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Dissection of Ras-Dependent Signaling Pathways Controlling Aggressive Tumor Growth of Human Fibrosarcoma Cells: Evidence for a Potential Novel Pathway

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Activation of multiple signaling pathways is required to trigger the full spectrum of in vitro and in vivo phenotypic traits associated with neoplastic transformation by oncogenic Ras. To determine which of these pathways are important for N-ras tumorigenesis in human cancer cells and also to investigate the possibility of cross talk among the pathways, we have utilized a human fibrosarcoma cell line (HT1080), which contains an endogenous mutated allele of the N-*ras* gene, and its derivative (MCH603c8), which lacks the mutant N-*ras* allele. We have stably transfected MCH603c8 and HT1080 cells with activating or dominant-negative mutant cDNAs, respectively, of various components of the Raf, Rac, and RhoA pathways. In previous studies with these cell lines we showed that loss of mutant Ras function results in dramatic changes in the in vitro phenotypic traits and conversion to a weakly tumorigenic phenotype in vivo. We report here that only overexpression of activated MEK contributed significantly to the conversion of MCH603c8 cells to an aggressive tumorigenic phenotype. Furthermore, we have demonstrated that blocking the constitutive activation of the Raf-MEK, Rac, or RhoA pathway alone is not sufficient to block the aggressive tumorigenic phenotype of HT1080, despite affecting a number of in vitro-transformed phenotypic traits. We have also demonstrated the possibility of bidirectional cross talk between the Raf-MEK-ERK pathway and the Rac-JNK or RhoA pathway. Finally, overexpression of activated MEK in MCH603c8 cells appears to result in the activation of an as-yet-unidentified target(s) that is critical for the aggressive tumorigenic phenotype.

The Ras superfamily of small GTPases, of which there are more than 80 mammalian members, comprises at least nine distinct branches. These include the Ras, Rab, RhoA, Ran, Rheb, Rad/Gen, Rin/Rif, and Arf families (2, 3). Ras family proteins constitute one of the three major branches of the Ras superfamily. Members of the Ras family, namely, H-ras, K-ras, and N-ras, have been implicated in human cancers. The association of mutated *ras* (H-, K-, and N-*ras*) genes with up to 30% of all human cancers suggests an important contribution of constitutively active Ras function to the development of human cancers (6, 28).

Early studies of the transforming activity of transfected oncogenes in mouse embryo fibroblasts led to the conclusion that a single *ras* oncogene, alone or in cooperation with another oncogene, e.g., *myc*, was sufficient to induce neoplastic transformation (1, 21, 37). It is now clear that mutations of multiple genes, including oncogenes, tumor suppressor genes, and DNA repair genes, are necessary to convert a normal cell into a neoplastic cell (20, 24, 44). The functional contribution of mutant, constitutively active Ras proteins to this progression remains unclear. It is clear from a variety of experimental approaches that mutant Ras has pleiotropic effects on the morphology and growth behavior of cells. These effects include shape changes associated with dissolution of actin microfilaments, a reduced requirement for serum growth factors, increased motility and invasiveness, anchorage-independent growth in vitro, and more aggressive tumor growth in vivo (15, 31, 37, 47).

Early studies of Ras signal transduction identified the linear transduction cascade of Ras→ Raf → MEK → ERK → Elk-1, culminating in transcriptional activation of genes involved in mitogenesis (22, 35). It has become increasingly clear, however, that this simple linear pathway represents only a minor component of a very complex signaling circuitry (5, 10). Recent evidence has indicated that Ras mediates its actions through interactions with multiple effectors, both Raf dependent and Raf independent. Additionally, there is accumulating evidence that components of the individual linear pathways engage in cross talk (5, 41).

