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Multiple p21ras effector pathways regulate nuclear factor of activated T cells

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The transcription factor, Nuclear Factor of Activated T cells (NFAT) is a major target for p21ras and calcium signalling pathways in the IL-2 gene and is induced by p21ras signals acting in synergy with calcium/calcineurin signals. One p21ras effector pathway involves the MAP kinase ERK-2, and we have examined its role in NFAT regulation. Expression of dominant negative MAPKK-1 prevents NFAT induction. Constitutively active MAPKK-1 fully activates ERK-2 and the transcription factor Elk-1, but does not substitute for activated p21ras and synergize with calcium/calcineurin signals to induce NFAT. Expression of dominant negative N17Rac also prevents TCR and p21ras activation of NFAT, but without interfering with the ERK-2 pathway. The transcriptional activity of the NFAT binding site is mediated by a complex comprising a member of the NFAT group and AP-1 family proteins. The induction of AP-1 by p21ras also requires Rac-1 function. Activated Rac-1 could mimic activated p21ras to induce AP-1 but not to induce NFAT. Moreover, the combination of activated MAPKK-1 and Rac-1 could not substitute for activated p21ras and synergize with calcium signals to induce NFAT. Thus, p21ras regulation of NFAT in T cells requires the activity of multiple effector pathways including those regulated by MAPKK-1/ERK-2 and Rac-1.

Keywords: NFAT/Rac-1/Ras/MAPKK-1/transduction/TCR

Introduction

The T cell antigen receptor (TCR) controls activation and the G0–G1 transition of T lymphocytes and regulates the production of a number of cytokines, including the growth factor interleukin-2 (IL-2). The regulation of IL-2 production by the TCR requires the co-ordinate action of multiple transcription factors that include Nuclear Factor of Activated T cells (NFAT), AP-1, NF-κB and Oct-1 (Schreiber and Crabtree, 1992; Rao, 1994). The intracellular signals that regulate these different transcription factors involve tyrosine kinase-induced calcium/calcineurin, protein kinase C (PKC) and p21ras-mediated signalling pathways (Weiss and Littman, 1994; Izquierdo-Pastor *et al.*, 1995). Inhibition of p21ras function in T cells by expression of a dominant negative p21ras mutant prevents induction of the IL-2 gene (Baldari *et al.*, 1992; Rayter *et al.*, 1992). The major target in the IL-2 gene for p21ras signalling pathways is NFAT (Woodrow *et al.*, 1993b). Thus, a constitutively active Ras mutant (p21-v-Ha-ras), when expressed in T cells, can synergize with a calcium/calcineurin signalling pathway to activate NFAT (Woodrow *et al.*, 1993a,b). p21ras regulatory effects on NFAT can be mimicked by phorbol esters that activate PKC or by expression of constitutively activated mutants of PKC (Genot *et al.*, 1995). The ability of phorbol esters to activate p21ras in T cells led to the proposal that PKC effects on NFAT were mediated by p21ras. However, it is now recognized that phorbol ester and p21ras induction of NFAT occur by different, independent mechanisms. In particular, p21ras but not PKC signals are essential for TCR induction of NFAT (Williams *et al.*, 1995). NFAT proteins are cytosolic in quiescent T cells but they translocate to the nucleus in TCR-activated cells and combine with newly induced AP-1 complexes to form a functional transcriptional factor unit (Flanagan *et al.*, 1991). There are multiple isoforms of the NFAT cytosolic subunit, NFATc, NFATp, NFAT3 and NFAT4, that have different patterns of cellular expression but are thought to share one property in that their nuclear translocation is controlled by calcium/calcineurin-dependent signals (Northrop *et al.*, 1994; Rao, 1994; Hoey *et al.*, 1995). The different NFAT proteins also share the ability to interact with AP-1 to bind co-operatively to the composite NFAT/AP-1 site in the IL-2 gene (Rao, 1994). The interaction with AP-1 is essential for NFAT transcriptional activity, and the role of p21ras in NFAT induction has been explained by the ability of p21ras signals to stimulate AP-1 activity in T cells (Rayter *et al.*, 1992; Woodrow *et al.*, 1993a). However, the p21ras effector pathways involved in NFAT regulation have not been defined and are the focus of the present report.

T lymphocytes express at least two MAP kinases, ERK-1 and ERK-2, that are stimulated by a p21ras pathway in response to TCR triggering (Izquierdo *et al.*, 1993). The MAP kinases are activated by a kinase cascade involving a MAP kinase kinase (MAPKK) that phosphorylates and stimulates the ERK-1 and -2 kinases directly (Marshall, 1994; Cobb and Goldsmith, 1995). The activity of the MAPKK is itself controlled by phosphorylation and, hence, a MAP kinase kinase kinase (MAPKKK) regulates ERK-1 and -2. Raf-1 is a p21ras effector that appears to be the MAPKKK that couples p21ras and hence receptors to the MAP kinases (Howe *et al.*, 1992; Warne *et al.*, 1993; Izquierdo *et al.*, 1994a; Leevers *et al.*, 1994). In neuronal cells, MAPKK-1 and ERK-1, -2 are critical mediators of p21ras functions (Cowley *et al.*, 1994). One regulatory substrate for the ERKs is the protein Elk-1, which is one of a family of proteins that can form a ternary complex with the transcriptional activator serum

response factor (SRF) (Marais *et al.*, 1993). Elk-1–SRF complexes are necessary and sufficient mediators of c-*fos* serum response element induction. Thus, p21ras regulation of the MAPKK/ERK pathway plays a key role in regulating c-*fos* gene expression (Hill and Treisman, 1995). The analysis of the role of the ERKs in the control of gene expression in fibroblasts has created a paradigm that the MAPKK/ERK pathway is a major route whereby p21ras regulates the transcriptional activity of immediate early genes such as c-*fos*. By analogy, there is the possibility that the p21ras/MAPKK pathway is the p21ras effector pathway for NFAT induction.

There has been one previous study on the role of MAPKK and the ERKs as p21ras effectors in T cells: this was a study in transgenic mice that showed that expression of a dominant negative MAPKK-1 mutant could mimic inhibitory p21ras mutants and suppress positive selection of thymocytes (Alberola-lla *et al.*, 1995). However, dominant negative Ras mutants can prevent the *in vitro* proliferation of thymocytes, whereas a dominant negative MAPKK-1 mutant does not, indicating that not all Ras functions in T cells are mediated by the MAP kinases cascade. The Ras-related GTPase Rac-1 has been described recently as another downstream effector of p21ras (Hall, 1994; Qiu *et al.*, 1995) Accordingly, the object of the present study was to explore the signalling connections between p21ras, the MAPKK/ERK cascade and Rac-1 in T cells in order to determine the role of these proteins in p21ras regulation of NFAT. The data show that expression of a dominant negative MAPKK-1 mutant can inhibit NFAT induction, but expression of a constitutively active MAPKK-1 mutant, which could activate ERK-2 in T cells, could not substitute for constitutively active p21ras and synergize with calcium-dependent signals to induce NFAT. These studies collectively suggest that the ERK pathway is a necessary but not sufficient p21ras effector pathway for NFAT induction in T cells. Expression of a dominant negative Rac-1 mutant had a pronounced inhibitory effect on Ras induction of NFAT, which suggests that Rac-1 is an effector for p21ras in NFAT regulation. In fibroblasts, Rac-1-mediated intracellular signals participate in activation of Jun kinases and transactivation of c-*jun* in a mechanism analogous to p21ras regulation of ERK-2–Elk-1 (Minden *et al.*, 1995). The present data show that expression of constitutively active Rac-1 can stimulate AP-1 transcriptional activity in T cells, demonstrating that Rac-1 can also stimulate transcription factor pathways in T cells. However, constitutively active Rac-1 mutants could not synergize with calcium signals to induce NFAT, indicating that Rac-1 is necessary but not sufficient for all p21ras-mediated signalling pathways in T cells. Accordingly, p21ras effects on NFAT are not mediated by a single linear signalling pathway but must involve multiple effectors that include Rac-1 and MAPKK-1.

