Oncogene (1999) 18, 1807–1817
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Differential contribution of the ERK and JNK mitogen-activated protein kinase cascades to Ras transformation of HT1080 fibrosarcoma and DLD-1 colon carcinoma cells

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Although an important contribution of ERK and JNK mitogen-activated protein kinase (MAPK) activation in Ras transformation of rodent fibroblasts has been determined, their role in mediating oncogenic Ras transformation of human tumor cells remains to be established. We have utilized the human HT1080 fibrosarcoma and DLD-1 colon carcinoma cell lines, which contain endogenous mutated and oncogenic N- and K-*ras* alleles, respectively, to address this role. Study of these cells is advantageous over Ras-transformed rodent model cell systems for two key reasons. First, the *ras* mutations occurred naturally in the progression of the tumors from which the cell lines were derived, rather than due to overexpression of an exogenously introduced gene. Second, although these tumor cells possess defects in multiple genetic loci, it has been established that mutated Ras contributes significantly to the transformed phenotype of these cells. Clonal variant lines of HT1080 and DLD-1 have been isolated which have lost the oncogenic *ras* allele and exhibit a corresponding impairment in growth transformation *in vitro* and *in vivo*. We found that upregulation of Raf/MEK/ERK and JNK correlated with expression of oncogenic Ras in HT1080, but not DLD-1 cells. Furthermore, inhibition of ERK activation in parental HT1080 cells caused the same changes in cell morphology and actin stress fiber organization seen with loss of expression of activated N-Ras(61K). Thus, we suggest that constitutive activation of the Raf/MEK/ERK and JNK pathways is necessary for Ras-induced transformation of HT1080 but not DLD-1 cells. These results emphasize that cell type differences exist in the signaling pathways by which oncogenic Ras causes transformation.

Keywords: Ras; ERK; JNK

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Received 17 July 1998; revised 30 September 1998; accepted 9 October 1998

Introduction

The association of mutated *ras* (H-, K- and N-ras) genes in 30% of all human cancers suggests an important contribution of aberrant Ras function to the development of human cancers (Barbacid, 1987; Bos, 1989; Clark and Der, 1993). Such a role is supported by experimental overexpression of mutated Ras proteins in cell culture-based and transgenic animal model systems. For example, overexpression of exogenously-introduced mutated *ras* genes has been shown to cause potent morphologic and growth transformation of a variety of rodent fibroblast and epithelial cell lines. Oncogenic *ras* can cooperate with other oncogenes to cause transformation of a variety of primary cells. Finally, the enhanced tumorigenesis seen in transgenic mice harboring mutated *ras* genes, also supports a key role of Ras in human carcinogenesis.

Since human cancers arise as a consequence of mutations in multiple genetic loci, the precise contribution of mutated *ras* to the malignant growth phenotype of human cancers remains to be fully elucidated. However, two studies of human tumor cells provide evidence that mutated *ras* genes contribute significantly to the aberrant growth properties of human tumor cells. First, Shirasawa *et al*. (1993) utilized homologous recombination to isolate variants of the DLD-1 and HCT116 human colon carcinoma cell lines that had lost the mutated K-*ras* allele. Loss of mutated *ras* caused a dramatic impairment in growth *in vitro* (e.g., colony formation in soft agar) and in tumor formation in nude mice. Second, we have shown that a variant of the HT1080 human fibrosarcoma cell line (designated MCH603c8), that had lost the short arm of chromosome one harboring the mutated N-*ras* allele (Anderson *et al*., 1994), corresponded to a significant impairment in its ability to grow in soft agar and to form tumors in nude mice (Plattner *et al*., 1996). This deletion occurred serendipitously during the microcell transfer of a copy of chromosome 1 from HT1080 cells that contained the wild type allele of N-*ras* into recipient HT1080 cells, i.e. the p-arm deletion occurred in the endogenous chromosome 1 containing the mutant allele. In those cells that received an intact chromosome 1 and retained the mutant N-*ras* allele, there was no change in their transformed or tumorigenic phenotypes (Anderson *et al*., 1994). Reintroduction of mutated N-*ras* into MCH603c8 resulted in a restoration of these growth properties.

Taken together, these studies clearly demonstrate that mutated *ras* contributes significantly to the transformed and tumorigenic growth properties of at least some human tumor cells.

The three human *ras* genes encode four Ras proteins that function as GDP/GTP-regulated binary switches (Boguski and McCormick, 1993). Ras proteins reside at the inner surface of the plasma membrane where they relay signals initiated by a wide variety of extracellular stimuli that control normal cell growth and differentiation to cytoplasmic signaling molecules (Egan and Weinberg, 1993; Khosravi-Far *et al*., 1998; Marshall, 1995). Upon stimulation, a rapid increase in the ratio of active, Ras-GTP occurs. This active state is transient and GTPase activating proteins (GAPs) stimulate conversion of Ras back to an inactive, GDP-bound state. Mutated *ras* genes encode oncogenic Ras proteins that contain single amino acid substitutions primarily at residues 12, 13 or 61 that render Ras insensitive to the action of Ras GAPs. Consequently, these mutant proteins persist as constitutively active, GTP-complexed proteins that cause chronic activation of growth-regulatory signaling pathways.

Ras mediates its actions through interaction with downstream effector targets that preferentially associate with active Ras-GTP (Khosravi-Far *et al*., 1998; Marshall, 1996). Considerable evidence supports the role of the Raf-1 serine/threonine kinase as a key effector of Ras function. Activated Ras-GTP causes activation of Raf-1, in part, by causing translocation of the normally cytosolic Raf-1 protein to the plasma membrane (Leevers *et al*., 1994; Stokoe *et al*., 1994), where a poorly understood series of events are initiated that cause full Raf-1 activation (Morrison and Cutler, 1997). Activated Raf then causes activation of the mitogen-activated protein kinase (MAPK) kinases MEK1 and MEK2, which in turn cause activation of the p42 and p44 MAPKs (also known as ERK2 and ERK1, respectively). Activated ERKs translocate into the nucleus where they phosphorylate and activate a variety of targets that include the Elk-1 transcription factor (Marais *et al*., 1993). Elk-1 causes transcriptional activation of a variety of growth-regulatory genes that include the c-*fos* proto-oncogene.

