Activation of the Raf/MAP kinase cascade by the Ras-related protein TC21 is required for the TC21-mediated transformation of NIH 3T3 cells

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TC21 is a member of the Ras superfamily of small GTP-binding proteins and, like Ras, has been implicated in the regulation of growth-stimulating pathways. Point mutations introduced into TC21 based on equivalent H-Ras oncogenic mutations are transforming in cultured cells, and oncogenic mutations in TC21 have been isolated from several human tumours. The mechanism of TC21 signalling in transformation is poorly understood. While activation of the serine/threonine kinases Raf-1 and B-Raf has been implicated in signalling pathways leading to transformation by H-Ras, it has been argued that TC21 does not activate Raf-1 or B-Raf. Since the Raf-signalling pathway is important in transformation by other Ras proteins, we assessed whether the Raf pathway is important to transformation by TC21. Raf-1 and B-Raf are constitutively active in TC21-transformed cells and the ERK/MAPK cascade is required for the maintenance of the transformed state. We demonstrate that oncogenic V23 TC21, like Ras, interacts with Raf-1 and B-Raf (but not with A-Raf), resulting in the translocation of the Raf proteins to the plasma membrane and in their activation. Furthermore, using point mutations in the effector loop of TC21, we show that the interaction of TC21 with Raf-1 is crucial for transformation.

Keywords: B-Raf/Raf-1/Ras/TC21/transformation

Introduction

The Ras superfamily of small GTP-binding proteins are regulators of a variety of cellular processes such as proliferation, differentiation, intracellular transport and the regulation of cellular architecture (Bos, 1997). The four mammalian Ras proteins (H-, N-, K4A- and K4B-Ras) have been of particular interest due to the high incidence of activated mutant forms of these proteins in human tumours (Barbacid, 1987). Recently, mutated forms of another Ras-related protein, TC21, were identified in cell lines derived from uterine sarcoma, ovarian and mammary tumours, tissues in which Ras is only rarely mutated (Chan *et al.*, 1994; Huang *et al.*, 1995; Barker and Crompton, 1998). TC21 proteins containing point mutations based on equivalent H-Ras oncogenic mutations are highly transforming in a variety of cell lines, including NIH 3T3 fibroblasts, Rat-1 fibroblasts, RIE-1 rat intestinal and MCF-10A human

mammary epithelial cells (Chan *et al.*, 1994; Graham *et al.*, 1994; Huang *et al.*, 1995; Clark *et al.*, 1996). Furthermore, injection of oncogenic TC21-expressing fibroblasts into nude mice results in the formation of highly aggressive tumours (Chan *et al.*, 1994; Graham *et al.*, 1994). These findings implicate TC21 in tumorigenesis and suggest that TC21, like Ras, may be a regulator of growth. TC21 expression in the mouse, unlike Ras, is not ubiquitous, but is high in ovaries, placenta and kidney (Graham *et al.*, 1996). TC21 expression in the untransformed MCF-10A human breast epithelial cell line is low, but seven out of nine human breast tumour cell lines tested were found to contain elevated levels of wild-type TC21 protein (Clark *et al.*, 1996). Upregulation of wild-type TC21 alone may be involved in growth stimulation; TC21 RNA and protein are highly induced upon releasing endothelial cells from cell cycle arrest (Kozian and Augustin, 1997).

TC21 has 55% amino acid identity with Ras proteins, but is 100% identical to Ras proteins in the core effector region (residues 32–40 in H-Ras and 43–51 in TC21), which has been characterized as the site of interaction of many Ras effectors (Nassar *et al.*, 1995; White *et al.*, 1995; Bos, 1997). The signalling pathways activated by TC21 and involved in transformation have not been defined. Activation of the Raf/MAP kinase pathway is essential for a variety of Ras-induced cellular events including proliferation, differentiation and morphological transformation (Cowley *et al.*, 1994; Dudley *et al.*, 1995). However, while ERK/MAP kinase activity is elevated in TC21-transformed fibroblasts (Graham *et al.*, 1994), Graham *et al.* (1996) were unable to demonstrate an elevation of Raf-1 or B-Raf activity in these cells. Furthermore, despite the conserved effector core region in TC21 (Drivas *et al.*, 1990), no interaction was detected between wild-type TC21 and Raf-1 and B-Raf in the yeast two-hybrid system (Graham *et al.*, 1996). On the basis of these results it was concluded that TC21-transformation proceeded through a Raf-independent mechanism.

We demonstrate by yeast two-hybrid analysis and co-immunoprecipitation that oncogenic V23 TC21 is capable of interacting with the serine/threonine kinases Raf-1 and B-Raf, but not with A-Raf. Furthermore, this interaction results in the membrane localization of the Raf proteins, a step that is essential to their activation. We also observe the constitutive activation of components of the MAP kinase cascade including ERK2, MEK1, Raf-1 and B-Raf in TC21-transformed cells. Inhibition of ERK activity in TC21-transformed cells leads to reversion of the transformed phenotype. Furthermore, we demonstrate that a point mutation in the effector region of TC21 that interferes with the interaction with Raf proteins also abolishes the ability of TC21 to transform cells. This transforming

ability can be rescued by the co-expression of a Raf mutant that can interact with the mutated TC21 protein, consequently demonstrating the importance of the TC21–Raf interaction for the transformation of NIH 3T3 fibroblasts.

