulser, Bio-Rad laboratories) and left for 10 min at room temperature. Cells were then transferred into 10 ml of RPMI medium supplemented with 10% FCS and cultured for 48 h prior to harvesting. After pelleting by centrifugation, cells were lysed and HA–ERK-1, HA–SAP K or Myc–MPK2 immunoprecipitated and assayed as previously described.

Preparation of recombinant bacterial fusion proteins

The fusion proteins for MEK, MEK(A207) and MEK(E217/E221) (from Chris Marshall, Chester Beatty Laboratories, London) were tagged at their N-terminus with GST and at their C-terminus with a hexahistidine sequence. The methodology for purification of these fusion proteins involved sequential affinity chromatography firstly with glutathione–agarose (Pharmacia) and then nickel–nitrilotriacetate–agarose (Quiagen) to permit purification of the full-length protein. This was carried out as described by Alessi *et al.* (1994). The ERK-1 fusion protein was tagged at its N-terminus with a hexahistidine sequence. This protein was purified by affinity chromatography using nickel–nitrilotriacetate–agarose, as described (Robbins *et al.*, 1993). The GST fusion proteins of SAP K(KR), SEK, SEK(KR), Jun and ATF2(C2) were all purified by binding to glutathione–agarose following a standard Pharmacia protocol. The purity of all fusion proteins was checked by SDS–PAGE and Coomassie blue staining and exceeded 75%. Purified proteins were dialysed against 50% glycerol, 50 mM Tris, pH 8, aliquoted and stored at –70 °C until use.

In vitro phosphorylation of MEK-1 and SEK-1

The indicated kinases were immunoprecipitated from lysates of transfected COS-1 cells using the appropriate antibodies, as described above for ERK-1. After washing in buffer A six times, the immunoprecipitated kinases were resuspended in 50 μl of buffer C (50 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$ and 10 μM ATP) plus 5 μg of a kinase-inactive GST fusion protein of MEK-1, GST–MEK-1(A207), or a kinase-inactive GST fusion protein of SEK-1, GST–SEK(KR) and 5 μCi of ATP. After a 30 min incubation at room temperature with constant gentle agitation, the reaction was stopped by addition of 2× Laemmli sample buffer. Labelled proteins were resolved by SDS–PAGE, transferred onto a PVDF membrane and autoradiographed. Immunoprecipitated kinases were subsequently detected by probing the PVDF membrane with the appropriate antibodies and ECL, following the manufacturer's instructions (Amersham International).

Coupled assay for the activation of MEK-1 in vitro

The protocol to assay MEK K activity was essentially the same as that described by Alessi *et al.* (1994) and Leevers *et al.* (1994). Tpl-2 or Raf were immunoprecipitated from lysates of transfected COS-1 cells with the appropriate antibody, using the same methodology described above for the immunoprecipitation of ERK-1. The immunoprecipitated kinases were then tested for their ability to activate recombinant GST–MEK-1 protein (0.1 μg) which in turn activated recombinant His–ERK-1 protein (1 μg), to phosphorylate MBP which was measured by binding to p81 paper (Whatman) and Cerenkov counting. The effect of co-transfection of COS-1 cells with V12Ras cDNA, or PDBu stimulation of COS-1 cells, on the MEK K activity of Tpl-2 or c-Raf-1 was quantified by normalizing the amount of Raf-1 or Tpl-2 in immunoprecipitates, determined by Western blotting, against the appropriate control.

Coupled assay for the activation of SEK-1 in vitro

Kinases were tested for their ability to activate recombinant SEK-1 fusion protein, GST–SEK-1, to phosphorylate recombinant, kinase-inactive SAP K fusion protein, GST–SAP K(KA), in a coupled assay.

The method used was based on that described by Yan *et al.* (1994). Briefly, immunoprecipitated kinases were first incubated in the presence or absence of 4 μg of GST–SEK-1 in 30 μl of buffer C plus 250 μM ATP for 15 min at room temperature with continous gentle agitation. Ten μl of supernatant from this reaction were then aspirated, mixed with 10 μl of buffer C and half of this mixture added to 2 μg of GST–SAP K(KA) in 40 μl of buffer C plus 2.5 μCi of [^{32}P]ATP. After incubation for 30 min at room temperature with gentle mixing, this reaction was terminated by addition of 2× Laemmli sample buffer and the labelled protein was resolved by SDS–PAGE and the blotted gel autoradiographed.

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References

Alessi,D.R., Saito,Y., Campbell,D.G., Cohen,P., Sithanandam,G., Rapp,U., Ashworth,A., Marshall,C.J. and Cowley,S. (1994) *EMBO J.*, **13**, 1610–1619.