The importance of the Raf/MEK/ERK kinase cascade in mediating Ras transformation has been demonstrated in NIH3T3 mouse fibroblasts. The ability of dominant negative mutants of Raf, MEK or ERK to block Ras transformation demonstrates that activation of this cascade is necessary for transformation (Brtva *et al*., 1995; Cowley *et al*., 1994; Kolch *et al*., 1991; Meloche *et al*., 1992; Troppmair *et al*., 1994; Westwick *et al*., 1994). The ability of constitutively activated mutants of Raf-1 or MEK to cause morphologic and growth transformation of NIH3T3 cells shows that activation of this pathway alone is sufficient to promote Ras transformation (Cowley *et al*., 1994; Mansour *et al*., 1994; Stokoe *et al*., 1994). However, recent observations revealed a more complex scenario where Ras causes transformation by activation of multiple effectors in addition to the three Raf kinases (Khosravi-Far *et al*., 1998; Marshall, 1996). First, it was shown that effector domain mutants of Ras that no longer bind Raf can still cause tumorigenic transformation of NIH3T3 cells (Khosravi-Far *et al*., 1996; White *et al*., 1995). Second, we observed that Ras transformation of RIE-1 and other epithelial cells required both activation of Raf-dependent and Raf-independent pathways (Gangarosa *et al*., 1997; Oldham *et al*., 1996, 1998). Third, Ras causes activation of a Raf-independent MAPK cascade that leads to activation of the Jun NH_2-terminal kinases (JNKs) (Minden *et al*., 1994; Olson *et al*., 1995) and inhibition of JNK activation also inhibits Ras transformation of NIH3T3 cells (Clark *et al*., 1997). Finally, the roster of candidate Ras effectors continues to grow and includes a diverse spectrum of proteins that show preferential binding to Ras-GTP (Khosravi-Far *et al*., 1998; Marshall, 1996). These include members of the family of phosphatidylinositol 3-kinase (PI3K) lipid kinases (Chantry *et al*., 1997, Rodriguez-Viciana *et al*., 1994), activators of the Ras-related Ral proteins (RalGDS, RGL, RGL2/Rlf) (Hofer *et al*., 1994; Kikuchi *et al*., 1994; Peterson *et al*., 1996; Spaargaren and Bischoff, 1994; Wolthuis *et al*., 1996), Ras GAPs (Boguski and McCormick, 1993), Rin1 (Han and Colicelli, 1995), and AF6 (Kuriyama *et al*., 1996; Van Aelst *et al*., 1994). Therefore, the precise contribution of the Raf/MEK/ERK pathway to Ras transformation of human tumor cells remains unresolved.

An emerging theme in signal transduction is that specific signaling molecules are likely to have distinct, cell type-specific functions (Khosravi-Far *et al*., 1998). *ras* mutations are most prevalent in epithelial cell-derived human carcinomas (Bos, 1989; Clark and Der, 1993). Thus, caution must be exercised in extrapolating observations made in the study of Ras transformation in experimental rodent fibroblast cell systems to human cancer cells. Furthermore, the relative ease in which rodent cells can be transformed contrasts dramatically with the highly resistant nature of human cells to transformation. Therefore, we have chosen to evaluate the role of MAPKs in Ras transformation utilizing two human tumor cell systems. As described above, it has been demonstrated that mutated *ras* is important for growth transformation of both HT1080 human fibrosarcomas and DLD-1 colon carcinoma cells. A valid criticism of experimental studies in rodent cell systems is that overexpression of an exogenously introduced mutated *ras* gene is used to cause transformation (Hua *et al*., 1997). The mutated N-*ras*(61K) and K-*ras*(13D) alleles present in HT1080 and DLD-1 cells, respectively, arose during the natural development of these tumor cells (Plattner *et al*., 1996; Shirasawa *et al*., 1993). The availability of variants of HT1080 and DLD-1 cells, that have lost their mutated *ras* alleles, allows for a comparative analysis to determine the signaling pathways that are activated by oncogenic Ras to cause tumorigenic transformation of these cells. In the present study, we found that constitutive activation of MAPKs correlated with the expression of oncogenic Ras in HT1080, but not DLD-1, cells. Furthermore, we found that inhibition of ERK activation in HT1080 cells caused the same reversion seen when the mutated N-*ras* allele is lost from these cells. We suggest that upregulated activation of MAPKs may be critical for Ras transformation of HT1080 but not DLD-1 cells.

Results

Oncogenic Ras expression correlates with constitutive activation of ERK1/2 in HT1080 fibrosarcoma, but not in DLD-1 colon carcinoma cells

Although constitutive upregulation of ERKs is required for oncogenic Ras transformation of NIH3T3 cells (Khosravi-Far *et al*., 1995), it has not been established whether it contributes to oncogenic Ras transformation of human tumor cells. To address this question, we assessed whether constitutive upregulation of $p42^{MAPK}$/ERK2 and $p44^{MAPK}$/ERK1 corresponded to the expression of mutated Ras in HT1080 and DLD-1 cells. We utilized two approaches to quantitate the levels of the phosphorylated and activated forms of ERK1 and ERK2. First we performed mobility shift assays, utilizing an antibody that recognized the phosphorylated (active) and unphosphorylated (inactive) forms of ERK1 and ERK2. Activated Erks show reduced electrophoretic mobility in SDS–PAGE. Second, we used an antibody that recognized only the phosphorylated and activated forms of ERK1 and ERK2. Untransformed and H-Ras(61L)-transformed NIH3T3 cells were included as negative and positive controls, respectively.

As we and others have described previously (Khosravi-Far *et al*., 1995), untransformed NIH3T3 cells do not exhibit detectable levels of activated ERKs, whereas H-Ras(61L)-transformed NIH3T3 cells show high levels of activated p42 ERK2 or p44 ERK1 (Figure 1). Similar results were seen in both the mobility shift (Figure 1a) and the activated ERK (Figure 1b) analyses. We observed that the parental HT1080 (6TG) cell line, which contains an oncogenic N-*ras*(61K) allele, possessed a 2.9-fold higher level of activated ERKs, when compared to the HT1080 variant (MCH 603c8) that had lost the mutated N-*ras* sequence (Figure 1a,b and c). This activation was due to activation of p42 ERK2, whereas no detectable activated p44 ERK1 was seen. However, transient activation of ERKs was seen in these cells when treated with EGF (Figure 1c), indicating that these cells were not defective in the signaling pathways that involve Ras activation of ERKs. Finally, isolates of MCH603c8 where we had re-established expression of mutant N-Ras(61K) (M8-10 and M5-8) (35) regained high levels of activated p42 ERK2 and p44 ERK1 that correlated with the low (M8-10) or high (M5-8) levels of N-Ras(61K) expression (2.4- and 4.7-fold, respectively) (Figure 1a,b). We conclude that constitutive upregulation of ERKs is associated with the expression of activated N-Ras in HT1080 cells.

We next determined whether constitutive ERK activation was associated with expression of mutated K-Ras(13D) in DLD-1 cells. The parental DLD-1 cell line, which contains one wild type and one mutated (13D) K-*ras* allele, was compared to knockout variants that had lost either the wild type (DKO1) or mutant allele (DKO3 and DKs8) (Shirasawa *et al*., 1993). Dks5 is a variant that was isolated during the homologous knockout procedure that retains both wild type and mutant K-*ras* alleles. Unlike the HT1080-derived cell lines, we observed no correlation between the presence of activated Ras and the level of ERK activation (Figure 1c). Clones that contained a disrupted mutant K-*ras* allele (DK03 and DKs8) showed similar levels of activated ERK2 as the parental DLD-1 cell line or as DLD-1 variant cell lines that retained the mutated K-*ras* allele (DK01 and DKs5) (Figure 1c). Immunoblots, using the antibody that recognizes only the phosphorylated forms of the ERKs, also showed comparable low levels in the parental DLD-1 and knockout clones (data not shown). An ERK immune complex kinase assay showed that all DLD-1 cell lines had similar