This multiplicity of functionally diverse effector targets is accompanied by a similar diversity of phenotypic consequences of Ras activation. The activation of the Raf-dependent signal transduction pathway results in growth promotion, following the transcription of genes required for mitogenesis. Several Raf-independent effectors have been shown to regulate the actin cytoskeleton and influence cell shape and motility. Rac induces peripheral actin accumulation and membrane ruffling, and RhoA is involved in the induction of the assembly of stress fibers and focal adhesions (15). Both Rac and Cdc42 assemble focal complexes; additionally, Cdc42 induces the formation of filopodia and Rac induces the formation of lamellipodia (2). Phosphoinositol 3-kinase (PI3K) is a lipid kinase that phosphorylates phosphoinositides and also has been implicated as a Ras effector (36). PI3K-induced activation of protein kinase B/Akt, which activates Bad via phosphorylation, leading to phosphorylation-induced inactivation of procaspase 9, constitutes an antiapoptotic survival signal (12).

This bewildering complexity of Ras effector functions has

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TABLE. 1. DN and constitutively active mutants used in this study

Mutant	Mutation	Mechanism	Reference
Dominant negative			
Raf C	Lacks Ras binding regions; contains kinase domain and MEK binding domain; encodes C-terminal fragment of human Raf-1	Forms a complex with endogenous MEK and inhibits its activation	8
MEK1(101KA)	Codon 101 (lysine) mutated to alanine	Phosphorylation site has been made catalytically inactive	19
Rac1(17N)	Codon 17 (serine) mutated to asparagine	Forms inactive complexes with Rac-specific GEFs	18
RhoA(19N)	Codon 19 (serine) mutated to asparagine	Forms inactive complexes with Rho-specific GEF(s)	18
Constitutively active			
Raf(22W)	N-terminal end of Raf-1 is truncated	Forms catalytically active Raf-1	49
MEK1ΔED	Codon 218 (serine) mutated to glutamic acid; codon 222 (serine) mutated to aspartic acid	Phosphorylation site (serine) has been mutated to make it catalytically active	25
Rac1(115I)	Codon 115 (asparagine) mutated to isoleucine	Forms catalytically-active Rac-1	18
RhoA(63L)	Codon 61 (glutamine) mutated to leucine	Forms catalytically active RhoA	18

made it difficult to determine those functions that are critical for neoplastic transformation. For example, it has been reported that interaction of activated Ras with Raf-1 alone is sufficient for transformation of Rat-2 cells (4). It was also reported that activating mutants of MEK were necessary and sufficient for neoplastic transformation of mouse NIH 3T3 cells (25). However, it has also been reported that Ras effector domain mutants that selectively activate Raf-independent pathways induce neoplastic transformation of NIH 3T3 cells (19, 47).

Most studies of the transforming effects of Ras or Ras effector proteins have employed transfection of the relevant oncogene(s) into rodent cells. The major reason for this is that rodent cells are readily transformed by these oncoproteins whereas normal human cells are refractory to their transforming effects (38, 44). Thus, it is possible that rodent experimental models may not accurately reflect the physiologic consequences of expression of mutant Ras and Ras-related proteins in human cells.

We have had a long-standing interest in the role of mutant Ras function in human cancers. Our earlier studies showed that expression of mutant Ras was not sufficient to neoplastically transform normal or immortalized human cells (7, 44). More recently, we have shown that deletion of endogenous mutant Ras alleles in human cancer cells does not result in loss of tumorigenicity (31). However, the loss of mutant Ras function resulted in profound pleiotropic phenotypic alterations in vitro, resulting in many features of reverse transformation. Furthermore, the kinetics of tumor growth are significantly affected; those cells lacking mutant Ras formed tumors more slowly. We also found interesting differences in the pathways activated by mutant Ras, depending on whether mutant N-ras or K-ras was involved (32).

We have extended these observations in the present study in an attempt to decipher which individual pathway(s) is critical for the transformed and aggressive tumorigenic phenotypes. Our model system is the HT1080 human fibrosarcoma cell line. These are pseudodiploid cells possessing a single mutant N-ras allele (27). We have isolated a variant, termed MCH603c8, in which the mutant N-ras allele has been deleted (31). The parental HT1080 cells exhibit typical features of a transformed cell in culture, including poor adherence, anchorage-independent growth, and disorganized actin and aggressive tumor formation. The MCH603c8 variants have more normal growth characteristics, including a flat adherent morphology, anchorage-dependent growth, and well-organized actin microfilaments. The cells are weakly tumorigenic—tumors are formed in all animals inoculated with the cells, but they grow significantly more slowly than HT1080 cells (31).