Results

Constitutive activation of the Raf/MAP kinase cascade in TC21-transformed cells

The MAP kinase cascade is an important transducer of signals from active Ras proteins in a variety of cellular functions including proliferation and differentiation. The MAP kinase ERK2 has been found to be constitutively active in Q72L TC21-transformed NIH 3T3 fibroblasts (Graham *et al.*, 1994), but the relevance of this observation to transformation by TC21 has not been assessed. Glycine to valine substitution at position 23 in TC21, based on a similar substitution at position 12 in H-Ras, results in a constitutively active protein capable of efficiently transforming a variety of different cell lines (Graham *et al.*, 1994; Clark *et al.*, 1996). Stable cell lines of NIH 3T3 cells transfected by Myc-tagged V23 TC21 were generated, selected on the basis of focus formation and used to assess the role of the ERK/MAP kinase cascade in TC21-induced transformation. The level of ERK/MAP kinase activity in these cell lines was determined by the specific immunoprecipitation of p42MAPK (ERK2) followed by the phosphorylation of the MAP kinase substrate myelin basic protein (MBP) (Figure 1A). In both the presence and absence of serum, V23 TC21-transformed cells have elevated levels of ERK2 activity compared with parental untransformed NIH 3T3 cells (Figure 1A). Total levels of ERK2 protein are similar in both cell lines under the different growth conditions (Figure 1B). In mammalian cells, ERKs are activated by phosphorylation by the MAP kinase kinases, MEK1 and 2 (Ahn *et al.*, 1991; Payne *et al.*, 1991; Nakielnny *et al.*, 1992). Immunoprecipitation of MEK1 followed by a coupled kinase assay showed that MEK1 activity is also elevated in the cytoplasmic fractions of TC21-transformed cells (Figure 1C).

The Raf serine/threonine kinases are major activators of MEK1 in Ras-induced transformation. However, a previous attempt to assay Raf activity in Q72L TC21-transformed NIH 3T3 failed to detect activation of Raf-1 or B-Raf (Graham *et al.*, 1996). In H-Ras-transformed cells, active Raf-1 locates mainly to the membranous fraction (Traverse *et al.*, 1993). To increase the sensitivity of the Raf-activation assay, we prepared membrane and cytosol fractions and assayed immunoprecipitates of Raf-1 and B-Raf using a coupled kinase assay (Marais *et al.*, 1995). Both Raf-1 and B-Raf were found to be constitutively active in TC21-transformed cells compared with parental untransformed cells (Figure 2). No elevation in A-Raf activity was detected in TC21-transformed NIH 3T3 cells. The elevation in Raf-1 and B-Raf activity in both TC21 and H-Ras transformed cells is particularly pronounced in the membrane fractions as has been described previously for Raf-1 in H-Ras-transformed cells (Traverse *et al.*, 1993). However, B-Raf protein has proved difficult to detect in particulate fractions of Ras-transformed cells (Jelinek *et al.*, 1996). This is thus the

Fig. 1. Activation of the MAP kinase pathway in TC21 transformed cells. ERK2 is constitutively active in V23 TC21-transformed NIH 3T3 cells. (A) V23 TC21-transformed and parental untransformed NIH 3T3 cells were transferred to serum-free media (–serum) or media supplemented with 10% serum (+serum) 4 h prior to harvesting. ERK2 activity was determined in immunoprecipitates by the incorporation of ^{32}P into MBP. (B) Whole-cell lysate was also separated on a 15% SDS–polyacrylamide gel and analysed by immunoblotting for ERK2 protein using the 124 rabbit polyclonal anti-ERK2 antibody (Leevers and Marshall, 1992). (C) Activation of MEK1 in TC21-transformed cells. MEK1 was immunoprecipitated from membrane-rich (P100) and cytoplasmic (S100) fractions of TC21- or N-Ras-transformed and untransformed NIH 3T3 cells. Immunoprecipitates were assayed for MAP kinase kinase activity on recombinant ERK2 and MBP. The average of duplicate samples is shown. Results for the V23 TC21 21–3 and N-Ras 149–169 cell lines are shown. The experiment was repeated twice with similar results.

first demonstration of which we are aware of the activation of B-Raf in the particulate fraction of transformed cells. In support of this are data to be described later, which demonstrate that TC21 recruits B-Raf to the plasma membrane (Figure 5).

A P100 **B S100**

Fig. 2. B-Raf and Raf-1 are constitutively active in TC21-transformed NIH 3T3 cells. B-Raf and Raf-1 were immunoprecipitated from 150 μ g of P100 membrane-rich (A) or S100 cytoplasmic (B) fractions of untransformed parental NIH 3T3, V23 TC21 and v-H-Ras stably expressing NIH 3T3 cell lines, and assayed by the sequential activation of MEK1 and ERK2, and MBP phosphorylation. Results for V23 TC21 21-3 and v-H-Ras V2 cell lines are shown. The experiment was repeated with two to four other independently isolated cell lines with similar results.