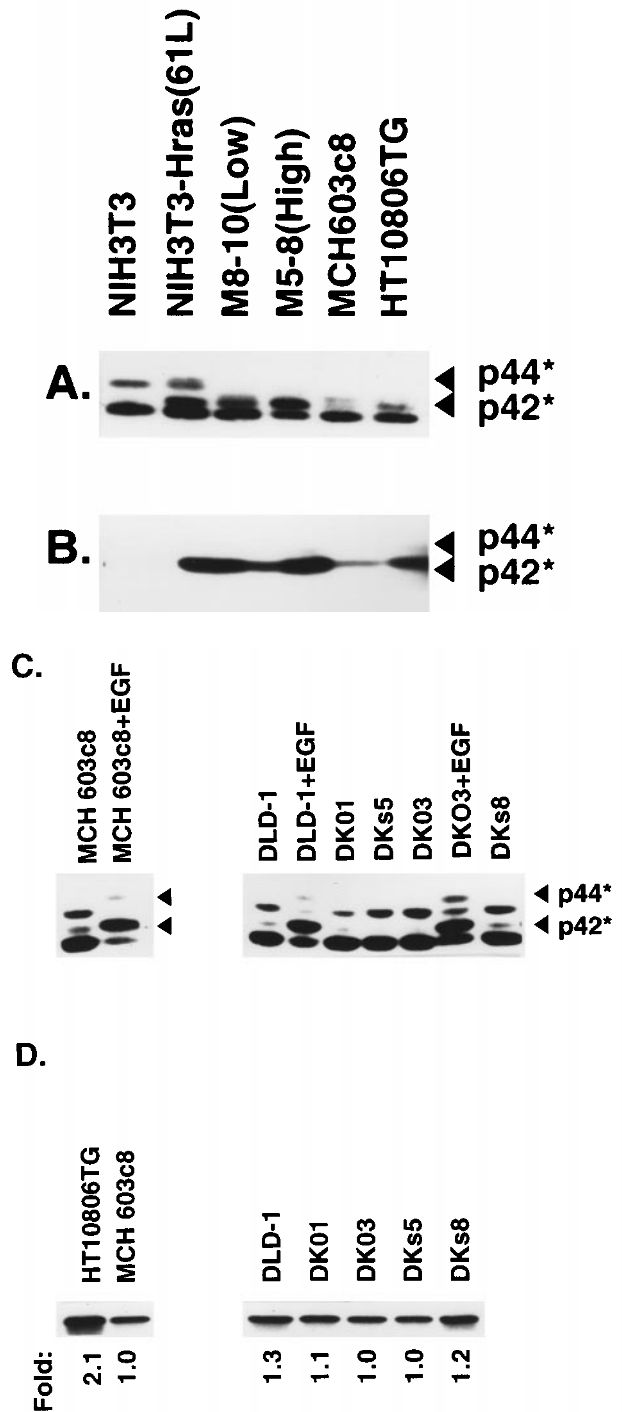

Figure 1 Constitutive activation of p42 and p44 ERKs correlates with expression of oncogenic Ras in HT1080, but not DLD-1, human tumor cells. ERK activity was assayed by Western blot analyses using (**a** and **c**) an ERK1/2-specific antibody or (**b**) a phospho-ERK antibody, or (**d**) by *in vitro* kinase assay. Arrows denote phosphorylated forms of ERK1 (p44) and ERK2 (p42)

levels of ERK activity, whereas HT1080 had 2.1-fold higher activity than MCH 603c8 (Figure 1d). Therefore, constitutive activation of ERKs did not correlate with oncogenic Ras expression in DLD-1 cells.

One possible explanation for the lack of ERK upregulation in cells expressing mutated K-Ras(13D) may be that these DLD-1 variant cell lines are impaired in the signaling components that link Ras with ERKs. To address this possibility, we stimulated serum-starved DLD-1 cells with epidermal growth factor (EGF), which is known to activate the Ras/ERK pathway. The parental DLD-1 and the K-Ras(13D)-deficient cell lines (DKO3 and DKs8) showed similar efficient stimulation of ERKs by EGF treatment (2.3–4.5-fold) (Figure 1c). Thus, we believe that the Ras/ERK pathway is intact in DLD-1 cells.

Elevated Raf-1 and MEK1 kinase activity are associated with the expression of oncogenic N-Ras(61K) in HT1080 fibrosarcoma cells

Since ERKs can be activated by signaling pathways independent of the Ras/Raf pathways, we performed Raf and MEK kinase assays in order to determine whether constitutive activation of Raf and MEK also correlated with the expression of mutated N-Ras(61K) in HT1080 cells. For these analyses, we immunoprecipitated endogenous Raf-1 and MEK1 from serum-starved cultures of the various HT1080 cell lines. The Raf-1 kinase assay was carried out using a coupled assay with recombinant MEK as an intermediate and kinase-deficient ERK protein as a substrate. The MEK assay utilized kinase-deficient ERK as a substrate.

Whereas MCH603c8 showed low Raf-1 and MEK1 kinase activities, the parental HT1080 cells contained high levels (twofold) of both kinases (Figure 2). In contrast, stable transfectants of MCH603c8, which expressed large amounts of exogenously introduced N-Ras(61K), possessed approximately threefold higher levels of Raf-1 and MEK1 kinase activity. Therefore, expression of oncogenic N-Ras(61K) correlated with elevated Raf, MEK, and ERK kinase activity in the HT1080-derived cell lines. Thus, ERK activation is likely due to oncogenic Ras upregulation of the Raf/MEK pathway.

Upregulation of the JNK MAPK is associated with expression of oncogenic Ras in HT1080 but not DLD-1 cells

In addition to ERKs, oncogenic Ras has been shown to cause constitutive activation of the JNKs in NIH3T3 cells (Khosravi-Far *et al*., 1996). JNK activation has also been shown to be required for Ras transformation of NIH3T3 cells (Clark *et al*., 1997). Therefore, we were interested in determining whether the JNK cascade was constitutively activated in human tumor cell lines containing mutant N- or K-*ras* alleles. For these analyses, endogenous JNK was immunoprecipitated from the different HT1080 and DLD-1 cell lines for use in an *in vitro* JNK kinase assay using recombinant GST-Jun as a substrate. Equal amounts of JNK protein were immunoprecipitated for each sample as assessed by immunoprecipitation and Western blotting (data not shown). Similar to ERK, we observed that elevated JNK activity also correlated with expression of N-Ras(61K) in HT1080 cells. The parental HT1080 cell line, as well as the variants of 603c8 with reconstituted N-Ras(61K) expression (M5-8, M8-10 and M8-17), all exhibited JNK activities that were elevated (2.0–2.6-fold) when compared to MCH603c8 cells (Figure 3a). In contrast, the various DLD-1-derived cell lines showed little difference in amount of JNK activity (Figure 3b). DK03 and DKs8, which lack expression of K-Ras(13D), showed the same level of JNK as the parental DLD-1 line and the variant that retained K-Ras(13D) expression (DKs5). Thus, activation of JNK correlated with the expression of oncogenic Ras in HT1080, but not DLD-1, cells. We also did not detect appreciable differences in p38 MAPK activity in any of the cell lines (data not shown).