Examination of the Ras signal transduction pathways in HT1080 cells showed that downstream members of all pathways examined, Raf dependent and Raf independent, have high constitutive activities (32). Conversely, MCH603c8 cells showed only low basal activity except for a lower level of constitutive ERK activity and constitutively activated protein kinase B/Akt and p38. The latter constitutive activities are probably due to the fact that both HT1080 and MCH603c8 secrete platelet-derived growth factor (PDGF), which binds to and activates its cognate receptor (R. Plattner and S. Gupta, unpublished observation), followed by activation of PI3K.

Utilizing either dominant-negative (DN) or constitutively active mutant cDNAs of members of the Raf, Rac, and RhoA pathways, we have downregulated or upregulated individual arms of the Ras signal transduction pathways in HT1080 and MCH603c8 cells, respectively. Distinct alterations in in vitro and in vivo phenotypic traits are seen and provide evidence for a possible novel signaling pathway that is required for the aggressive tumorigenic phenotype.

MATERIALS AND METHODS

Molecular constructs. The mutants used in this study are listed in Table 1. The expression construct pCMV(hyg)Raf(22W) was derived from pZipRaf(22W), which encodes an NH_2-terminally truncated human Raf-1 that is catalytically active (49). The construct pCGN(hyg)RafC encodes truncated Raf, which lacks the Ras binding sequences (8) and is catalytically inactive. The construct pmc1(hyg)MEK1ΔED encodes a mutated MEK cDNA in which two serine codons in the regulatory site, namely, codons 218 and 222, have been mutated to glutamic and aspartic acid, respectively (25). The mutant MEK also has an NH_2-terminal deletion of amino acids 31 to 52. This region acts as a nuclear export signal, and this signal directs cytoplasmic localization or nuclear exclusion of MEK (13). This results in a catalytically active protein that has been shown to have potent transforming activity for mouse NIH 3T3 cells. The construct pCMV(neo) MEK-KA(101A) encodes a mutant MEK in which the ATP binding site has been mutated, rendering it catalytically inactive (30). pCMV(neo)Rac(17N) encodes a mutated Rac in which codon 17 (serine) has been changed to asparagine, rendering it catalytically inactive (18). Also, pCMV(hyg)Rac1 (115I) encodes a mutated Rac in which codon 115 (asparagine) has been changed to isoleucine, resulting in a catalytically active protein (18). pCMV (hyg)RhoA(63L) was derived from pZip-RhoA(63L), which encodes RhoA, where codon 63 has been changed from glutamine to lysine, rendering it constitutively active. pCMV(neo)RhoA(19N) encodes RhoA, where codon 19 has been changed from serine to asparagine and is catalytically inactive (18).

Cell culture and stable transfection. The HT1080 cell line contains one mutant and one wild-type N-*ras* allele (27, 31). MCH603c8 contains only wild-type N-*ras* (31). The cell lines were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Life Technologies). The HT1080 and MCH603c8 cell lines transfected with the various mutant cDNAs were maintained in their respective antibiotic selection media prior to experimentation. Subconfluent (70%) 100-mm-diameter dishes of MCH603c8 cells or HT1080 cells were transfected with 5 μg of linearized DNA or vector control DNA, using 30 μl of Lipofectin (Gibco BRL) in OPTIMEM medium (Gibco BRL). Clones from each transfection were selected and maintained in medium containing the relevant selective antibiotic (either 800 μg of Geneticin [Gibco BRL]/ml or 36 U of hygromycin B [Calbiochem]/ml).