Activation of the MAP kinase pathway is required for TC21-induced transformation

Given the activation of components of the ERK/MAP kinase cascade in TC21-transformed cells as compared with untransformed cells, we reasoned that this pathway contributed to the characteristics of the TC21-induced transformed state and was required for its maintenance. The importance of the activation of the MAP kinase cascade in TC21-transformed cells was assessed using the specific MEK1 inhibitor PD098059. TC21-transformed NIH 3T3 cells characteristically have elongated spindle shapes with very few or no visible stress fibres. As has been previously characterized for H-Ras-transformed cells (Dudley *et al.*, 1995), exposure to PD098059 results in the flattening of TC21-transformed cells and the appearance of stress fibres (Figure 3A). The effect of inhibiting the MAP kinase cascade on TC21-induced DNA synthesis was assessed by the incorporation of bromodeoxyuridine (BrdU). Serum-starved conditions were used, because under these conditions proliferation is dependent on the TC21 oncogene rather than on serum components. Treatment with PD098059 strongly inhibits DNA synthesis in both Ras and TC21-transformed NIH 3T3 cells (Figure 3B). Similar results were also found

A **B**

Cell line	Percentage of cells incorporating BrdU (sample size)		Percentage inhibition of DNA synthesis
	DMSO control	PD098059	
V23 TC21	74.0% (173)	21.3% (122)	71%
D12 H-Ras	85.4% (123)	50.5% (606)	41%

Fig. 3. The MAP kinase pathway is required for transformation by TC21. TC21 or H-Ras-transformed NIH 3T3 cells plated on collagen dishes and growing in 5% serum/DMEM were exposed to 30 μ M of PD098059 or to an equal volume of DMSO for 41 h. (A) The staining of polymerized actin in the V23 TC21 6B-2C and D12 H-Ras 1C cell lines is shown. The experiment was repeated three times with similar results. (B) The effect of PD098059 on DNA synthesis was determined by transferring the cells to serum-free media 16 h prior to the addition of PD098059, and supplementing the culture media with 10 mM BrdU 18 h after the addition of PD098059. Under these conditions untransformed NIH 3T3 cells do not synthesize DNA during the BrdU-labelling period. Results for V23 TC21 2A-4-7 and D12 H-Ras 3-2 cell lines are shown. The experiment was repeated three times on independently isolated cell lines (with an average inhibition of DNA synthesis by PD098059 of 72% in TC21-transformed cells and of 61% in H-Ras-transformed cells).

with a second MEK inhibitor, U0126 (Favata *et al.*, 1998; data not shown). Inhibition of the MAP kinase cascade through the microinjection of a dominant-negative mutant of MEK1, S222A MEK1 (Alessi *et al.*, 1994; Cowley *et al.*, 1994), also inhibited transformation by TC21 in terms of both morphology and DNA synthesis (data not shown). The constitutive activation of the MAP kinase cascade in TC21-transformed cells is thus required for both their morphological transformation and proliferation in serum-free conditions.

TC21 interacts with Raf-1 and B-Raf, resulting in Raf translocation to the plasma membrane

Raf proteins interact with a region on Ras termed the core effector domain, which is absolutely conserved in TC21. Some residues outside this region are also involved in the interaction and one of these, arginine 41 in H-Ras is substituted for a threonine residue in TC21 (Nassar *et al.*, 1995). It is therefore unclear from analysis of the sequences of H-Ras and TC21 alone whether TC21 could interact with Raf proteins. Recently it was suggested, based on yeast two-hybrid analysis, that TC21 does not interact with Raf proteins (Graham *et al.*, 1996). Given the presence

of active Raf-1 and B-Raf at the plasma membrane of TC21-transformed cells, we decided to re-assess the ability of TC21 to interact with the Raf proteins using both two-hybrid analysis and co-immunoprecipitation (Figure 4). We performed two-hybrid analysis on yeast co-expressing V23 TC21 Δ CAAX–GAL4-activation domain and Raf–GAL4 DNA-binding domain fusion proteins. The CAAX membrane localization motif was deleted in the TC21 fusion protein in order to prevent the localization of the fusion protein to the plasma membrane. V23 TC21 was found to interact with the N-terminus of Raf-1 and in contrast to previous work (Graham *et al.*, 1996), we observed the interaction of TC21 with full-length B-Raf. No interaction was detected between TC21 and full-length A-Raf (Figure 4A).

To confirm these interactions we co-expressed V23 TC21 and Raf proteins in NIH 3T3 mouse fibroblasts, immunoprecipitated the tagged TC21 protein and assayed for the co-immunoprecipitation of Raf proteins by kinase assays and immunoblotting (Figure 4B and C). Both Raf-1 and B-Raf protein and activity co-immunoprecipitate with V23 TC21, confirming the results obtained by two-hybrid analysis. As has been previously demonstrated, co-expression of H-Ras and Raf-1 leads to only a low level of Raf-1 activation. However, when active Ras and Src are co-expressed there is a synergistic activation of Raf-1 (Williams *et al.*, 1992; Marais *et al.*, 1995). To aid the detection of Raf-1 activity associated with TC21 we co-expressed Raf-1 in the presence of TC21 and Src. Figure 4B shows that Raf-1 activity co-immunoprecipitating with TC21 can be further enhanced by the expression of active Src. These data also suggest that at least a proportion of Raf-1 protein remains bound to H-Ras or TC21 after further activation by Src.

The association of Raf proteins with Ras results in their membrane localization (Traverse *et al.*, 1993; Marais *et al.*, 1995, 1997). The elevation of Raf-1 and B-Raf activity in the plasma membrane of TC21-transformed cells (Figure 2A) and the ability of TC21 to interact with these kinases (Figure 4) suggests that Raf proteins are also translocated to the plasma membrane in the presence of active TC21. To confirm this, we co-expressed Myc-tagged TC21 with Raf-1 or B-Raf in Madin–Darby canine kidney (MDCK) epithelial cells and analysed the localization of the Raf proteins by immunocytochemistry (Figure 5). In line with our observed elevation in Raf activity at the plasma

Fig. 4. TC21 interacts with B-Raf and Raf-1 but not with A-Raf.