Results

The effects of inhibitory and active MAPKK-1 mutants on NFAT induction

Activation of p21ras in T cells can be prevented by expression of an inhibitory mutant of p21ras, N17Ras (Izquierdo *et al.*, 1993). Expression of N17Ras in T cells also suppresses TCR activation of the MAP kinases ERK-1 and -2, which correlates with the inhibitory effects of N17Ras on TCR activation of NFAT (Izquierdo *et al.*, 1993; Woodrow *et al.*, 1993b). The MAP kinases ERK-1 and -2 are activated by a kinase (MAPKK) that phosphorylates and stimulates the ERK-1 and -2 kinases directly. MAPKK is activated by protein phosphorylation, and mutation of phosphorylation sites at position 217 and/or 221 on MAPKK-1 from serine to alanine generates an inhibitory molecule that can suppress growth factor-mediated activation of the ERK kinase cascade (Cowley *et al.*, 1994). Accordingly, in initial experiments to explore whether regulation of ERKs explains p21ras control of NFAT, the effects of the inhibitory mutant of MAPKK 221A (DN MAPKK) on TCR regulation of NFAT were analysed. To assess the activity of the transcription factor NFAT, we employed transient transfection protocols and quantitation of the expression of a CAT reporter gene whose activity is regulated by an enhancer corresponding to the NFAT binding site. The data in Figure 1A show that this NFAT reporter gene is not expressed in unstimulated Jurkat cells but is induced in cells stimulated with the antibody UCHT-1 that triggers the TCR–CD3 complex. TCR induction of NFAT can be mimicked by the combination of stimuli that activate PKC and elevate intracellular calcium levels such as phorbol esters and calcium ionophores. PKC and p21ras independently induce NFAT and similarly independently activate ERK-2 (Izquierdo *et al.*, 1993; Williams *et al.*, 1995). There is thus the possibility that PKC and Ras signals for NFAT induction converge at the level of the MAP kinases. The data in Figure 1A show that the phorbol ester phorbol 12,13 dibutyrate (Pdbu) has no stimulatory effect on NFAT when used as a single stimulus, and the calcium ionophore ionomycin has only a weak inductive effect on NFAT. However, the combination of calcium ionophore and Pdbu synergizes to give maximal NFAT activity. Expression of the inhibitory MAPKK-1 mutant in T cells inhibits TCR induction of NFAT and also suppresses Pdbu and ionomycin induction of NFAT (Figure 1A). In four experiments, the inhibitory effect of the dominant negative MAPKK mutant on the TCR/NFAT response ranged from 77 to 50%, whereas the inhibitory effects of this mutant on Pdbu plus ionomycin responses ranged from 30 to 40%.

To monitor ERK activation in cells transfected with the dominant negative MAPKK-1 mutant, the inhibitory mutant was co-expressed in T cells with ERK–2tag, an epitope-tagged ERK-2 construct. Activated ERK-2 is phosphorylated and, as we have shown previously, activated ERK–2tag isolated from Pdbu- or TCR-activated T cells has reduced electrophoretic mobility in SDS–PAGE gels as compared with ERK–2tag isolated from quiescent cells (Izquierdo *et al.*, 1994a,b) (Figure 1B). Accordingly, the activation of ERK–2tag can be monitored sensitively by analysis of its electrophoretic mobility. The data in Figure 1B show that co-transfecting the dominant negative MAPKK-1 mutant with ERK–2tag prevents ERK–2tag phosphorylation as induced by either TCR or phorbol ester.

In further experiments, we examined the consequences of expressing a constitutively activated MAPKK-1 mutant on NFAT induction. Mutation of residues 217 and 221 on MAPKK-1 from serine to glutamic acid generates a constitutively active enzyme, MAPKK-1 217E/221E (MAPKK*). The expression of this constitutively active

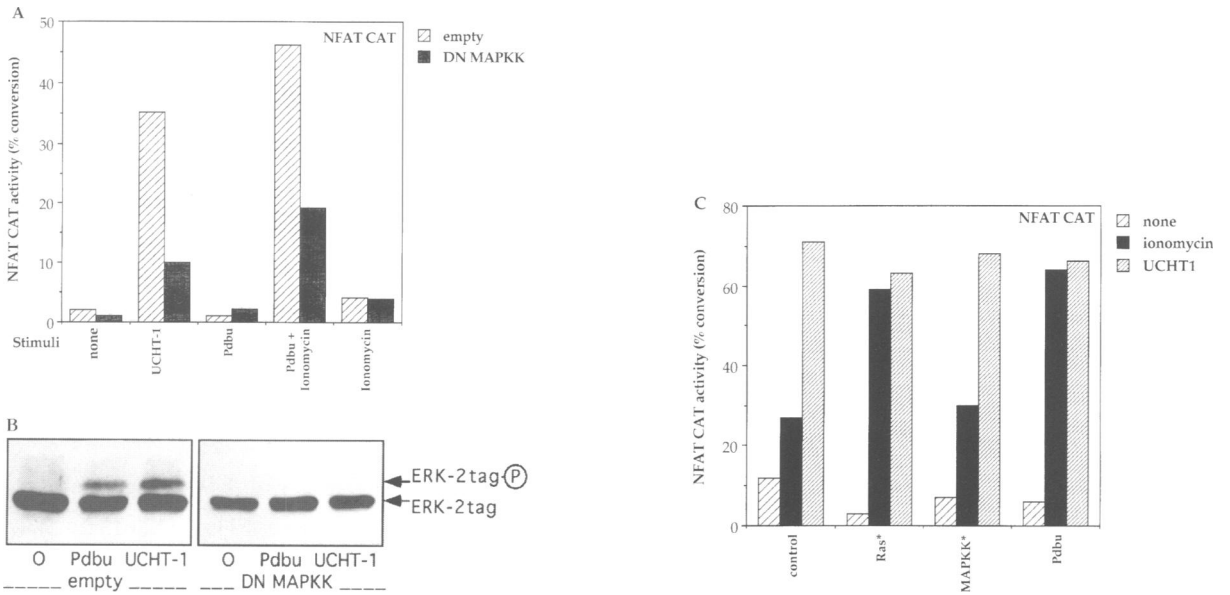

Fig. 1. The effects of an inhibitory MAPKK mutant on NFAT induction. (**A**) 10^7 Jurkat cells were co-transfected by electroporation with the NFAT–CAT reporter construct (15 μg) and the empty vector or the plasmid encoding the dominant negative MAPKK-1 mutant (20 μg of $pEXV_3$ MAPKK 221A, DN MAPKK) as described in Materials and methods. At 6 h after transfection, transfected cells were either left unstimulated, or stimulated with UCHT-1 (10 μg/ml), Pdbu (50 ng/ml), ionomycin (0.5 μg/ml) or a combination of Pdbu and ionomycin for 16 h before extracts were made, and CAT reporter activity was assessed. Data show the percentage conversion of chloramphenicol into the acetylated product. Results from one experiment representative of four are presented. (**B**) Jurkat cells were co-transfected with the pEF-BOS ERK–2tag construct (18 μg) together with the empty vector or with the inhibitory MAPKK-1 mutant (20 μg of $pEXV_3$ MAPKK 221A). At 16 h after transfection, cells were left unstimulated or were stimulated with UCHT-1 (10 μg/ml) or Pdbu (50 ng/ml) for 10 min. Total cellular extracts were made and run on SDS–PAGE (15% acrylamide–0.075% bisacrylamide). Data show Western blot analysis of ERK–2tag with the 9E10 antibody. (**C**) Jurkat cells were co-transfected with the NFAT–CAT (15 μg) reporter construct and the empty vector or the constitutively active Ras mutant (15 μg of $pEXV_3$ v-Ha-ras, Ras*) or constitutively active MAPKK-1 mutant (15 μg of $pEXV_3$ MAPKK 217E/221E, MAPKK*). After 6 h, cells transfected with the empty vector were either left unstimulated or were stimulated with Pdbu (50 ng/ml). All sets of transfected cells were then either left unstimulated or were stimulated with ionomycin (0.5 μg/ml) or UCHT-1 (10 μg/ml). CAT assay was performed as in (A). Data are shown as the percentage conversion of chloramphenicol into the acetylated product. Results from one experiment representative of five are presented.

MAPKK-1 will activate the MAP kinases ERK-1 and -2 and has been shown to substitute for activated p21ras in many cellular responses in fibroblasts and neuronal cells (Cowley *et al.*, 1994). The data in Figure 1C are representative of five similar experiments and show that NFAT responses were only marginally induced by the activated MAPKK-1 mutant in combination with ionomycin. No further induction of the NFAT–CAT reporter construct could be seen by increasing or decreasing the amount of activated MAPKK-1 DNA transfected (data not shown). In contrast, the constitutively active Ras mutant, v-Ha-ras, which has no effect on NFAT alone, synergizes strongly with ionomycin for NFAT induction. The data in Figure 1C thus show that the activated MAPKK-1 mutant does not mimic activated p21ras for NFAT induction.