Activation of Raf/MEK/ERK and JNK pathways in HT1080 cells resulted in constitutive activation of the TCF/Elk-1 transcription factor

The ERK and JNK MAPKs are activators of various nuclear transcription factors. For example, ERK causes phosphorylation and activation of Elk-1 (Marais *et al*., 1993), and JNK phosphorylates and activates Elk-1 and Jun (Whitmarsh *et al*., 1995). To

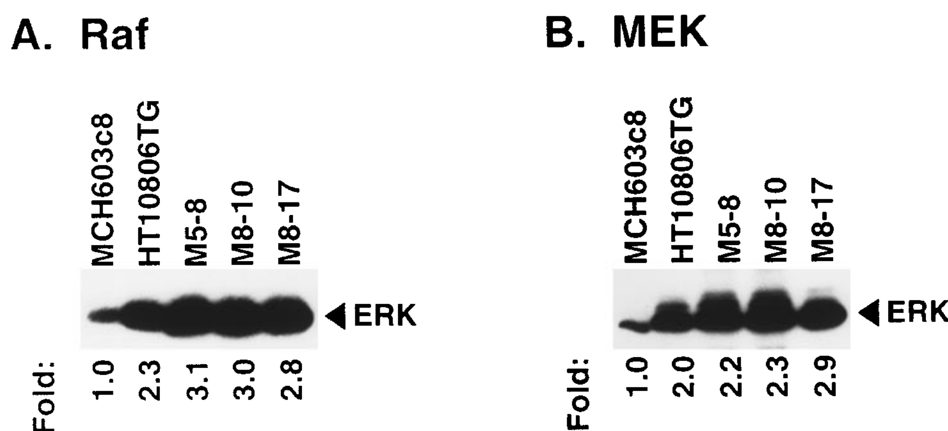

Figure 2 Raf and MEK kinases are constitutively activated in HT1080-derived cells containing an oncogenic N-*ras* allele. *In vitro* Raf-1 (**a**) and MEK (**b**) kinase assays were performed on the indicated cells as described in Materials and methods. Data shown are representative of three independent experiments

determine whether the level of ERK and JNK activation correlated with enhanced activation Elk-1, we performed transient expression assays using the various HT1080 and DLD-1 cell lines. An expression plasmid that encodes a chimeric Gal-Elk-1 protein containing the DNA binding domain of the yeast Gal4 protein fused to the transactivation domain of Elk-1 was cotransfected with a reporter construct where luciferase gene expression was controlled by a promoter containing tandem Gal4 DNA binding sites.

Parental HT10806TG cells showed a threefold higher level of Elk-1 activity than that seen with MCH603c8 (Figure 4). Consistent with the kinase assays, the N-*ras*(61K) transfectants of MCH603c8 showed elevated levels of Elk-1 activity that were higher than those seen for HT10806TG. Transient expression of exogenously introduced N-*ras*(61K) resulted in twofold activation of Elk-1 in MCH 603c8, whereas Elk-1 activity was not elevated further by the additional expression of N-Ras(61K) in cells that already possessed oncogenic N-Ras(61K) expression (data not shown). Thus, we found that Elk-1 activation correlated with the elevated ERK and JNK activity seen in oncogenic N-Ras(61K)-expressing versions of HT1080 cells. Use of a similar Gal-Jun chimeric expression construct with a Gal4-luciferase reporter also resulted in reduced c-Jun activation in MCH603c8 cells as compared to HT1080; re-expression of mutant N-Ras (61K) in MCH603c8 cells restored elevated Jun activity (data not shown).

In contrast to HT1080, there was no correlation between Elk-1 activity and K-*ras*(13D) mutation status in DLD-1 cell lines (Figure 4). In fact, the highest activity was seen in the DKs8 knockout cell line that had lost the mutated K-*ras*(13D) allele. Therefore, consistent with what we observed in the ERK and JNK kinase assays, K-Ras(13D) expression did not correlate with increased Elk-1 activity.

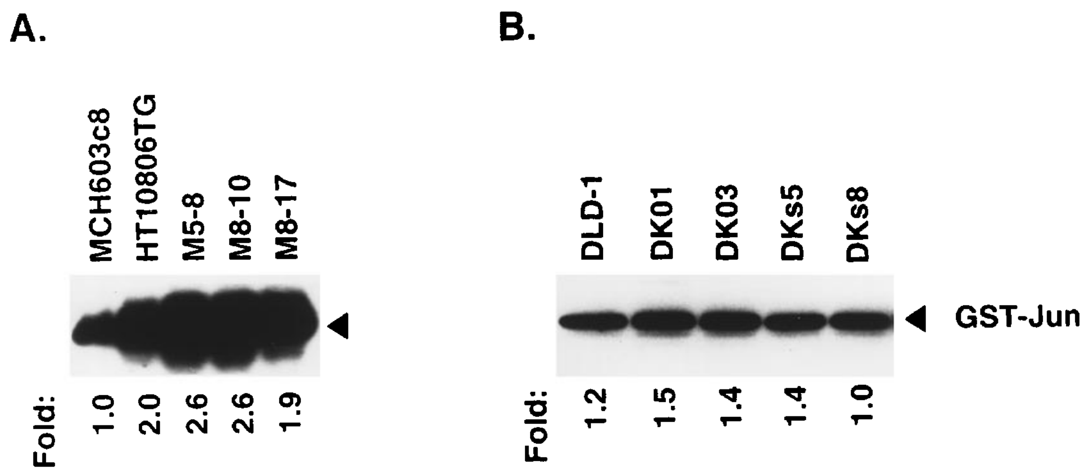

Figure 3 Constitutive activation of JNK correlates with the expression of activated Ras in HT1080, but not DLD-1, cells. *In vitro* JNK kinase assays were done with the indicated (**a**) HT1080- or (**b**) DLD-1-derived cell lines. Data shown are representative of three independent experiments

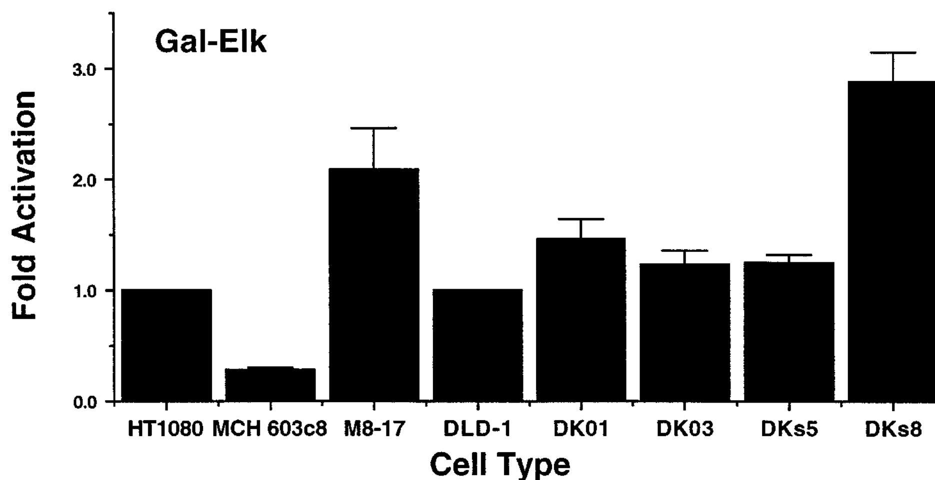

Figure 4 Increased Elk-1 activity is correlated with expression of activated Ras in HT1080, but not DLD-1 cells. HT1080- and DLD-1-derived cells were cotransfected with the pMMLV-Gal-Elk fusion construct together with a 5×-Gal-Luc reporter. Luciferase activity was normalized to protein concentration using Lowry DC (Biorad). Luciferase activity was expressed as a percentage of the luciferase activity obtained after the simultaneous transfection with pGL2, a control construct which contains a SV40 promoter upstream of a luciferase gene. Fold activation was determined by comparing activities from HT1080-derived and DLD-1 derived cell lines to each parental cell line, respectively. Luciferase assays were performed in triplicate, in multiple independent assays (3–5 times) using independent preparations of DNA with similar results