(A) V23 TC21 Δ CAAX interacts with full-length B-Raf but not with A-Raf in the yeast two-hybrid system. Yeast were transformed with DNA for the indicated GAL4 fusion protein combinations and grown under selection for the presence of both plasmids. Three independent yeast colonies were assayed for β -galactosidase activity by filter assay, blue colour indicating the presence of β -galactosidase activity and hence the interaction of the GAL4 fusion pair. (B) Raf-1 and B-Raf protein and activities co-immunoprecipitate with TC21. Glu-tagged V23 TC21 or G12R H-Ras was immunoprecipitated from low-detergent lysates of NIH 3T3 cells transiently co-expressing the Glu-tagged V23 TC21 or G12R H-Ras with Myc-tagged B-Raf or Raf-1, in the presence or absence of an active form of Src, Y527F. Immunoprecipitates were either assayed for MAP kinase kinase activity (B) or separated by SDS–PAGE and Western blotted for co-immunoprecipitated Raf protein using the 9E10 anti-Myc epitope monoclonal antibody (Evan *et al.*, 1985) (C). The average kinase activity of duplicate samples is shown. The experiment was repeated three times with similar results.

Fig. 5. Translocation of Raf-1 and B-Raf to the plasma membrane by TC21. MDCK cells were microinjected with 50 µg/ml of an expression vector for Raf-1 or B-Raf alone or in conjunction with 50 µg/ml of an expression vector for Myc-tagged V23 TC21 or Myc-tagged V23 E48G TC21. Cells were stained 14 h after microinjection for Myc-tagged TC21 and for the Raf proteins using specific antibodies. Samples were analysed by confocal fluorescence microscopy.

membrane of TC21-transformed cells, Raf-1 and B-Raf become translocated to the plasma membrane in the presence of active TC21 (Figure 5).

Glutamate 37 in the effector region of Ras has been identified as a mediator of hydrogen-bond interactions with the Ras-binding domain of Raf-1 (Nassar *et al.*, 1995). Mutation of this residue to glycine interferes with the interaction of Raf-1 with H-Ras, but not with the interaction of Ras with other Ras effectors such as RalGDS and AF6 (Van Aelst *et al.*, 1994; White *et al.*, 1995; Khosravi Far *et al.*, 1996; Rodriguez Viciano *et al.*, 1997). Mutation of the equivalent residue (position 48) in TC21 abolishes the translocation of both Raf-1 and B-Raf to the plasma membrane by TC21 (Figure 5), suggesting that similar interactions may be involved in the association of H-Ras or TC21 and Raf-1. No interaction between V23 E48G TC21 and Raf-1 or B-Raf could be detected by yeast two-hybrid analysis or co-immunoprecipitation (data not shown).

TC21 activates Raf-1 and B-Raf

The data presented above suggest that TC21 can interact directly with Raf-1 and B-Raf and hence induce their membrane translocation and activation. Confirmation of the ability of TC21 to activate the Raf proteins was obtained using NIH 3T3 cells transiently transfected with expression vectors for the Raf proteins and for V23 TC21. Lysates normalized for levels of exogenously expressed Raf protein were either assayed directly for B-Raf activity (Figure 6A), or first immunoprecipitated for Raf-1 protein and then assayed for Raf-1 activity (Figure 6B). Co-expression of V23 TC21 results in the activation of both B-Raf and Raf-1. In whole-cell lysate assays, B-Raf or Raf-1 activation by H-Ras is 1.5- to 2-fold higher than by TC21 (Figure 6B; data not shown). In accordance with the yeast two-hybrid results, so far we have not been able to detect any activation of A-Raf by TC21 in transient expression assays (data not shown).

Fig. 6. Activation of B-Raf and Raf-1 by TC21. (A) B-Raf is activated by V23 TC21. Myc-tagged B-Raf was expressed in NIH 3T3 cells alone or with R12 H-Ras or V23 TC21. B-Raf protein levels in the different samples were normalized to each other using the 9E10 anti-Myc epitope monoclonal antibody (Evan *et al.*, 1985) and the kinase activities in whole-cell lysates determined in a coupled 'pull-down' assay with glutathione *S*-transferase (GST)–MEK1 and ERK2. ERK2 activity was assayed on MBP. Fold-activation with respect to B-Raf alone above background is shown underneath. The average of duplicate experiments is shown in each case. The experiment was repeated four times with similar results (B) Raf-1 is activated by V23 TC21 but not by V23 E48G TC21. Myc-tagged wild-type, S257L or R89L S257L Raf-1 were expressed in NIH 3T3 cells alone, or together with V23 TC21 or V23 E48G TC21. The exogenously expressed Myc-tagged Raf was immunoprecipitated from whole-cell lysates normalized for levels of Raf-1 protein as above. Immunoprecipitates were assayed for Raf activity on MEK1, ERK2 and MBP as before. Fold-activation with respect to Raf alone are shown above each bar. The experiment was repeated twice with similar results.