To ensure that the failure of the active MAPKK-1 217E/221E mutant to regulate NFAT in T cells did not reflect the inability of MAPKK-1 to stimulate the ERKs, experiments to monitor ERK-2 activity in cells expressing the activated MAPKK-1 mutant were performed. In initial experiments, the activated MAPKK-1 mutant was co-expressed in T cells with ERK–2tag. The data in Figure 2A show that ERK–2tag isolated from T cells co-transfected with activated MAPKK-1 mutant has a reduced mobility in SDS–PAGE similarly to the ERK-2 isolated from activated T cells. To confirm that this mobility shift of ERK–2tag correlates with activation of the enzyme and to demonstrate that the activated MAPKK-1 could stimulate ERK-2 activity, kinase assays using myelin basic protein (MBP) as a substrate were carried out on ERK–2tag immunoprecipitates isolated from activated MAPKK-1 or control transfected cells. We have used this system previously to demonstrate that expression of activated p21ras in T cells is sufficient to stimulate ERK-2 kinase activity (Izquierdo *et al.*, 1993). TCR induction of ERK-2 is transient and peaks at 10–15 min post-stimulation. In the experiment presented in Figure 2B, it is this peak TCR response that is shown, whereas in the transient transfection experiments the sustained effects of either activated p21ras or MAPKK-1 mutants on ERK-2 are monitored. Expression of activated MAPKK-1 mutant induced an ~5-fold increase in ERK-2 kinase activity in T cells. The activated p21ras induction of ERK-2 activity is 50% of the maximal response induced by the TCR, which is consistent with our previous quantitation of ERK-2 activity in T cells expressing activated p21ras (Izquierdo *et al.*, 1993). The increase in ERK-2 activity in cells expressing activated MAPKK-1 mutant was to a level which is equivalent to the effects of activated p21ras on ERK-2 activity (Figure 2B).

The effector function of the transfected activated MAPKK-1 mutant on the endogenous ERK in Jurkat cells was also examined by assessing its ability to regulate phosphorylation of the transcription factor Elk-1, which is a cellular substrate for ERK-2 (Marais *et al.*, 1993). Elk-1 is one of a family of proteins that can form a ternary complex with the transcriptional activator SRF and thus can play a key role in regulation of c-*fos* gene expression

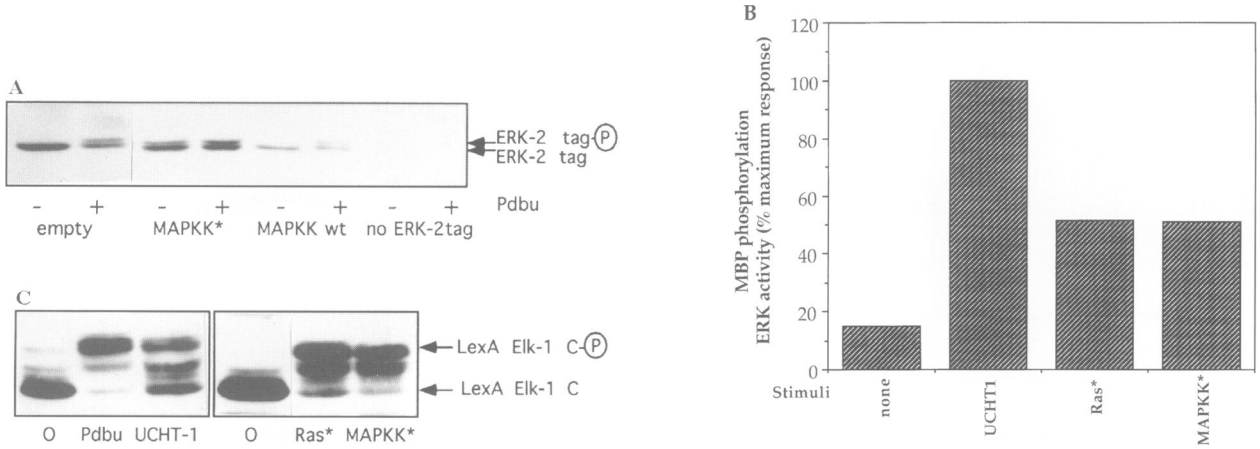

Fig. 2. The activated MAPKK mutant is able to activate ERK. (**A**) Jurkat cells were co-transfected with the pEF-BOS ERK–2tag construct (12.5 μg) together with the empty vector, or with the activated MAPKK-1 mutant (12.5 μg of $pEXV_3$ MAPKK 217E/221E) or the wild-type MAPKK-1 (12.5 μg $pEXV_3$ MAPKK wt). At 16 h post-transfection, cells were left unstimulated or were stimulated with Pdbu 50 ng/ml for 10 min. Total cellular extracts were made and run on SDS–PAGE. Data show Western blot analysis of ERK–2tag with the 9E10 antibody. (**B**) Jurkat cells were co-transfected with the pEF BOS ERK–2tag construct (15 μg) together with 25 μg of the empty vector or with the activated Ras (25 μg of $pEXV_3$ v-Ha-ras) or MAPKK-1 (25 μg of $pEXV_3$ MAPKK 217E/221E) mutants. At 16 h post-transfection, cells were left unstimulated or were stimulated with UCHT-1 (10 μg/ml) for 10 min. Cells were lysed, the ERK–2tag was immunoprecipitated with the 9E10 antibody and kinase activity was assessed in a kinase assay using MBP as substrate. Radioactivity incorporated into MBP was quantified with the phosphoimager (Molecular Dynamics). Data show the MBP kinase activity in ERK–2tag precipitates presented as a percentage of the maximal response obtained by TCR stimulation. (**C**) Jurkat cells were co-transfected with 15 μg of the plasmid encoding the fusion protein, pEF N Lex–Elk-1C, together with 20 μg of the empty vector or with the activated Ras (20 μg of $pEXV_3$ v-Ha-ras) or MAPKK-1 (20 μg of $pEXV_3$ MAPKK 217E/221E) mutants. At 16 h post-transfection, cells were transfected with the empty vector and were either left unstimulated or were stimulated with UCHT-1 (10 μg/ml) or Pdbu (50 ng/ml) for 30 min. Total cell extracts were made and run on SDS–PAGE. Data show Western blot analysis of LexA–Elk-1C with LexA antibodies.

(Hill and Treisman, 1995). It has been shown previously that activators of the ERK kinase cascade can induce phosphorylation of the Elk-1 C-terminus and potentiate Elk-1 transcriptional activity (Price *et al.*, 1995b). To monitor this phosphorylation, a fusion protein comprising the C-terminus of Elk-1 linked to the LexA repressor (LexA–Elk-1C) was co-transfected into T cells with activated p21ras or MAPKK-1 mutants. The phosphorylation of LexA–Elk-1C reduces its electrophoretic mobility in SDS–PAGE gels. Accordingly, the data in Figure 2C show the reduced electrophoretic mobility of LexA–Elk-1 in Pdbu- or TCR-activated cells compared with control unstimulated cells. LexA–Elk-1C isolated from T cells expressing either activated p21ras or activated MAPKK-1 mutant has a similar reduced electrophoretic mobility (Figure 2C). The activated MAPKK and activated p21ras had equivalent stimulatory effects on the transcriptional activity of Elk-1 (data not shown).

In summary, the data in Figures 1 and 2 show that expression of a dominant negative mutant of MAPKK-1 suppresses TCR induction of NFAT. The data demonstrate also that expression of an activated MAPKK-1 mutant in T cells is sufficient for ERK-2 activation and sufficient for phosphorylation of the transcription factor Elk-1. However, expression of a constitutively active MAPKK-1 mutant is not sufficient to substitute for p21ras for NFAT induction. Accordingly, to explain p21ras regulation of NFAT in T cells, it is necessary to invoke the existence of alternative Ras effector pathways.

The effects of inhibitory and activated mutants of Rac-1 on NFAT induction

The Ras-related GTPase Rac-1 has been identified as a downstream effector of p21ras in fibroblast transformation (Qiu *et al.*, 1995). The role of Rac-1 in NFAT regulation in T cells was explored by monitoring the effects of a dominant negative Rac-1 mutant, N17Rac, and a constitutively active Rac-1 mutant, V12Rac (Rac*) (Nobes and Hall, 1995). The data in Figure 3A show that expression of the dominant negative N17Rac molecule inhibits NFAT induction by the TCR and also, although to a lesser extent, suppresses the NFAT response induced by phorbol ester and calcium ionophore. In an experiment that investigated the N17Rac dose dependency of NFAT induction, it appeared that Pdbu and ionomycin induction of NFAT comprised an N17Rac-sensitive and -insensitive component (Figure 3B). To examine the selectivity of the effects of the dominant negative mutant of Rac-1 on TCR signalling, the effects of N17Rac-1 on TCR and Pdbu activation of ERK-2 were determined. Accordingly, T cells were transfected with ERK–2tag either alone or together with N17Rac. The data in Figure 3C show that TCR or Pdbu stimulation or transfection of activated p21ras induces ERK-2 activation while expression of activated Rac mutant has no effect on ERK-2 activity. Co-expressing N17Rac in these cells did not affect p21ras, TCR or Pdbu activation of ERK-2, showing that the inhibitory effects of N17Rac did not result from inhibition of ERK-2 (Figure 3C).