Transient overexpression of K-Ras(13D) causes activation of ERK2 in DLD-1 cells

The lack of MAPK upregulation in K-Ras(13D)-expressing cells argues that Ras-mediated transformation is promoted by upregulation of other signaling pathways in DLD-1 cells. However, there is evidence that the K-Ras(13D) mutant protein is not as potent as other activated forms of K-Ras. For example, 13D mutations are observed in colonic tumors from individuals who develop cancer at a later age (Perucho, unpublished observation). In addition, K-Ras(13D) exhibits a much weaker transforming activity than K-Ras(12V) when expressed in rodent fibroblasts (Perucho, unpublished observations). Thus, our observed lack of MAPK upregulation associated with expression of K-Ras(13D) may simply reflect the possibility that this activated mutant is not as potent as other activated K-Ras proteins in causing ERK activation. To evaluate this possibility, we compared the ability of different activated forms of K-Ras4B to cause activation of ERK in DLD-1 cells.

For these analyses, we utilized expression plasmids where wild type (13G) and mutant K-Ras4B proteins were expressed from the human genomic K-*ras* promoter (Winter and Perucho, 1986). Mutant proteins included the weakly transforming 13D protein, as well as the highly transforming mutants harboring substitutions at residue 12 (12C, 12D and 12V). This approach was chosen to minimize over-expression of the exogenously introduced gene sequences. Transient expression of wild type K-Ras4B (13G) or vector DNA did not cause activation of cotransfected HA-ERK2 (Figure 5, data not shown). In contrast, expression of all four mutant K-Ras proteins in the parental DLD-1 cells led to HA-ERK2 activation. Essentially identical results were seen when the mutant proteins were expressed in wild type allele-deficient DLD-1 cells (data not shown). We also observed that all mutant K-Ras proteins showed similar activation of Elk-1 in DKs8 cells (Figure 5b). Taken together, these results suggest that the failure of K-Ras(13D)-expressing cells to cause MAPK upregulation is not due to an inherent defect of signaling in this pathway.

Suppression of the Raf/MEK/ERK pathway caused morphologic reversion of HT1080 fibrosarcoma cells and restoration of actin filaments

Our observation that the Raf/MEK/ERK pathway was constitutively activated in fibrosarcoma cells that expressed the oncogenic N-*ras*(61K) allele suggests that upregulation of this kinase cascade may be necessary for N-Ras(61K)-mediated transformation of HT1080 cells. To evaluate this possibility, we utilized the PD098059 MEK inhibitor, which has been shown to specifically block ERK, but not JNK or p38, activation (Alessi *et al*., 1995).

We first determined whether treatment with PD098059 could effectively reduce ERK activation in HT1080. Serum-starved cultures were treated overnight with different concentrations of inhibitor and the level of ERK activation was then determined. Significant reductions in both activated p42 and p44 ERKs were seen when HT1080 cells were treated with 20 μM PD098059 (Figure 6). At 75 μM, only a low level of

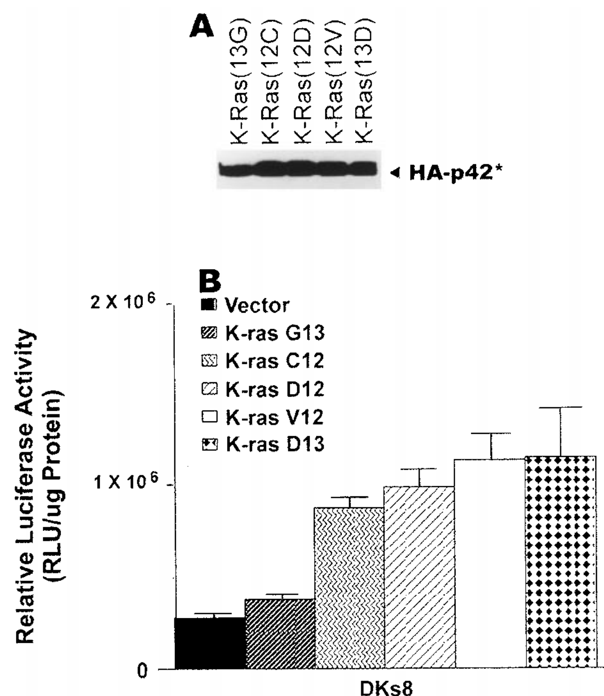

Figure 5 All mutant forms of K-Ras4B cause activation of ERK. Mini-genomic mutant (12C, 12D, 12V, 13D) and wild type (13G) K-*ras* constructs, where expression is regulated by the human K-*ras* promoter, were transiently cotransfected in DK01 cells together with HA epitope-tagged ERK (HA-ERK2). HA-ERK2 phosphorylation was assessed by direct Western blot mobility shift assay using an anti-HA epitope antibody (**a**). Mini-genomic wild type, or mutant K-*ras* constructs, or vector alone (300 ng) were transiently cotransfected with pMMLV-Gal-Elk fusion construct (250 ng) and 5 × Gal-Luc (2.5 μg) reporter into DKs8 cells (**b**). Luciferase activity was standardized to protein concentration using Lowry DC (Biorad). The assays were performed in triplicate, multiple times (3–5), using different lots of K-Ras expression vector DNA with similar results

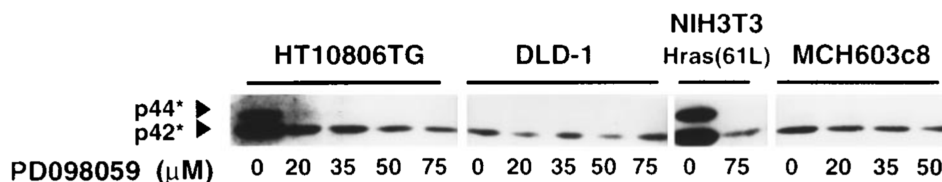

Figure 6 Treatment with the PD098059 MEK inhibitor causes reduction of constitutive ERK phosphorylation in parental HT1080 but not DLD-1 cells. The indicated cells were serum-starved and treated overnight with the indicated concentration of PD098059. The cells were lysed and Western blot analysis was performed using the anti-active ERK antibody (Promega)