Transformation by TC21 requires interaction with Raf-1

Our data strongly suggest that the interaction of TC21 with Raf-1 leads to its activation. Furthermore, the inhibitory effects of PD098059 indicate that activation of the MAP kinase cascade is required for TC21-induced transformation. To confirm that this requirement is mediated by the direct activation of Raf by TC21, we used an effector mutant rescue approach first developed for analysing the H-Ras–Raf-1 interaction (White *et al.*, 1995). White *et al.* (1995) demonstrated that the effector mutant of Ras, E37G, fails to interact with or activate Raf-1. However, they were able to select a mutant of Raf-1 (S257L) that restored interaction

with E37G H-Ras. Co-expression of S257L Raf-1 with V12 E37G H-Ras resulted in transformation, providing a strong argument for the requirement for the H-Ras–Raf-1 interaction for transformation.

The equivalent E48G mutation in TC21 also interferes with the Raf–TC21 interaction and the activation of Raf-1 by TC21 (Figures 5 and 6B). We tested the effect of this mutation on the transformation potential of V23 TC21. V23 TC21 transforms NIH 3T3 cells very efficiently, producing large, highly refractile foci. V23 E48G TC21, however, failed to transform NIH 3T3 cells (Figure 7A). V23 TC21 and V23 E48G TC21 express equally well in NIH 3T3 cells (data not shown).

The failure of V23 E48G TC21 to transform NIH 3T3 cells may be a consequence of its inability to interact with and activate Raf-1. However, the interaction of other TC21 effectors may also be compromised by this mutation. Since the S257L mutation in Raf-1 restores a degree of interaction between V12 E37G H-Ras and Raf-1 (White *et al.*, 1995), we assessed whether the interaction of Raf-1 with V23 E48G TC21 was restored by the S257L Raf mutation. Unlike wild-type V23 TC21, V23 E48G TC21 is unable to translocate Raf-1 to the plasma membrane (Figure 6B). However, V23 E48G TC21 was able to translocate S257L Raf-1 to the plasma membrane (Figure 7B). The translocation of S257L Raf-1 by V23 E48G TC21 is not as marked as by wild-type V23 TC21, suggesting that the S257L mutation does not allow full reconstitution of the wild-type interaction. Confirmation that translocation is dependent on the interaction of TC21 with Raf was obtained by insertion of the R89L mutation into S257L Raf-1. This mutation blocks the ability of Raf-1 to interact with Ras-GTP (Fabian *et al.*, 1994). R89L S257L Raf-1 did not translocate to the plasma membrane in the presence of V23 or V23 E48G TC21, indicating that translocation of S257L Raf-1 by V23 E48G TC21 is due to the restoration of the interaction between these two proteins.

We also tested whether the partial restoration of the interaction between V23 E48G TC21 and Raf-1 by the S257L mutation in transiently transfected NIH 3T3 cells allows the activation of S257L Raf-1 in the presence of V23 E48G TC21. Figure 6B shows that S257L, but not wild-type or R89L S257L Raf-1, was activated in cells co-expressing V23 E48G TC21. Consistent with the observed decreased ability of V23 E48G TC21 to translocate S257L Raf-1 to the plasma membrane as compared with V23 TC21, the activation of Raf-1 by V23 E48G TC21 is only partially restored by the S257L mutation in Raf-1.

We then analysed whether the co-expression of S257L Raf-1 could rescue the transformation of NIH 3T3 cells by V23 E48G TC21. In agreement with the inability of V23 E48G TC21 to interact with and activate wild-type Raf-1, the co-expression of wild-type Raf-1 has no effect on transformation by V23 E48G TC21. However, co-expression of S257L Raf-1 with V23 E48G TC21 led to transformation (Figure 7C). Insertion of the R89L mutation in S257L Raf-1, which abolishes the translocation and activation of S257L Raf-1 by V23 E48G TC21, also abolishes the co-operation in focus formation (Figure 7C), therefore providing a strong argument for the requirement of the TC21–Raf-1 interaction in the transformation of NIH 3T3 cells.

Fig. 7. Transformation by TC21 requires interaction with Raf-1. **(A)** V23 E48G TC21 is defective for the transformation of NIH 3T3 cells. Low-passage NIH 3T3 cells were transfected with increasing amounts of expression vectors for V23 TC21 or V23 E48G TC21. The cells were equally divided over two plates 24 h after transfection and focus formation was assessed after 14 days growth in 5% serum/DMEM. Samples were fixed in 4% formaldehyde and stained with crystal violet. The experiment was repeated six times with similar results. **(B)** V23 E48G TC21 translocates S257L but not R89L S257L Raf-1 to the plasma membrane. MDCK cells were microinjected with Myc-tagged S257L or R89L S257L Raf-1 expression vectors alone or together with Glu-tagged V23 E48G TC21 or V23 TC21 constructs. Cells were stained 14 h after microinjection for the expression of the tagged proteins. The experiment was repeated with similar results. **(C)** Co-expression of S257L but not of wild-type Raf-1 rescues transformation by V23 E48G TC21. NIH 3T3 cells were transfected with 30 or 0.3 ng of expression vectors for wild-type, S257L or R89L S257L Raf-1. Differing amounts of expression plasmids for V23 and V23 E48G TC21 were used in order to keep the assay in a linear range. Foci were counted after 14 days growth in 5% serum/DMEM. The experiment was repeated four times with similar results.