To explore further the role of Rac-1 in NFAT regulation, the effects of an activated Rac-1 mutant (V12Rac) on transcription factor activity were compared with the effects of the activated p21ras mutant. p21ras can regulate the transcription factor AP-1 activity in a variety of cells including T cells (Rayter *et al.*, 1992; Woodrow *et al.*, 1993b). To compare p21ras and Rac-1 regulation of the activity of AP-1, we quantified the expression of an AP-1–CAT reporter gene. The AP-1 reporter gene can be induced

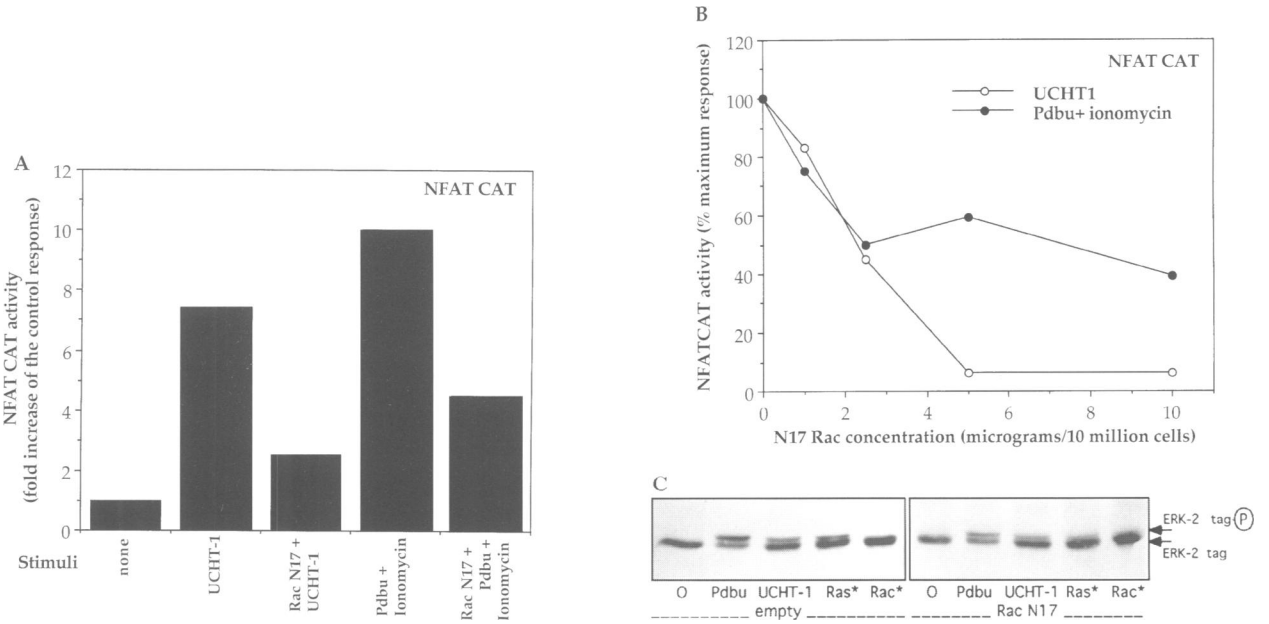

Fig. 3. The effects of an inhibitory Rac mutant on NFAT induction. (**A**) Jurkat cells were co-transfected with the NFAT–CAT reporter construct (12 μg) and 5 μg of the empty vector or the dominant negative Rac mutant-containing plasmid (5 μg of pcDNA1 N17Rac). At 6 h post-transfection, cells were either left unstimulated or were stimulated with UCHT-1 (10 μg/ml) or a combination of Pdbu (50 ng/ml) and ionomycin (0.5 μg/ml) for 16 h before extracts were made, and CAT reporter activity was assessed. Data show fold induction of NFAT–CAT activity. Results from one experiment representative of five are presented. (**B**) Jurkat cells were co-transfected with the NFAT–CAT reporter construct (12 μg) and the empty vector or different concentrations of the inhibitory Rac mutant (pcDNA1 N17Rac), and the experiment was carried out as in (A). Data are shown as a percentage of the maximum NFAT–CAT induction obtained with UCHT-1 stimulation in the absence of the inhibitory Rac mutant. (**C**) Jurkat cells were co-transfected with the ERK–2tag construct (18 μg) together with 8 μg of the empty vector, or plasmids encoding active Ras (8 μg of pEF-BOS v-Ha-ras) or the active Rac (8 μg of pEFplink V12Rac, Rac*) mutants, in the absence or presence of 5 μg of the inhibitory Rac mutant (pcDNA1 N17Rac). At 16 h post-transfection, cells transfected with the empty vector or with the plasmid encoding the inhibitory mutant were either left unstimulated or were stimulated with UCHT-1 (10 μg/ml) or Pdbu (50 ng/ml) for 10 min. Total cellular extracts were made and run on SDS–PAGE. The data show Western blot analysis of ERK–2tag with the 9E10 antibody.

by Pdbu, TCR triggering with UCHT1 antibodies and constitutively active p21ras protein (Figure 4A). AP-1 activity is also induced by the activated Rac-1 mutant (Figure 4A). Further experiments demonstrate that TCR and Pdbu induction of AP-1 activity in T cells are prevented by expression of the dominant negative Rac-1 mutant N17Rac (Figure 4B). These data show that Rac-1 activity is essential for TCR and Ras regulation of AP-1 in T cells. Moreover, expression of constitutively activated Rac-1 is sufficient to stimulate levels of AP-1 activity comparable with that seen in cells expressing activated p21ras mutants (Figure 4A). To compare p21ras and MAPKK-1 regulation of the activity of AP-1, we quantified the expression of the AP-1–CAT reporter gene in cells expressing either constitutively active p21ras protein or activated MAPKK-1. The data in Figure 4C show that AP-1–CAT is not induced by expression of activated MAPKK-1 mutant in T cells.

Since AP-1 is a component of the active NFAT complex in T cells, we also assessed the effects of activated Rac-1 on NFAT responses in T cells. The data in Figure 5A show that activated Rac-1 could not mimic activated p21ras and synergize with calcium signals for NFAT induction. No NFAT induction could be seen when using the activated Rac mutant, in conditions for which activated Ras was effective (Figure 5B) and under conditions where activated Rac-1 could induce AP-1. Given that neither expression of activated MAPKK-1 nor activated Rac-1 substitute for p21ras for NFAT, we investigated the possibility of a synergy between MAPKK-1 and Rac-1. Data presented in Figure 5C show that the combination of activated Rac-1 and activated MAPKK could not synergize with calcium signals to induce NFAT. No further induction of the NFAT–CAT reporter construct could be seen when transfecting different ratios of the two plasmids (data not shown). The data in Figures 1–5 collectively show that MAPKK-1 and Rac-1 function are each important but not sufficient, neither alone nor in combination, for TCR and p21ras regulation of NFAT.

p21ras regulation of NFAT and AP-1 in T cells requires Rac-1 function

To position Rac-1 in p21ras signalling pathways, the effect of the dominant negative Rac-1 mutants on signals generated by the constitutively active p21ras mutant were monitored. Expression of N17Rac-1 did not prevent expression of the constitutively active p21ras mutant in T cells (data not shown). Moreover, activated Rac-1 did not induce activation of ERK-2 (Figure 3C), nor did expression of N17Rac prevent p21ras- or Pdbu-mediated activation of ERK-2 (Figure 3C). Results presented in Figure 6A show that expression of N17Rac did not prevent p21ras-mediated phosphorylation of Elk-1, which is consistent with the failure of the dominant negative Rac-1 to prevent p21ras-mediated activation of the ERK-2 pathway (Figure 3C). Activators of the ERK kinase cascade can induce transcriptional activation by the Elk-1 C-terminus (Price *et al.*, 1995b; Hill and Treisman, 1995). To monitor Elk-1 transcriptional activity, LexA–Elk-1C was co-transfected into T cells with a LexA operator-controlled CAT reporter