Alvarez,E., Northwood,I.C., Gonzalez,F.A., Latour,D.A., Seth,A., Abate,C., Curran,T. and Davis,R.J. (1991) *J. Biol. Chem.*, **266**, 15277–15285.

Aoki,M., Hamada,F., Sugimoto,T., Sumida,S., Akiyama,T. and Toyoshima,K. (1993) *J. Biol. Chem.*, **268**, 22723–22732.

Cano,E. and Mahadevan,L.C. (1995) *Trends Biochem. Sci.*, **20**, 117–122.

Chan,A.M.-L., Chedid,M., McGovern,E.S., Popescu,N.C., Miki,T. and Aaronson,S.A. (1993) *Oncogene*, **8**, 1329–1333.

Coso,O.A., Chiariello,M., Kalinec,G., Kyriakis,J.M., Woodgett,J. and Gutkind,J.S. (1995) *J. Biol. Chem.*, **270**, 5620–5624.

Cowley,S., Paterson,H., Kemp,P. and Marshall,C.J. (1994) *Cell*, **77**, 841–852.

Dent,P., Haser,W., Haystead,T.A.J., Vincent,L.A., Roberts,T.M. and Sturgill,T.W. (1992) *Science*, **257**, 1404–1407.

Derijard,B., Hibi,M., Wu,I.-H., Barrett,T., Su,B., Deng,T., Karin,M. and Davis,R.J. (1994) *Cell*, **76**, 1025–1037.

Derijard,B., Raingeaud,J., Barrett,T., Wu,I.-H., Han,J., Ulevitch,R.J. and Davis,R.J. (1995) *Science*, **267**, 682–685.

Erikson,R.L. (1991) *J. Biol. Chem.*, **266**, 6007–6010.

Errede,B. and Levin,D.E. (1993) *Curr. Opin. Cell Biol.*, **5**, 254–260.

Evan,G.I., Lewis,G.K., Ramsay,G. and Bishop,J.M. (1985) *Mol. Cell. Biol.*, **5**, 3610–3616.

Feig,L.A. and Cooper,G.M. (1988) *Cell*, **71**, 335–342.

Field,J., Nikawa,J.-I., Broek,D., MacDonald,B., Rodgers,L., Wilson,I.A., Lerner,R.A. and Wigler,M. (1988) *Mol. Cell. Biol.*, **8**, 2159–2165.

Gupta,S., Campbell,D., Deriijard,B. and Davis,R.J. (1994) *Science*, **267**, 389–393.

Han,J., Lee,J.-D., Bibbs,L. and Ulevitch,R.J. (1994) *Science*, **265**, 808–811.

Herskowitz,I. (1995) *Cell*, **80**, 187–197.

Hibi,M., Lin,A., Smeal,T., Minden,A. and Karin,M. (1993) *Genes Dev.*, **7**, 2135–2148.

Hill,C.S. and Treisman,R. (1995) *Cell*, **80**, 199–211.

Howe,L.R., Leevers,S.J., Gomez,N., Nakielny,S., Cohen,P. and Marshall,C. (1992) *Cell*, **71**, 335–342.

Izquierdo,M., Bowden,S. and Cantrell,D. (1994) *J. Exp. Med.*, **180**, 401–406.

Kayne,P.S. and Sternberg,P.W. (1995) *Curr. Opin. Gene Dev.*, **5**, 38–43.

Kyriakis,J.M., App,H., Zhang,X., Banerjee,P., Brautigan,D.L., Rapp,U.R. and Avruch,J. (1992) *Nature*, **358**, 417–421.

Kyriakis,J.M., Banerjee,P., Nikolakaki,E., Dai,T., Rubie,E.A., Ahmad, M.F., Avruch,J. and Woodgett,J.R. (1994) *Nature*, **369**, 156–160.

Lange-Carter,C.A., Pleiman,C.M., Gardner,A.M., Blumer,K.J. and Johnson,G.L. (1993) *Science*, **260**, 315–319.

Lee,J.C. *et al.* (1994) *Nature*, **372**, 739–746.

Lee,K.S. and Levin,D.E. (1992) *Mol. Cell. Biol.*, **12**, 172–182.

Leevers,S.J. and Marshall,C.J. (1992) *EMBO J.*, **11**, 569–574.

Leevers,S.J., Paterson,H.F. and Marshall,C.J. (1994) *Nature*, **369**, 411–414.

Lenormand,P., Sardet,C., Pages,G., L'Allemain,G., Brunet,A. and Pouyssegur,J. (1993) *J. Cell Biol.*, **122**, 1079–1088.

Lin,A., Minden,A., Martinetto,H., Claret,F.-X., Lange-Carter,C., Mercurio,F., Johnson,G.L. and Karin,M. (1995) *Science*, **268**, 286–290.