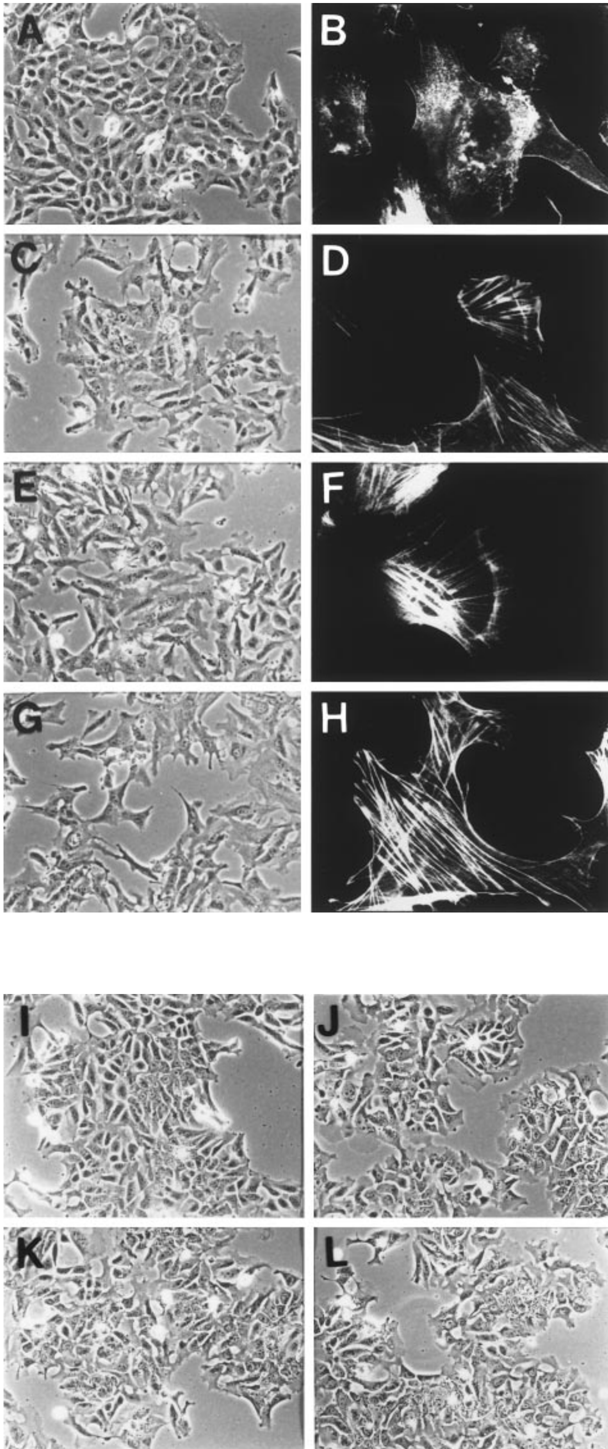

Figure 7 Treatment of HT10806TG causes a reversion in morphology and actin filament organization similar to that seen with loss of N-Ras(61K) expression. HT10806TG (**a–d**) and MCH 603c8 (**e–h**) cells were treated for two days either with DMSO alone (**a**, **b**, **e** and **f**) or with 50 μM (**c** and **d**) or 75 μM (**g** and **h**) PD098059. Changes in cell morphology were visualized with phase contrast microscopy (50 × magnification). Actin organization was visualized by staining with Texas Red-phalloidin and viewed with fluorescence using Texas-Red excitation/emission filters (200 × magnification), and CCD camera imaging (Oncor). The experiment was performed three times using various additional concentrations of MEK inhibitor (data not shown). Treatment of DLD-1-derived cell lines with PD098059 has little effect on cellular morphology; DLD-1 (**i** and **j**) and DKs8 (**k** and **l**) cells were treated either with DMSO alone (**i** and **k**) or with 50 μM PD098059 (**j** and **l**), and cellular morphology was visualized with phase-contrast microscopy (50 × magnification)

activated p42 was detected in HT1080 or H-Ras(61L)-transformed NIH3T3 cells. Western blot analyses of total ERK expression in the treated cells confirmed that the reduction in activated ERKs was not due to reduced levels of ERK expression (data not shown).

The low level of activated p42 ERK2 activity present in DLD-1 cells was not detectably reduced by treatment with up to 75 μM PD098059. Similarly, only modest (30%) reduction of ERK activation was seen in MCH603c8 cells treated with 50 μM PD098059. Therefore, both DLD-1 and MCH603c8 may possess MEK-independent activation of ERKs. Alternatively, MEK inhibition may not be complete even at high concentrations of inhibitor.

Treatment of parental HT10806TG cells with PD098059 caused a dramatic change in morphology (Figure 7). The cells exhibited a more adherent appearance after treatment with as little as 20 μM of PD098059. When treated with 50 μM of inhibitor, HT10806TG cells were indistinguishable in appearance from untreated MCH603c8 cells. Thus, inhibition of ERK activation mimicked the loss of oncogenic Ras expression in HT1080 cells. Interestingly, treatment of MCH603c8 with PD098059 also caused additional flattening (Figure 7k), whereas treatment of DLD-1-derived cells had little effect on morphology (Figure 7i–l).

We have shown previously that loss of the mutated N-*ras* allele caused an appearance of well organized actin stress fibers in MCH603c8 cells (Plattner *et al*., 1996). Therefore, we determined whether blocking ERK activation alone could similarly restore actin stress fiber formation. Treatment of HT10806TG with PD098059 caused dose-dependent enhancement in the actin fibers, with the appearance of organized stress fibers similar to those seen in untreated MCH603c8 cells (Figure 7). Interestingly, treatment of MCH603c8 cells caused a change in the appearance of its actin fibers into prominent cable-like fibers which were obvious at low drug concentrations (35 μM) and were striking at higher drug concentrations (75 μM) (Figure 7h). It is possible that the PD098059 inhibitor may have effects on cytoskeletal architecture unrelated to inhibition of MEK activity. We, therefore, stably transfected HT1080 cells with the MEK dominant negative expression vector, MEK-KA. Clones expressing MEK-KA had significantly decreased ERK activity, approximating that of MCH603c8 (data not shown), and exhibited the reappearance of actin fibers (Figure 8).

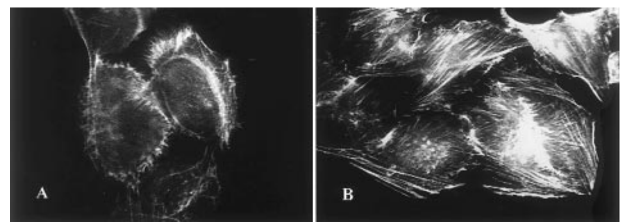

Figure 8 Actin filament organization in HT1080 (**a**) and HT1080/MEK-KA dominant negative stable transfectant (**b**). Well organized filaments are seen only in the stable transfectant cells, (800 × magnification)

Discussion

The importance of the Raf/MEK/ERK pathway in mediating Ras transformation of human tumor cells remains unresolved (Khosravi-Far *et al*., 1998). Experimental studies using rodent fibroblast or epithelial cells have reached conflicting conclusions. Furthermore, it is unclear whether the observations from these studies provide a valid assessment of the importance of this kinase cascade in promoting the Ras-induced transformed and tumorigenic phenotype in human tumor cells. Finally, whereas N-*ras* and K-*ras* are most frequently mutated in human cancers, much of our knowledge on Ras signaling and transformation has been derived from studies using H-Ras-transformed cells (Clark and Der, 1993). In the present study, we utilized two human tumor cell systems, where the contribution of oncogenic Ras to transformation has been established, in order to evaluate the role of the ERK and JNK pathways in cellular transformation. Parental and variant populations of HT1080 human fibrosarcoma and DLD-1 human colon carcinoma cells that differ in their Ras mutation status were evaluated (Plattner *et al*., 1996; Shirasawa *et al*., 1993). Whereas the expression of mutated N-Ras(61K) correlated with constitutive upregulation of the Raf/MEK/ERK and JNK pathways in HT1080 cells, no such correlation was observed for the expression of mutated K-Ras(13D) in DLD-1 cells. Additionally, we found that inhibition of ERK activation caused a reversion of the transformed phenotype of HT1080 cells. Taken together, our observations suggest that constitutive Raf/MEK/ERK and JNK pathway activation is necessary for maintenance of the full transformed phenotype of HT1080 but not DLD-1 cells.