Discussion

Activation of the ERK/MAP kinase cascade in TC21-transformed cells

TC21 shares only 55% amino acid identity with Ras proteins. However, mutated forms of TC21 show the same ability to transform fibroblast and epithelial cell lines as Ras (Chan *et al.*, 1994; Graham *et al.*, 1994; Clark *et al.*, 1996). Discovery of constitutively active mutants of TC21 in human tumours further suggests that TC21 may regulate growth pathways *in vivo* (Chan *et al.*, 1994; Huang *et al.*, 1995). The Raf/MAP kinase pathway plays a central role in the control of proliferation by Ras proteins. The data presented here show that the Raf/MAP kinase pathway also plays a crucial role in the transformation of NIH 3T3 cells by TC21. We not only identify the constitutive activation of ERK2 in TC21-transformed NIH 3T3 cells, as previously reported (Graham *et al.*, 1994), but also the constitutive activation of MEK1 and of the Raf proteins, Raf-1 and

B-Raf. Furthermore, inhibition of the ERK/MAP kinase pathway in TC21-transformed cells interferes with the induction of DNA synthesis and the morphological alterations induced by TC21. Others have failed to see Raf-1 or B-Raf activity in whole-cell lysates from TC21-transformed NIH 3T3 cells (Graham *et al.*, 1996). However, usage of membrane fractions and a three-step cascade kinase assay, where the read-out is ERK activity, result in a more sensitive assay than the assay used by Graham *et al.* (1996), where Raf activity in whole-cell lysates is measured by the ability of MEK to phosphorylate kinase dead ERK.

Activation of Raf-1 and B-Raf by interaction with TC21

The presence of activated components of the ERK/MAP kinase pathway in TC21-transformed cells could be a consequence of a direct activation by TC21. We demonstrate here that TC21 directly interacts with the Raf

proteins Raf-1 and B-Raf *in vivo*, translocating them to the plasma membrane and leading to their activation.

Yeast two-hybrid assays and the co-immunoprecipitation of proteins co-expressed in mammalian cells clearly demonstrate that TC21 associates with Raf-1 and B-Raf. Previous reports, suggesting that TC21 did not interact with the Raf proteins, assayed this interaction in the yeast two-hybrid system using wild-type CAAX-containing TC21–GAL4 fusion proteins (Graham *et al.*, 1994). Study of the H-Ras–Raf interaction using the yeast two-hybrid system demonstrated that no interaction can be detected between H-Ras and Raf if the H-Ras fusion protein used had an intact CAAX box (Van Aelst *et al.*, 1993). Deletion or mutation of the CAAX box is required for the interaction to be detected by this system, presumably to facilitate the nuclear localization of the fusion protein.

In accordance with the observed interaction between TC21 and the Raf proteins Raf-1 and B-Raf, and the constitutive activation of these kinases in TC21-transformed cells, we also find that TC21 leads to the activation of Raf-1 and B-Raf in transiently transfected NIH 3T3 cells. B-Raf and Raf-1 activation by TC21 is generally weaker than that seen with Ras in the same assay. This may be a consequence of a weaker interaction between TC21 and the Raf proteins. However, due to the cascade nature of the MAP kinase cascade, even low levels of activation of Raf proteins may be sufficient for a full response. Furthermore, recent results argue that too high a level of activation of the Raf/ERK signalling pathway may be detrimental to cell transformation by inducing the expression of high levels of cyclin-dependent kinase inhibitors (Sewing *et al.*, 1997; Woods *et al.*, 1997). Indeed, under conditions where oncogenic H-Ras does not induce cell-cycle entry, oncogenic TC21 induces DNA synthesis in an ERK/MAP kinase pathway-dependent fashion (M.Rosário, unpublished data).

We also demonstrate that the interaction between TC21 and Raf is necessary for transformation using a point mutation (E48G) in the core effector domain of TC21. This mutation interferes with the ability of TC21 to transform cells, but transformation can be rescued by co-expression of a mutant Raf-1 (S257L) that is capable of interacting with the E48G TC21 mutant protein (Figure 7).

It has been suggested that the co-operation of a similar Raf mutant, S257A, with the equivalent H-Ras mutant (V12 E37G) in transformation is due not to the restoration of the Ras–Raf interaction but due to the higher basal activity of S257A versus wild-type Raf-1 (Jaitner *et al.*, 1997). However, V23 E48G TC21 was able to translocate S257L Raf-1 but not wild-type Raf-1 to the membrane, suggesting that this mutation does partially restore interaction with TC21 (Figure 7B). Furthermore, a possible higher basal activity of S257L Raf-1 is unlikely to be the reason for the co-operation seen with V23 E48G TC21, since combining S257L and the R89L mutation, which interferes with the interaction of Raf with Ras or TC21 (Figure 7B; Fabian *et al.*, 1994), abolishes the activation of S257L Raf-1 by V23 E48G TC21 and co-operation in focus assays (Figures 6B and 7C). This strongly suggests that the restoration of transformation in V23 E48G TC21 expressing cells by co-expression of S257L Raf-1 but not of wild-type or R89L S257L Raf-1 is due to the differential

ability of these Raf-1 mutants to interact with V23 E48G TC21.

These results show that, as for Ras, activation of the ERK/MAP kinase pathway by TC21 via interaction with Raf proteins is critical for transformation by TC21. However, given the conserved nature of the core effector domain of TC21, it is possible that other known Ras effectors are also effectors of TC21 signalling. Indeed, RalGDS, an effector for Ras (Albright *et al.*, 1993; Wolthuis *et al.*, 1998), has been shown to interact with TC21 (Lopez Barahona *et al.*, 1996). The role of this effector in transformation by Ras or TC21, however, is unclear. Like the equivalent Ras effector mutation, V23 E48G TC21 retains the ability to interact with members of the RalGDS family suggesting therefore that activation of this pathway is insufficient for transformation (M.Rosário, unpublished data).