Lin,L.L., Wartmann,M., Lin,A.Y., Knopf,J., Seth,A. and Davis,R. (1993) *Cell*, **72**, 269–278.

Livingstone,C., Patel,G. and Jones,N. (1995) *EMBO J.*, **14**, 1785–1797.

Makris,A., Patriotis,C., Bear,S.E. and Tsichlis,P.N. (1993) *J. Virol.*, **67**, 4283–4289.

Mansour,S.J., Matten,W.T., Hermann,A.S., Candia,J.M., Rong,S., Fukasawa,K., Van de Woude,G.F. and Ahn,N.G. (1994) *Science*, **265**, 966–970.

Marais,R., Light,Y., Paterson,H.F. and Marshall,C.J. (1995) *EMBO J.*, **14**, 3136–3145.

Marshall,C.J. (1994) *Curr. Opin. Genet. Dev.*, **4**, 82–89.

Marshall,C.J. (1995) *Cell*, **80**, 179–185.

Minden,A., Lin,A., McMahon,M., Lange-Carter,C., Derijard,B., Davis,R.J., Johnson,G.L. and Karin,M. (1994) *Science*, **266**, 1719–1723.

Miyoshi,J., Higashi,T., Mukai,H., Ohuchi,T. and Kakunaga,T. (1991) *Mol. Cell. Biol.*, **11**, 4088–4096.

Nebreda,A.R., Hill,C., Gomez,N., Cohen,P. and Hunt,T. (1993) *FEBS lett.*, **333**, 183–187.

Ohara,R., Miyoshi,J., Aoki,M. and Toyoshima,K. (1993) *Jap. J. Cancer Res.*, **84**, 518–525.

Okazaki,K. and Sagata,N. (1995) *Oncogene*, **10**, 1149–1157.

Owaki,H., Varma,R., Gillis,B., Bruder,J.T., Rapp,U.R., Davis,L.S. and Geppert,T.D. (1993) *EMBO J.*, **12**, 4367–4373.

Patriotis,C., Makris,A., Bear,S.E. and Tsichlis,P.N. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 2251–2255.

Patriotis,C., Makris,A., Chernoff,J. and Tsichlis,P.N. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 9755–9759.

Rhodes,N., Connell,L. and Errede,B. (1990) *Genes Dev.*, **4**, 1862–1874.

Robb,R.J., Munck,A. and Smith,K.A. (1981) *J. Exp. Med.*, **154**, 1455–1474.

Robbins,D.J., Zhen,E., Owaki,H., Vanderbill,C.A., Ebert,D., Geppert, T.D. and Cobb,M.H. (1993) *J. Biol. Chem.*, **268**, 5097–5106.

Rouse,J., Cohen,P., Trigon,S., Morange,M., Alonso-Llamazares,A., Zamanillo,D., Hunt,T. and Nebreda,A.R. (1994) *Cell*, **78**, 1027–1037.

Sanchez,I., Hughes,R.T., Mayer,B.J., Yee,K., Woodgett,J.R., Avruch,J., Kyriakis,J.M. and Zon,L.I. (1994) *Nature*, **372**, 794–798.

Schaap,D., van der Wal,J., Howe,L.R., Marshall,C.J. and van Blitterswijk,W.J. (1993) *J. Biol. Chem.*, **268**, 20232–20236.

Schneider,C., Newman,R.A., Sutherland,D.R., Asser,U. and Greaves, M.F. (1982) *J. Biol. Chem.*, **257**, 10766–10769.

Stokoe,D., Campbell,D.G., Nakielny,S., Hidaka,H., Leevers,S.J., Marshall,C.J. and Cohen,P. (1992) *EMBO J.*, **11**, 3985–3994.

Su,B., Jacinto,E., Hibi,M., Kallunki,T., Karin,M. and Ben-Neriah,Y. (1994) *Cell*, **77**, 727–736.

Troppmair,J., Bruder,J.T., Munoz,H., Lloyd,P.A., Kyriakis,J., Banerjee,P., Avruch,J. and Rapp,U.R. (1994) *J. Biol. Chem.*, **269**, 7030–7035.

Wang,Y., Xu,H.-P., Riggs,M., Rodgers,L. and Wigler,M. (1991) *Mol. Cell. Biol.*, **11**, 3554–3563.

Wassarman,D.A., Therrien,M. and Rubin,G.M. (1995) *Curr. Opin. Gene Dev.*, **5**, 44–50.

Yan,M., Dai,T., Deak,J.C., Kyriakis,J.M., Zon,L.I., Woodgett,J.R. and Templeton,D.J. (1994) *Nature*, **372**, 798–800.

Zheng,C.-F. and Guan,K.-L. (1994) *EMBO J.*, **13**, 1123–1131.

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