Our results with HT1080 fibrosarcoma cells are similar to observations with Ras-transformed NIH3T3 rodent fibroblasts (Khosravi-Far *et al*., 1995). Oncogenic Ras activation of the Raf/MEK/ERK pathway has been shown to be necessary and sufficient to cause growth and morphologic transformation of NIH3T3 cells (Cowley *et al*., 1994; Kolch *et al*., 1991; Mansour *et al*., 1994; Meloche *et al*., 1992; Westwick *et al*., 1994). We found that constitutive activation of Raf, MEK and ERKs correlated with the expression of activated N-Ras in HT1080 cells. Whereas the loss of N-Ras(61K) expression in MCH603c8 cells corresponded to a decreased activation of the Raf/MEK/ERK pathway, reintroduction of N-Ras(61K) expression caused restoration of upregulated activation of this kinase cascade. Inhibition of ERK activation in HT1080 cells by treatment with a MEK inhibitor caused the same reversion of cellular morphology and actin organization that was associated with loss of N-Ras(61K) expression in MCH603c8 cells. The same restoration of actin microfilaments was seen in HT1080 cells stably transfected with the MEK-KA dominant negative plasmid. Significant decreases in p42 and p44 ERK activities accompanied expression of this dominant-negative MEK cDNA. These results suggest that the Raf/MEK/ERK pathway is a key mediator of at least some aspects of the transformed phenotype caused by oncogenic Ras in HT1080 cells.

Although Ras activation of Raf alone is sufficient to cause transformation of NIH3T3 cells (Stokoe *et al*., 1994), Ras effector domain mutants that are impaired in Raf binding can still cause tumorigenic transformation of NIH3T3 cells. Thus, Ras transformation of NIH3T3 cells is mediated by activation of both Raf-dependent and Raf-independent effector pathways (Graham *et al*., 1996; Khosravi-Far *et al*., 1996; White *et al*., 1995). At present, it is unclear whether activation of the Raf/MEK/ERK pathway alone is sufficient for oncogenic Ras-mediated transformation of HT1080 cells. However, we have preliminary observations that overexpression of constitutively activated mutants of Raf-1 (Raf/CAAX and Raf22W) failed to restore tumorigenic growth potential to the oncogenic Ras-deficient MCH603c8 cells (Gupta, unpublished observations). Therefore, we anticipate that Ras activation of both Raf-dependent and Raf-independent pathways will be important for oncogenic Ras transformation of HT1080 cells. Ras causes activation of JNK via a Raf-independent pathway (Minden *et al*., 1994; Olson *et al*., 1995). Inhibition of JNK activation has been shown to block Ras transformation of NIH3T3 cells (Clark *et al*., 1997). Whether JNK activation is also important for Ras transformation of HT1080 cells will be important to determine.

In contrast to our observations with HT1080 cells, we found that expression of oncogenic K-Ras(13D) did not correlate with an upregulation of either ERKs or JNKs. These results suggest that upregulation of MAPKs is not required for Ras transformation of DLD-1 cells. Thus, we suggest that K-Ras might upregulate other effector-mediated pathways that promote growth transformation of these epithelial cells. This may well include the PI3-K pathway or, indeed, one or more as yet unidentified pathways. We are currently examining these possibilities. Although it is possible that the lack of upregulation of MAPKs may reflect the weaker potency of the oncogenic K-Ras(13D) protein, we found that this mutant could cause a transient upregulation of ERKs that was comparable to that caused by more potent forms of oncogenic K-Ras4B protein (12D, 12V and 12C). Furthermore, we have found that overexpression of K-Ras(12V) under the control of a strong CMV promoter had a similar ability to activate ERK and Elk-1 as N-Ras(61K) (data not shown). Therefore, we do not believe that the lack of a correlation between the expression of oncogenic K-Ras(13D) and upregulation of MAPKs is due to differences in signaling between N-Ras and K-Ras4B. Instead, we believe that this reflects cell type differences in the signaling pathways that Ras utilizes to cause transformation of the fibroblastic HT1080 and the epithelial cell-derived DLD-1 cells. This possibility is consistent with our recent observations that Ras transformation of NIH3T3 mouse fibroblasts and RIE-1 rat epithelial cells is mediated by the activation of very distinct signaling pathways (Gangarosa *et al*., 1997; Oldham *et al*., 1996, 1998).

In summary, our analyses of HT1080 and DLD-1 cells provide the first evaluation of the contribution of MAPKs to oncogenic Ras transformation of human tumor cells. The lack of a correlation between the expression of oncogenic Ras and upregulation of MAPKs seen in DLD-1 cells argues that cell type differences will exist regarding the signaling pathways that promote Ras transformation. Establishing the

importance of MAPK pathways in promoting oncogenic Ras transformation of human tumor cells is important in light of the intense drug discovery efforts to develop inhibitors of these kinase cascades as novel anti-cancer agents. Our study suggests that upregulated MAPKs will be important for some, but not all, Ras mutation positive cancers. Further evaluation of the importance of the ERK and JNK pathways in human tumors where Ras mutations are frequently seen (e.g., lung, colon, and pancreatic carcinomas) will be required to further clarify whether upregulation of these pathways are cell-type specific as our data suggest. More studies such as this clearly are warranted before anti-ERK or other anti-Ras pathway therapies can be considered as potential cancer treatments.

Material and methods

Molecular constructs

The mammalian expression construct pCMVneo-N-*ras*(61K) encodes an oncogenic form of N-Ras(61K) and was generated and characterized previously (Plattner *et al*., 1996). Mini-genomic expression constructs that encode wild type (pMLG13) or mutant human K-Ras4B (pMLC12, pMLD12, pMLV12, and pMLD13 encode proteins with 12C, 12D, 12V and 13D mutations, respectively), where expression is controlled from the human K-*ras* promoter, have been described (Winter and Perucho, 1986; Perucho, manuscript in preparation). The pLNCMKEA plasmid (provided by M Weber) encodes a hemagglutinin (HA) epitope-tagged ERK2 protein (Reuter *et al*., 1995). The MEK-KA (104A) plasmid is a MEK dominant-negative construct that is mutated in the ATP-binding site. Plasmids for use in luciferase reporter assays (5X-Gal-Luc and pMMLV-Gal-Elk) were obtained from M Karin and R Treisman, respectively. The 5X-Gal-Luc construct contains five yeast Gal4 DNA binding sites in a minimal promoter that controls expression of the luciferase gene. The pMMLV-Gal-Elk construct encodes a fusion protein where the Elk-1 DNA binding domain is replaced by the DNA binding domain of the yeast Gal4 protein (Marais *et al*., 1993). The pGL2 control expression plasmid contains the luciferase gene under control of the constitutive SV40 promoter (Clontech).