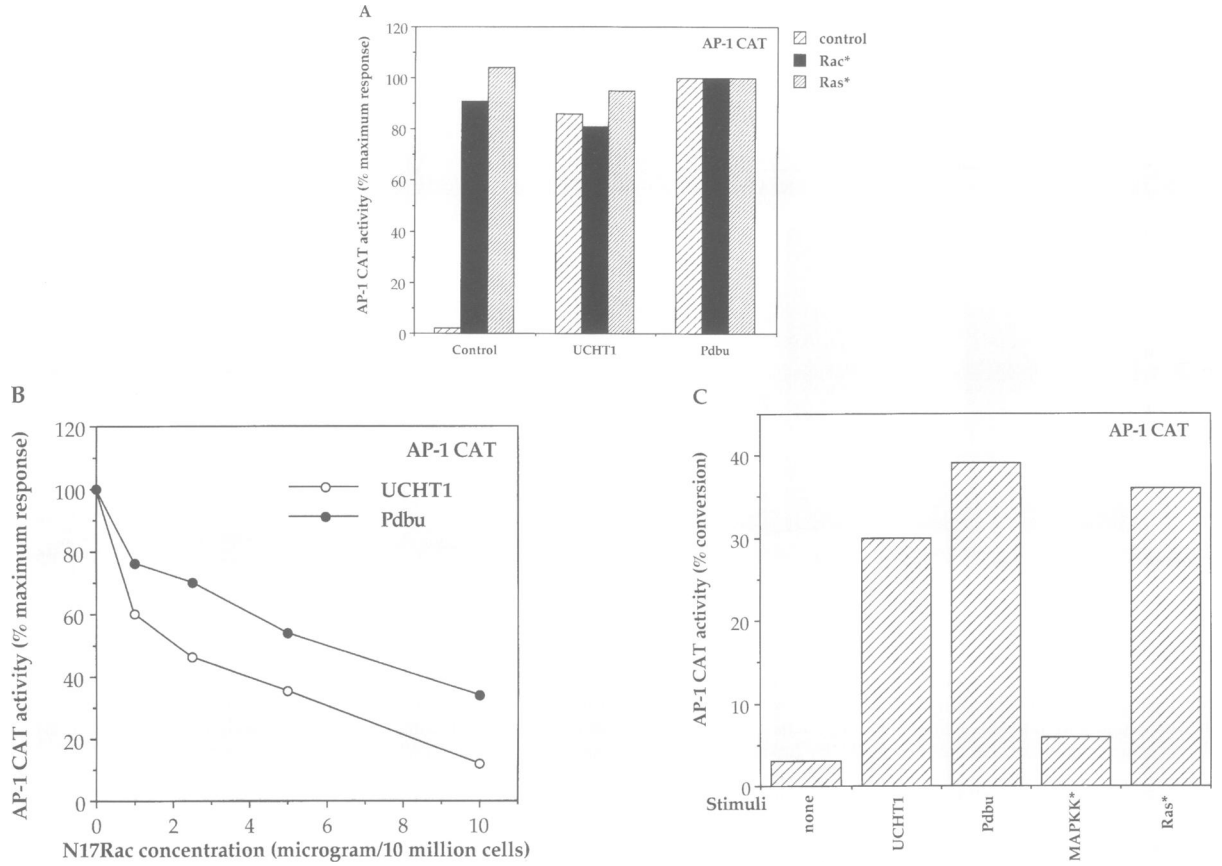

Fig. 4. The effects of Rac and MAPKK mutants on AP-1 induction. (**A**) Jurkat cells were co-transfected with the AP-1–CAT reporter construct (2 μg) and 8 μg of the empty vector or the plasmid encoding the constitutively active Ras (pEF-BOS v-Ha-ras) or active Rac (pEFplinkV12Rac) mutants. After 6 h, transfected cells were either left unstimulated or were stimulated with either UCHT-1 (10 μg/ml) or Pdbu (50 ng/ml) for 16 h before extracts were made, and CAT reporter activity was assessed. Data are presented as a percentage of the maximum AP-1–CAT response induced by Pdbu. (**B**) Jurkat cells were co-transfected with the AP-1–CAT reporter construct (2 μg) and the empty vector or different concentrations of the plasmid encoding the inhibitory Rac mutant (pcDNA1 N17Rac), and the experiment was carried out as in (A). Data are shown as a percentage of the maximum response obtained with Pdbu stimulation in the absence of the inhibitory Rac mutant. (**C**) Jurkat cells were co-transfected with the AP-1–CAT reporter construct (2 μg) and 15 μg of the empty vector or the constitutively active Ras mutant (15 μg of $pEXV_3$ v-Ha-ras) or constitutively active MAPKK-1 mutant (15 μg of $pEXV_3$ MAPKK 217E/221E). At 6 h post-transfection, cells transfected with the empty vector were either left unstimulated or were stimulated with either UCHT-1 (10 μg/ml) or Pdbu (50 ng/ml) for 16 h before extracts were made, and CAT reporter activity was assessed. Data are shown as a percentage of the conversion of chloramphenicol into the acetylated product.

gene (LexA–OP.tkCAT). LexA–Elk-1C transcription activity is low in unactivated T cells but can be induced by co-expression of a constitutively active p21ras, v-Ha-Ras, or by stimulation of T cells with Pdbu (Figure 6B). Expression of N17Rac did not suppress Pdbu or v-Ha-ras induction of LexA–Elk-1C. In contrast, in the same experiment, N17Rac inhibits the AP-1 response induced by the constitutively active p21ras (Figure 6C) and also prevents NFAT induction induced by activated p21ras plus calcium ionophore (Figure 6D). These data thus indicate that Rac-1 function is required for p21ras induction of AP-1 and NFAT but Rac-1 function is not necessary for p21ras activation of ERK-2 and Elk-1.

Expression of activated Rac-1 did not activate ERK-2 in T cells (Figure 3C) nor was Rac-1 function necessary for TCR- and p21ras-mediated stimulation of ERK-2 (Figure 3C). Nevertheless, it has been described that Rac-1 plays a role in AP-1 regulation via control of Jun kinases such as JNK/SAPK. Elk-1 has also been described to be a substrate for the JNK/SAPK family (Whitmarsh *et al.*, 1995; Zinck *et al.*, 1995), and to explore if Rac-1 could regulate transcription factor phosphorylation in T cells, we examined the effects of activated Rac-1 on LexA–Elk-1C phosphorylation. The data in Figure 6A show that expression of V12Rac-1 in T cells induced phosphorylation of LexA–Elk-1C comparable with that induced by activated p21ras. Rac-1 function is not required for p21ras induction of Elk-1 (Figure 6A and B) which is mediated via ERK-2. Nevertheless, these results indicate that, in T cells, Rac-1 has the potential to regulate a kinase cascade that can converge with the ERK-2 pathway on the transcription factor Elk-1.

Discussion

The MAP kinase ERK-2 has been proposed to be a major effector of the p21ras signal transduction pathways in a variety of cell systems including the differentiation of PC12 cells and the transformation of fibroblasts. The present study has used constitutively active and inhibitory mutants of a MAP kinase kinase (MAPKK-1) to examine the role of this pathway in TCR signal transduction. Activated mutants of MAPKK-1 can stimulate ERK-2 kinase activity in T cells and can also induce phosphoryl-