It is possible that Ras and TC21 activate a similar set of pathways, but become activated in different tissues or under different biological contexts. Unlike Ras, TC21 does not appear to be ubiquitously expressed in the mouse (Chan *et al.*, 1994; Graham *et al.*, 1996). Endothelial cells have recently been shown to induce TC21 expression upon release from density-dependent cell cycle arrest (Kozian and Augustin, 1997). To date, extracellular signals that activate TC21 have not been identified. However, TC21 is reported to have a very high GDP/GTP exchange rate, but weak intrinsic GTPase activity (Graham *et al.*, 1996), and induction of the protein may thus be the major mechanism for the regulation of TC21.

Materials and methods

Plasmids

The cDNA for TC21 in which glycine 23 was replaced with valine was cloned into the plasmid Myc-pEFPlink.2 which incorporates a Myc-epitope tag (EQKLISEEDL) at the N-terminus of the protein (Marais *et al.*, 1995) and into pEFgPlink.2 which incorporates a Glu tag (EFMPME) at the N-terminus of the protein (Rausch and Marshall, 1997). Equivalent V23 E48G TC21 5' Myc and 5' Glu-tagged constructs were generated using PCR-directed mutagenesis to replace the glutamate residue at position 48 with glycine. PCR-directed mutagenesis was also used to delete the last four codons of the G23V TC21 and K-Ras cDNA sequences, which encode the membrane localization CAAX box, for cloning into the yeast two-hybrid expression vector pACTII (Clontech) in order to generate GAL4-activation domain fusion proteins. cDNA for full-length B-Raf, full-length A-Raf and for the N-terminus of Raf-1 (amino acids 1–304) were cloned into the yeast expression vector pYTH9 (Clontech) in order to generate GAL4-binding domain fusion proteins. All other constructs have been described previously (Marais *et al.*, 1995, 1997).

Cell culture and transfection

Untransformed and transformed NIH 3T3 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 or 5% calf serum, respectively. MDCK cells were grown in DMEM supplemented with 10% fetal calf serum (FCS). DNA transfections were performed using lipofectAMINE (Gibco-BRL Life Technologies) as described previously (Marais *et al.*, 1995), with the exception that 0.7 µg of total DNA was used with 3 µl of lipofectAMINE. In transient transfections the cells were grown in 10% serum/DMEM for 48 h before harvesting. To generate TC21 or Ras-transformed cell lines or for focus formation assays, transfected cells were transferred to two 10 cm Petri dishes 24 h after transfection and grown for 14 days in DMEM supplemented with 5% serum. Foci were either picked and re-grown for the generation of stable cell lines or counted, stained (1% crystal violet, 70% ethanol) and photographed. Selection of transformed subclones was on the basis of the uniform expression of the oncogene by immunocytochemistry. All transformed cell lines were further subcloned

at least once before use. The 149-169 cell line, expressing N-Ras, is a tertiary transfectant from a transfection with DNA from human rhabdomyosarcoma cells (Cowley *et al.*, 1994).

Microinjections and immunofluorescence analysis

Cells were transferred to collagen-coated dishes for microinjection. Microinjections were performed on a Zeiss Microinjection Workstation (Carl Zeiss, Oberkochen) as previously described (Marais *et al.*, 1995). Samples were fixed in 4% formaldehyde 16 h post-injection, permeabilized in 0.2% Triton X-100 (15 min), blocked in 10% FCS (20 min) and incubated for 1 h in a mixture of 2 µg/ml A14 rabbit polyclonal anti-Myc antibody (Santa Cruz) and 11 µg/ml RAG4f rat monoclonal anti-Glu tag antibody or in a mixture of 5 µg/ml 9E10 mouse monoclonal antibody and 1.5 µg/ml rabbit polyclonal anti-B-Raf antibody (Santa Cruz), or 5 µg/ml mouse monoclonal anti-Raf-1 antibody (Transduction Laboratories). Appropriate fluorescein isothiocyanate (FITC) and Texas Red-conjugated secondary antibodies (Jackson Immuno-Research Laboratories) were used for detection of the specific immunostaining. Samples were mounted under a glass coverslip in Moviolt mountant [20% w/v Moviolt (Calbiochem) in 50% glycerol] containing *p*-phenylenediamine (Sigma) and analysed with a Bio-Rad MRC 1024 confocal imaging system equipped with a Nikon eclipse 400 microscope. DNA synthesis was determined by the uptake from the culture medium and incorporation into newly synthesized DNA of BrdU. Staining for incorporated BrdU was performed using an anti-BrdU FITC-conjugated monoclonal antibody according to the manufacturer's instructions (Boehringer Mannheim).

Preparation of cell lysates and analysis

Cells were lysed and assayed for Raf activity as previously described (Marais *et al.*, 1995). Cell lysates were normalized for levels of Raf protein by Western blotting with the 9E10 anti-Myc tag antibody (Evan *et al.*, 1985), using [¹²⁵I]protein A as the detection system and quantification by PhosphorImager analysis (Molecular Dynamics). Normalized whole-cell lysates were used either directly in a 'pull-down' assay with GST-MEK1, ERK2 and MBP (Sigma) as previously described (Marais *et al.*, 1995), or first immunoprecipitated for the Myc-tagged Raf using the 9E10 mouse monoclonal anti-Myc tag antibody (Evan *et al.*, 1985). Immunoprecipitates were washed sequentially in Raf reactivation buffer containing no ATP (Marais *et al.*, 1995) but containing 1 M, 0.1 M KCl or no salt, and then assayed by incubation with 20 µl of Raf reactivation buffer containing 6.5 µg/ml MEK1, 100 µg/ml ERK2 and 0.8 mM ATP for 30 min at 30°C (Alessi *et al.*, 1994; Marais *et al.*, 1995). The reaction was stopped by the addition of Raf reactivation buffer containing 6 mM EGTA. 10 µl of this reaction was tested for ERK activity by incubation with 40 µl of MBP buffer containing 50 µCi of [³²P]ATP (5000 Ci/mmol; Amersham) as described previously (Marais *et al.*, 1995). Background radioactivity in all cases was determined using lysates transfected with empty vector.