Cell culture

The HT1080 cell line contains one wild type and one oncogenic (Q61K) N-*ras* allele (Anderson *et al*., 1994; Plattner *et al*., 1996). The microcell hybrid cell line MCH603c8 was produced from HT10806TG by microcell-mediated chromosome transfer of a copy of an HT1080-derived chromosome 1 containing the wild type allele of N-ras. At some point subsequent to the transfer, the endogenous p-arm of chromosome 1 containing the mutant allele of N-*ras* was deleted (Anderson *et al*., 1994). Thus, MCH603c8 contains two wild type N-*ras* alleles and no oncogenic N-*ras* allele. M5-8, M8-10, and M8-17 represent clonal isolates of MCH 603c8 cells that were stably transfected to re-establish expression of N-Ras(61K) and have been characterized previously (Plattner *et al*., 1996). The DLD-1 human colon carcinoma cell line contains one oncogenic (G13D) and one wild type allele of K-*ras* (Shirasawa *et al*., 1993). Variant clones where either the mutant (DK03, Dks8) or wild type (DK01) K-*ras* allele was inactivated by homologous recombination have been characterized and described previously. DKs5 represents a DLD-1 variant that was isolated during the homologous recombination procedure where both wild type and mutant K-*ras* alleles were retained. NIH3T3 cells transformed by overexpression of activated H-Ras(61L) have been described previously (Khosravi-Far *et al*., 1995).

The parental HT10806TG cell line and all DLD-1-derived cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (gibco/BRL), whereas parental and Ras-transformed NIH3T3 cells were grown in DMEM supplemented with 10% calf serum (Hyclone). MCH603c8 was grown in growth medium supplemented with HAT (hypoxanthine/thymidine/methotrexate) (Plattner *et al*., 1996). MCH603c8 variants stably transfected with an exogenous N-*ras*(61K) gene (M5-8, M8-10, and M8-17) were maintained in growth medium supplemented with HAT and 600 μg/ml G418 (Geneticin, gibco/BRL). MCH603c8 and transfected variants were weaned off of HAT selection prior to analysis as described previously (Plattner *et al*., 1996).

Luciferase reporter assays

To measure Elk-1 activation, cells were transiently transfected by liposome-mediated transfection (lipofectin, Gibco/BRL) for 6 h with 2.5 μg of 5 $\times$ Gal-Luc reporter and 0.25 μg of the pMMLV-Gal-Elk expression constructs. The pGL2 plasmid (2.5 μg) was transfected to control for differences in transfection efficiencies between cell lines. After transfection, cells were serum-starved by incubation overnight in DMEM containing 0.25% serum, and lysed 24 h later in 1 $\times$ Cell Lysis reagent (Promega). The lysate was analysed for luciferase activity (Moonlight 2010 luminometer; Analytical Luminescence Laboratory) and normalized for protein concentration (Lowry DC reagent; Biorad).

ERK expression, activation, and inhibition analyses

Subconfluent (40–60%) cultures were serum-starved for 24 h followed by either no stimulation or stimulation with epidermal growth factor (EGF) (10 ng/ml for 5 min at 37°C). Stimulated and unstimulated cells were lysed in modified RIPA buffer #1 containing 50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM sodium chloride, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and phosphatase inhibitors (1 mM PMSF, 10 μg/ml aprotinin, leupeptin, pepstatin). Ten μg of total cell extracts was resolved on 8% sodium dodecyl sulfate polyacrylamide gels (SDS–PAGE) and transferred onto Immobilon membranes (Millipore).

For endogenous ERK mobility shift assays, blots were incubated with the ERK-1 (sc-94, Santa Cruz Biotech) primary antibody which recognizes phosphorylated (active) and unphosphorylated (inactive) forms of the p44 ERK1 and p42 ERK2 proteins, followed by horseradish peroxidase-conjugated (HRP) goat anti-rabbit IgG secondary antibody (Santa Cruz Biotech) and chemiluminescence detection (Pierce).

To assay the effect of the different K-Ras4B mutants on HA-ERK2 phosphorylation, cells were transiently transfected with 2 μg of either a vector, wild type or mutant K-*ras* construct together with 2 μg of HA-ERK2 using liposome-mediated transfection. Cells were serum-starved, lysed, and resolved on 8% SDS–PAGE gels as above. Immobilon membranes were incubated with an anti-HA epitope monoclonal primary antibody (Babco) followed by anti-mouse IgG-HRP (Santa Cruz Biotech), secondary antibody, and chemiluminescence detection.

In order to assess the phosphorylation state of ERK proteins, antibodies specific for the phosphorylated forms of the ERK proteins (phosphospecific MAPK antibody (New England Biolabs), anti-active MAPK antibody (Promega) also were utilized.

To determine the contribution of ERK activation to transformation, we blocked ERK activation by using the PD098059 MEK inhibitor. This inhibitor has been shown to specifically block ERK, but not JNK or p38 activation (Alessi *et al*., 1995). PD098059 (New England Biolabs) was dissolved in dimethylsulfoxide (DMSO). Two days after plating, cells were serum-starved overnight in medium containing various concentrations of PD098059. Control cultures, treated with only DMSO, were also included. The next day, the cells were lysed and Western blots were performed using anti-active MAPK antibody (Promega). To further analyse the contribution of MEK activation, a dominant-negative MEK (MEK-104A) plasmid was transfected as previously described (Plattner *et al*., 1996). Multiple clonal populations were isolated and inhibition of ERK activity was assessed by Western blot analysis as described above.

Kinase assays

Kinase assays were performed as described previously (Graham *et al*., 1996) on serum-starved cultures of HT1080 and DLD-1-derived cell lines. Raf-1, MEK, or ERK was immunoprecipitated with anti-Raf-1 rabbit polyclonal antibody (C-12; Santa Cruz Biotech), MEK1 mouse monoclonal antibody (Transduction Laboratories) or anti-ERK1 rabbit polyclonal antibody (C16; Santa Cruz Biotech), respectively. The Raf-1 immunocomplex assay was carried out in a coupled assay using MEK as an intermediate (Reuter *et al*., 1995). The MEK immunocomplex assay was carried out by incubating immunoprecipitated MEK with 5 μg of kinase-deficient ERK protein for 30 min in the kinase reaction mix. The ERK immunocomplex assay was carried out by incubating immunoprecipitated ERK with 4 μg of myelin basic protein in a kinase assay for 20 min at 30°C.

For JNK activation assays, lysates containing approximately 30 μg of proteins were incubated for 4 h with 1–5 μg of GST-c-Jun (1–79) to pull down JNK as described previously (Westwick and Brenner, 1995). Alternatively, JNK was immunoprecipitated with JNK antibody (C17; Santa Cruz Biotech), and incubated in a kinase reaction with 2 μg of purified GST-cJun. All kinase reactions were stopped by addition of 2 × SDS–PAGE sample buffer. Proteins were separated on 15% SDS–PAGE and visualized by autoradiography.

Actin cytoskeleton staining

Actin stress fibers were visualized by staining with Texas Red-conjugated phalloidin (Molecular Probes). Two days after plating, cells were treated with PD098059, an inhibitor of MEK activity (Alessi *et al*., 1995), for 2 days at various concentrations, or incubated with an equivalent amount of DMSO alone. Cells were fixed in 3.7% paraformaldehyde, stained with phalloidin (0.005 U/μl) for 20 min at room temperature, and mounted in Prolong Fade antifade (Molecular Probes). Clones that had been stably transfected with the MEK dominant negative plasmid were processed in a similar fashion.

Acknowledgements
This work was supported by NIH grants to EJS (CA19401 and CA69515) and to CJD (CA42978, CA63071, and CA69577). We thank Michael Anderson for isolating the MCH603c8 cell line, Adrienne Cox, John Westwick and Simona Raffioni for their invaluable technical advice, and Jennifer Parrish for preparation of figures.

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