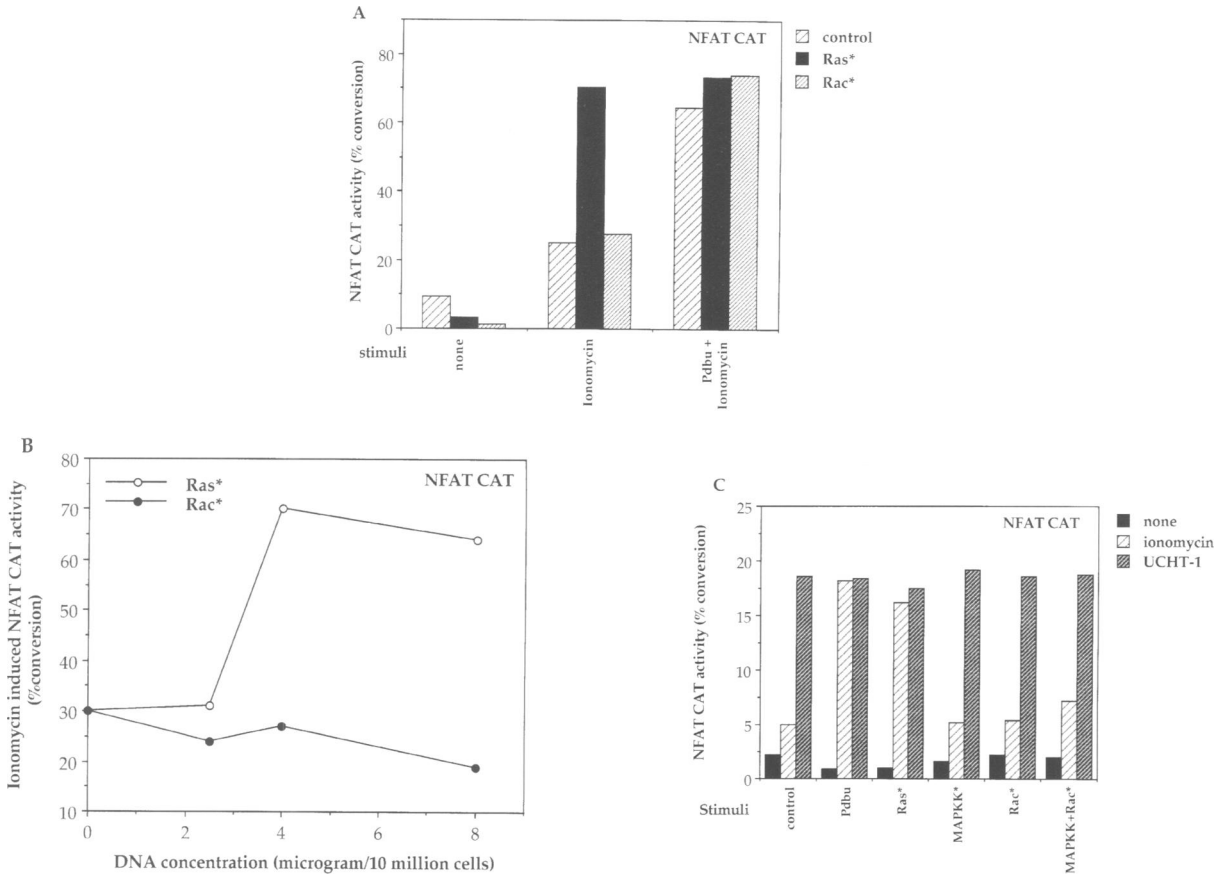

Fig. 5. Comparison of the effects of the activated Rac and Ras mutant on NFAT induction. (**A**) Cells were co-transfected with the NFAT–CAT reporter construct (12 μg) and 8 μg of the empty vector or the plasmid encoding activated Ras (8 μg of pEF-BOS v-Ha-ras) or activated Rac (8 μg of pEFplinkV12Rac) mutants. Transfected cells were either left unstimulated or were stimulated with ionomycin (0.5 μg/ml) or a combination of Pdbu (50 ng/ml) and ionomycin (0.5 μg/ml), 6 h post-transfection and for 16 h before extracts were made, and CAT reporter activity was then assessed. Data show the percentage conversion of chloramphenicol into the acetylated product. Results from one experiment representative of four are presented. (**B**) Jurkat cells were co-transfected with the NFAT–CAT reporter construct (12 μg) and the empty vector or different concentrations of the plasmid encoding the activated Ras (pEF-BOS v-Ha-ras) or activated Rac (pEFplinkV12Rac) mutants, and the experiment was carried out as in (A). Data are shown as a percentage of the conversion of chloramphenicol into the acetylated product. (**C**) Plasmids encoding the activated Ras (15 μg of $pEXV_3$ v-Ha-ras), the activated MAPKK-1 (15 μg $pEXV_3$ MAPKK 217E/221E) or the activated Rac (15 μg of pEFplinkV12Rac) mutants, or a combination of the activated MAPKK-1 (15 μg of $pEXV_3$ MAPKK 217E/221E) and activated Rac-1 (15 μg of pEFplinkV12Rac) mutants were co-transfected with the NFAT–CAT reporter construct (12 μg). Cells transfected with the empty vector were either left unstimulated or were stimulated with Pdbu (50 ng/ml). Transfected cells were then either left unstimulated or stimulated with ionomycin (0.5 μg/ml) or UCHT-1 (10 μg/ml) for 16 h before extracts were made, and CAT reporter activity was assessed. Data show the percentage conversion of chloramphenicol into the acetylated product. Results from one experiment representative of four are presented.

ation of the ternary complex factor Elk-1, demonstrating that MAPKK-1 is an effector molecule in the regulation of the transcription factor Elk-1 in T cells as in fibroblasts (Hill and Treisman, 1995). The transcription factor NFAT is controlled by the TCR by a mechanism involving a synergistic interaction of p21ras and calcium signals. Experiments using an inhibitory MAPKK-1 mutant indicate that the activation of the MAPKK-1/ERK-1, -2 cascade plays a role in TCR activation of NFAT because TCR stimulation of NFAT was prevented by the inhibitory form of MAPKK-1. However, activated MAPKK-1 could not substitute for p21ras signals and synergize with calcium signals to stimulate NFAT. A comparison of the effects of inhibitory p21ras or MAPKK-1 mutants on thymocyte development in transgenic mice concluded recently that p21ras functions in T cells cannot be mediated solely by the ERK-2 signalling cascade (Alberola-lla *et al.*, 1995; Swan *et al.*, 1995). The failure of the activated MAPKK-1 to substitute for activated p21ras in NFAT induction confirms this idea and thus clearly demonstrates that additional p21ras effector pathways must exist in T cells.

Recently, the p21ras-related GTPase Rac-1 has been found to be an important mediator of oncogenic transformation by Ras (Qiu *et al.*, 1995). Here we describe a role for Rac-1 in transcription factor regulation in T cells. A dominant negative mutant of Rac-1 did not suppress TCR or p21ras activation of the MAP kinase ERK-2 but could suppress TCR and p21ras induction of NFAT and AP-1. These data are consistent with the existence of a Rac-1-controlled signalling pathway operating in parallel with the ERK-2 pathway to mediate p21ras induction of AP-1 and NFAT. Importantly, experiments with a constitutively active Rac-1 mutant showed that Rac-1 was a sufficient signal for activation of AP-1, although the active Rac-1 mutant could not substitute for the activated p21ras mutant for NFAT induction. Expression of activated MAPKK-1 in T cells could stimulate ERK-2, and the activated Rac-1 mutant can stimulate parallel kinase signal-