Cell lysates for the determination of endogenous ERK activity were prepared in 20 mM Tris pH 8.0, 40 mM sodium pyrophosphate, 50 mM NaF, 5 mM MgCl₂, 100 µM Na₃VO₄, 10 mM EGTA, 1% v/v Triton X-100, 0.5% w/v sodium deoxycholate, 20 mg/ml leupeptin, 20 mg/ml aprotinin and 1 mM PMSF as previously described (Howe *et al.*, 1992). ERK2 was immunoprecipitated using the polyclonal antiserum 122 (Leevers and Marshall, 1992). Immunoprecipitates were assayed for ERK2 activity as described previously (Rausch and Marshall, 1997).

Cell lysates for the analysis of co-immunoprecipitated Ras and Raf proteins were prepared using Ras lysis buffer (30 mM HEPES pH 7.5, 1% v/v Triton X-100, 10% glycerol, 10 mM NaCl, 5 mM MgCl₂, 25 mM NaF, 1 mM EGTA) supplemented with inhibitors (1 mM Na₃VO₄, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml pepstatin A, 10 mM benzamidine, 0.5 µg/ml microcystin LR). Lysates were used immediately after preparation in immunoprecipitations with the RAG4f rat monoclonal anti-Glu tag antibody using equal levels of total protein. Co-immunoprecipitates were washed once in the Ras lysis buffer and three times in Ras wash buffer (50 mM HEPES pH 7.5, 0.1% v/v Triton X-100, 10% v/v glycerol, 100 mM NaCl, 5 mM MgCl₂, 20 mM NaF), before being analysed for co-immunoprecipitating Myc-tagged Raf protein either by Western blotting with the 9E10 mouse monoclonal antibody or for Raf activity in a coupled kinase assay. Coupled kinase assays were carried out by the incubation of immunoprecipitates with 30 µl of Raf kinase buffer containing 6.5 µg/ml MEK1 and 0.8 mM ATP for 30 min at 30°C. The reactivated MEK1 supernatant was transferred to a new tube containing 3 µg of recombinant ERK2 and 12 nM ATP and incubated at 30°C for 30 min. Reactions were stopped by the addition of 3 µl of

120 mM EDTA and 12 µl of this reaction was assayed for ERK activity by incubation with 40 µl of MBP buffer as described above.

Cell fractions were prepared as described by Traverse *et al.* (1993), with the exception that the washed membrane P100 pellets were dissolved in MD buffer [50 mM Tris-HCl pH 7.9, 0.5% v/v NP-40, 0.1% w/v sodium deoxycholate, 0.05% w/v SDS, 20 mM *N*-octyl glucopyranoside, 0.5 mM EDTA, 0.5 mM EGTA, 5 mM sodium pyrophosphate, 25 mM sodium β-glycerophosphate, 10% glycerol, 0.1% v/v β-mercaptoethanol, 1 mM Na₃VO₄, 1 mM *p*-aminoethoxy-benzene sulfonyl fluoride (PMSF), 1 mM benzamidine, 10 µg/ml pepstatin, 10 µg/ml leupeptin, 0.5 µg/ml microcystin LR]. S100 cytoplasmic fractions were adjusted to the MD buffer composition by addition of appropriate volumes of NP-40, sodium deoxycholate, SDS, *N*-octyl glucopyranoside, EGTA, sodium pyrophosphate, sodium β-glycerophosphate, β-mercaptoethanol and inhibitors. MEK1, Raf-1, B-Raf and A-Raf were immunoprecipitated from cellular fractions using an anti-MEK1 antibody (Transduction Laboratories), C19 rabbit polyclonal anti-B-Raf (Santa Cruz), C12 rabbit polyclonal anti-Raf-1 (Santa Cruz) and C20 rabbit polyclonal anti-A-Raf (Santa Cruz) antibodies, respectively. Immunoprecipitates were washed three times in MD buffer and once in Raf kinase buffer. Raf activities were determined in coupled kinase assays as described above. MEK1 activity was determined by incubation with Raf kinase buffer containing 100 µg/ml ERK2 and 0.8 mM ATP and ERK2 activity was assayed on MBP as described above.

Yeast two-hybrid analysis

Plasmids were co-transfected into yeast (*Saccharomyces cerevisiae* Y190 strain; HIS3⁺, Leu⁻, Trp⁻ Gal4:LacZ, Gal4:HIS3) as described by Gietz and Woods (1994). Transformed yeast were grown on Leu⁻ Trp⁻ selection media for 3–4 days. Three independent colonies from each transfection were patched onto nitrocellulose filters and grown on Leu⁻ Trp⁻ selective media overnight. Yeast grown on the filters were then assayed for β-galactosidase activity with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal; Boehringer Mannheim) as described previously (Van Aelst *et al.*, 1993).

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