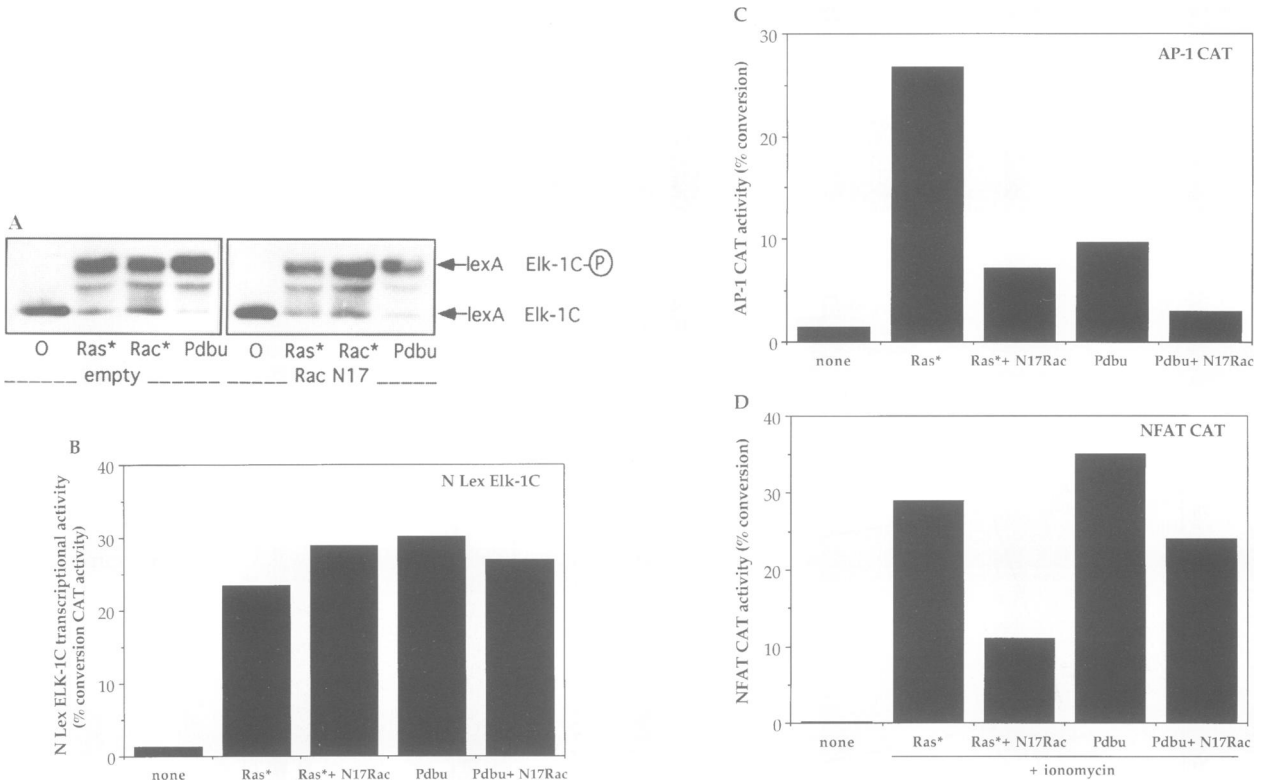

Fig. 6. p21 ras regulation of NFAT and AP-1 requires Rac-1 function. (**A**) Jurkat cells were co-transfected with the pEF N Lex–Elk-1C fusion protein expression construct (12 μg) together with 8 μg of the empty vector or with the plasmid encoding activated Ras (8 μg of pEF-BOS v-Ha-ras) or activated Rac (8 μg of pEFplinkV12Rac) mutants and in the absence or presence of the plasmid encoding the inhibitory Rac mutant (8 μg of pcDNA1 N17Rac). At 16 h post-transfection, cells transfected with the empty vector were either left unstimulated or were stimulated with Pdbu (50 ng/ml) for 30 min. Total cell extracts were made and run on SDS–PAGE. The data show Western blot analysis of LexA–Elk-1C with LexA antisera. (**B**), (**C**) and (**D**) Jurkat cells were co-transfected with the MLV N Lex–Elk-1C (6 μg) fusion protein expression construct and its reporter construct LexA–OP.tkCAT (3 μg) (B), AP-1–CAT (2 μg) (C) or NFAT–CAT (12 μg) (D) reporter constructs together with 15 μg of the empty vector or with the plasmid encoding the activated Ras mutant (15 μg of pEF-BOS v-Ha-ras) and in the absence or presence of 2.5 μg of the plasmid encoding the inhibitory Rac mutant (pcDNA1 N17Rac). At 6 h post-transfection, cells transfected with the empty vector or with pcDNA1 N17Rac alone were either left unstimulated or were stimulated with Pdbu (50ng/ml) for 16 h. CAT assays were carried out as before.

ling pathways that could phosphorylate and transcriptionally activate Elk-1 in T cells. Nevertheless, the combination of activated MAPKK-1 and Rac-1 could not substitute for activated p21ras and synergize with calcium signals to induce NFAT. There must, therefore, be at least one more p21ras effector required for NFAT induction (Figure 7). There are several additional candidate p21ras effectors including the GTPase Rho (Prendergast *et al.*, 1995), and also the GTPase Ral (Hofer *et al.*, 1994; Spaargaren and Bischoff, 1994), whose function in NFAT regulation remain to be investigated. p21ras effects on the MAPKK-1/ERK-2 cascade in T cells are mediated by the serine kinase Raf-1 (Izquierdo *et al.*, 1994a). It is not clear whether or not there are multiple Raf-1 effector pathways in T cells but, in fibroblasts, Raf-1 and Rac-1 signals synergize to mediate cellular transformation by p21ras (Qiu *et al.*, 1995). We currently are exploring functional interactions between Raf-1 and Rac-1 in NFAT induction. In this context, Jurkat cells expressing constitutively active Raf-1 show enhanced levels of IL-2 production but there has not been a direct analysis of the role of Raf-1 in NFAT regulation (Owaki *et al.*, 1993).

Questioning how p21ras would regulate Rac-1, a couple of recent reports raise some possibilities. Thus, a report has described phosphatidyl inositol 3′ kinase (PtdIns 3 kinase) as an upstream regulator of Rac-1 in fibroblasts, particularly in regulating cell adhesion and morphology (Rodriguez-Viciana *et al.*, 1994; Nobes and Hall, 1995). Also, PtdIns 3 kinase can bind directly to activated p21ras, and function as an effector molecule for p21ras in neuronal cells (Rodriguez-Viciana *et al.*, 1994). There is, therefore, the possibility that PtdIns 3 kinase could be the link between Ras and Rac-1. Preliminary experiments show that PtdIns 3 kinase inhibitors (wortmannin and LY294002) fail to show any inhibitory effect on TCR induction of NFAT (E.Genot, K.Reif and D.A.Cantrell, unpublished data). The failure of PtdIns 3 kinase inhibitors to regulate NFAT is not compatible with a role for PtdIns 3 kinase as a mediator of p21ras effects on NFAT. Accordingly, the signalling links between p21ras, PtdIns 3 kinase and Rac-1 in T cells may be different from those described in other cell lineages. However, this question has to be investigated further with different and complementary approaches. Concerning the Rac-1 effector pathway for NFAT induction, Rac-1-regulated protein kinases have now been identified (Manser *et al.*, 1994; Martin *et al.*, 1995), but the role of these protein kinases in Rac-1 effector pathways in T cells remains to be discovered.

The transcriptional activity of the NFAT binding site used in the present experiments is mediated by a complex comprising a member of the NFAT group of DNA binding proteins and AP-1 family proteins (Rao, 1994). The AP-1

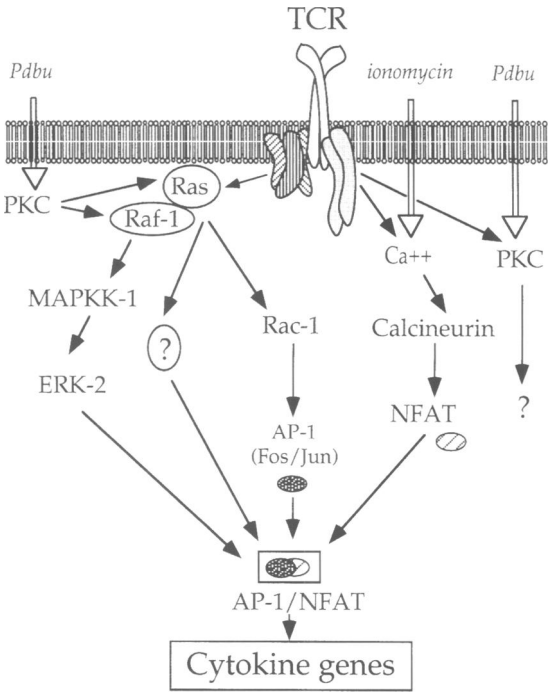

Fig. 7. Schematic representation of signalling pathways predicted to induce NFAT. Our model for NFAT induction shows calcium/calcineurin signals regulating NFAT translocation from the cytosol to the nucleus. p21ras /Rac-1 regulation of NFAT involves regulation of AP-1. MAPKK-1 signalling pathways are also required for NFAT induction and, since MAPKK-1 does not regulate AP-1, we would predict that MAPKK-1 pathways regulate NFAT itself or the NFAT–AP-1 complex. To explain why the combination of activated MAPKK-1 and activated Rac-1 do not substitute for activated p21ras for NFAT induction, we have to evoke the existence of at least one more p21ras signalling pathway. The contribution of PKC to NFAT regulation is complicated because the TCR activates PKC but this is not required for NFAT induction. Phorbol esters can regulate NFAT, but this occurs by an independent PKC-mediated pathway that converges on MAPKK-1 and Rac-1.

complex is composed of dimers of members of the Fos and Jun family of proteins. The exact nature of the AP-1 component of NFAT in T cells has not yet been established clearly. Boise *et al.* have described that a Fra-1–JunB heterodimer is the inducible nuclear component of the NFAT binding activity, while Northrop *et al.* have reported a dimer composed of c-Fos (or Fra-1) and either c-Jun or JunD, suggesting that the nuclear subcomponent may be represented by a heterogeneous population of AP-1–NFAT complexes (Boise *et al.*, 1993; Northrop *et al.*, 1993). It is not clear whether the AP-1 reporter gene used in the present experiments measures the activity of the AP-1 components of NFAT or of distinct AP-1 family members. Nevertheless, the present AP-1 reporter gene data are valuable because they show that, in T cells, Rac-1 function is both necessary and can be sufficient for AP-1 induction and that Rac-1 can mediate Ras effects on AP-1. Moreover, the activated Rac-1 mutants were equivalent to the activated Ras mutants in the context of AP-1 induction. This role of Rac-1 in AP-1 regulation could thus explain Rac-1 requirement for NFAT induction. Accordingly, to consider the possible Rac-1 effector pathways in T cells, it is necessary to focus on the mechanisms for AP-1 induction. In general, the activation of AP-1 results from increased expression of Fos and Jun family proteins and the phosphorylation of Fos and Jun proteins which up-regulates their transcription activity. Several parallel MAP kinase signalling pathways involving ERK-1, -2, JNK-1, -2 and MPK2/p38 and a Ras-regulated Fos kinase can all potentially contribute to AP-1 activation (Deng and Karin, 1994; Cobb and Goldsmith, 1995; Coso *et al.*, 1995; Hill and Treisman, 1995). The TCF Elk-1 which contributes to increased c-*fos* expression via complex formation with the ubiquitous transcription factor SRF is a substrate for the ERKs and JNK-1, -2 (Marais *et al.*, 1993; Whitmarsh *et al.*, 1995). c-*jun* is also transcriptionally regulated by MAP kinase pathways, is a direct substrate for JNK-1 and can be activated by JNK-1-mediated phosphorylation (Dérijard *et al.*, 1994). Recent studies suggest that Rac-1 plays a central role in the activation of the MAP kinases JNK and p38 that is exactly analogous to the role of p21ras in the ERK pathways (Coso *et al.*, 1995; Minden *et al.*, 1995). The present data show that Rac-1 signals are necessary and sufficient to induce Elk-1 phosphorylation in T cells, which indicates that Rac-1 signals regulate transcriptional factor kinase pathways in T cells. Future studies will establish whether Rac-1 effects on Elk-1 phosphorylation in T cells are mediated by JNK-1/SAPK, p38 or a novel protein kinase pathway. By analogy with other models for transcription factor regulation by p21ras-related GTPases, then Rac-1 effects on NFAT could be mediated by one of the MAP kinase pathways. Clearly Rac-1 does not regulate ERK-2 in T cells, but it is possible that the role of Rac-1 in TCR induction of NFAT involves JNK-1/SAPK or the p38 MAP kinase. It is possible, moreover, that the role of Rac-1 in TCR induction of NFAT will be analogous to the role of Rac-1 in serum regulation of SRF transcriptional activity in fibroblasts, which occurs by an unknown Rho/Rac-1-controlled signalling pathway that does not involve ERK-2, JNK-1 or p38 (Hill *et al.*, 1995).

Rac-1 may mediate p21ras regulation of AP-1 and NFAT in T cells but, if the signalling connections between p21ras and Rac-1 in T cells are as complicated as those in fibroblasts, then it is unlikely that p21ras and Rac-1 will function solely in a linear signalling cascade and it is more likely that Rac-1 and p21ras will function in both parallel and convergent pathways. Rac-1 has diverse roles in many cellular systems. For example, in neutrophils, Rac-1 is involved in the activation of the NADPH oxidase complex (Xu *et al.*, 1994). In fibroblasts, Rac-1 plays a critical role in the regulation of the actin cytoskeleton (Nobes and Hall, 1995) but can also regulate transcriptional activity of SRF and controls c-*jun* transcriptional activity via activation of the c-Jun kinases JNK-1/SAPK (Coso *et al.*, 1995; Hill *et al.*, 1995; Minden *et al.*, 1995). In this context, the activation of JNK-1 in T cells is an integration point for signals from the co-stimulatory receptor CD28- and TCR-mediated calcium/calcineurin signals (Su *et al.*, 1994). It will thus be of interest to determine in future studies whether Rac-1 is an integration point for TCR and CD28 signals for JNK-1/SAPK activation.

In summary, the present study has examined p21ras effector pathways in a T cell line Jurkat in an attempt to characterize the signals contributed by p21ras for activation of NFAT. One p21ras effector pathway involves MAPKK-1, which is shown by the present data to be both necessary and sufficient for activation of ERK-2 and the

transcription factor Elk-1 in Jurkat cells. Rac-1 is a sufficient and necessary signal for AP-1 activation and also appears to be involved in p21ras effector pathways in Jurkat, since p21ras-mediated induction of AP-1 and NFAT requires Rac-1 function. MAPKK-1- and Rac-1-mediated signals may be sufficient for either ERK-2 or AP-1 activation but, either alone or in combination, they cannot substitute fully for p21ras signals for induction of NFAT. There must, therefore, be at least one more p21ras effector pathway that is required for NFAT induction. The Jurkat T cell line used in the present study has proved to be a valuable model system for studies of NFAT regulation. It would thus seem likely that there will be multiple Ras effectors in normal T cell populations that contribute to NFAT regulation and hence cytokine gene induction. This is analogous to the transformation of fibroblasts where Ras effects are mediated by multiple effectors. The Rac-1 effector pathway for NFAT induction is not yet characterized. Nevertheless, the NFAT family of transcription factors are important in the regulation of cytokine genes and hence important for a successful immune response. The present data predict that Rac-1 and Rac-1 signalling pathways would be important targets for immune intervention.

Materials and methods

Reagents

Ionomycin (Ca^{2+} salt) and phorbol-12,13 dibutyrate were from Calbiochem Corp. (UK). [^{14}C]Acetyl coenzyme A (at 50–60 mCi/mmol) was from Amersham International (Buckinghamshire, UK). Other reagents were from Sigma. Monoclonal antibodies UCHT-1 and 9E10 reactive with human CD3 and the c-Myc epitope used a tag for ERK-2 were affinity purified from hybridoma supernatants at I.C.R.F. Rabbit antisera specific for LexA were a gift of Richard Treisman and Alberto Gandarillas (ICRF). Polyclonal rabbit anti-mouse and goat anti-rabbit antibodies are from Amersham International (Buckinghamshire, UK).

Plasmid constructs

The reporter construct AP-1–CAT, containing three copies of the sequence for the AP-1 site inserted in pBLCAT2, has been described previously (Williams *et al.*, 1995). LexA–OP.tkCAT contains two copies of the LexA reporter, NFAT–CAT contains three copies of the NFAT binding site TAAGGAGGAAAAACTGTTTCATACAGAAGGCG upstream of the IL-2 minimal promoter driving the reporter gene *CAT* (Verweij *et al.*, 1990). Plasmids directing expression of wild-type MAPKK-1: $pEXV_3$ MAPKKwt, constitutively active MAPKK-1: $pEXV_3$ MAPKK 217E/221E, the dominant negative MAPKK-1: $pEXV_3$ MAPKK 221A and constitutively active p21ras: pEF-BOS v-Ha-ras or $pEXV_3$ v-Ha-ras, pEF BOS ERK–2 tag, MLV N-LexA–Elk-1C fusion protein or pEF N-LexA–Elk-1C fusion protein, have already been described (Medema *et al.*, 1991; Cowley *et al.*, 1994; Leevers *et al.*, 1994; Price *et al.*, 1995a). The active and dominant negative mutants of Rac-1 (V12Rac-1 and N17Rac-1) have also been described previously (Nobes and Hall, 1995). All plasmids were purified by equilibrium centrifugation in CsCl–ethidium bromide gradients by standard procedures.

Cells and transfections

J-TAg, a subline of the human T acute lymphocytic lymphoma cell line Jurkat, was maintained in RPMI supplemented with 10% heat-inactivated fetal calf serum (FCS), at 37°C in 5% CO_2 in humidified air. Cells were transfected via electroporation (Gene pulser: Bio-Rad, UK) as previously described. Briefly, cells were pulsed (1×10^7 cells/0.5 ml) in complete medium at 960 μF and 320 V. Cells transfected with similar plasmid mixtures were pooled and re-aliquoted before stimulation. Transfected cultures were incubated for 6 h, and stimuli, when required, were applied for an additional 16 h. The amounts of DNA transfected in each experiment were kept constant by adding control plasmids as indicated.

Western blot analysis and MBP kinase assays

Cells cultured in complete medium were harvested at different times after transfection and washed three times in RPMI alone. SDS was added to 1% final concentration. After vortexing, extracts were heated for 5 min at 100°C. Debris were pelleted by ultracentrifugation for 30 min at 90 000 r.p.m. in the TL100.2 rotor of a Beckman centrifuge. Proteins were acetone precipitated from supernatants for 10 min on ice and resuspended in reducing Laemmli sample buffer. Disruption of the pellet was completed by sonication. Extracts prepared from equivalent numbers of living cells were subjected to SDS–PAGE on 10% gels (10% acrylamide–0.27% bisacrylamide), unless otherwise indicated. Proteins were transferred to polyvinyldifluoridine membranes (Immobilon, Millipore) by overnight Western blot. Membranes were 'blocked' in phosphate-buffered saline (PBS)–0.05% Tween 20, (PBST) plus 5% milk for 2 h. One μg/ml of 9E10 antibody or a 1/1000 dilution of LexA antisera was allowed to react with the membrane overnight at 4°C. After extensive washes, membranes were incubated with a dilution of anti-mouse (1/4000) or anti-rabbit IgG (1/10 000) in PBST for 1 h at room temperature. After the washes in PBST, and PBS, membrane-bound antibodies were visualized by use of ECL Western blotting detection reagents (Amersham) and Kodak XS1 films. ERK–2 tag immunoprecipitations with 9E10 antibody and MBP assays were carried out as described previously (Izquierdo *et al.*, 1993).

CAT assays

CAT assays were performed using the liquid scintillation method of Sleigh *et al.* using modifications (Sleigh, 1986). Briefly, 5×10^6 cells were lysed in 50 μl of 0.5% NP-40, 10 mM Tris pH 8, 1 mM EDTA 150 mM NaCl for 10 min on ice. Cellular debris was pelleted and lysates were heated at 68°C for 10 min before use. Assay conditions were 150 mM Tris pH 8, 0.05 μCi [^{14}C]acetyl coenzyme A and 2 mM chloramphenicol. After 18 h at 37°C, chloramphenicol was extracted with ethylacetate, and the amount of radioactivity in the acetylated products and non-acetylated substrate of each reaction was determined by liquid scintillation counting of organic and aqueous phases respectively. The amount of chloramphenicol acetylation is calculated as a percentage.

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