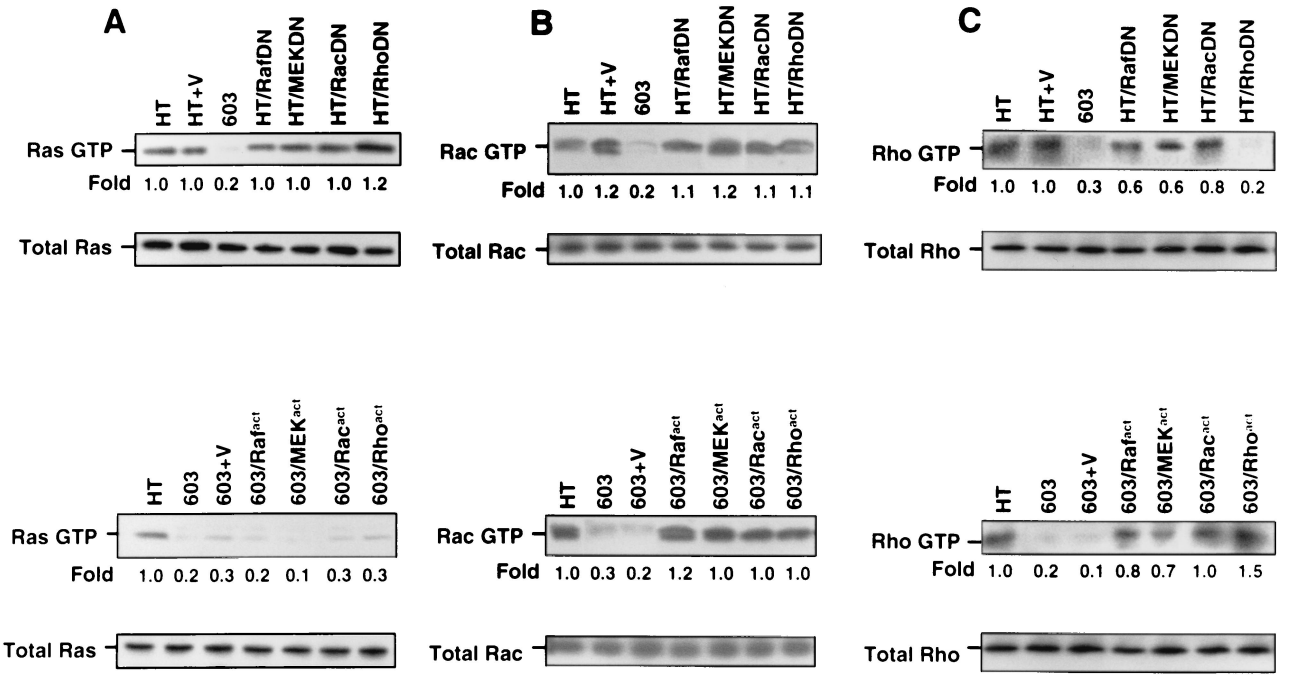

FIG. 1. Pull-down assays of activated Ras, Rac, and RhoA. The GTP-bound forms of Ras, Rac, and RhoA were pulled down with glutathione *S*-transferase fusion proteins, corresponding to the Ras binding domain of Raf-1, PBD of human PAK-1, and C21 binding domain of RhoA, respectively, conjugated to agarose beads. The Ras-GTP, Rac-GTP, and RhoA-GTP proteins bound to the beads were identified using anti-Ras (A) anti-Rac (B), and anti-RhoA (C) antibodies, respectively, in a Western immunoblot. Immunoblot analysis of total cell lysates identified the levels of total protein. HT, HT1080 cells; 603, MCH603c8 cells; V, vector-only control.

Growth kinetics. To assess in vitro growth kinetics, 10^4 cells were seeded in triplicate in T25 flasks containing DMEM–10% FCS. The cells were harvested on various days and counted in a Coulter counter. The growth medium was replenished at regular intervals.

Growth in soft agar. Cells (10^4 or 10^6) were seeded in suspension in a 0.3% top agar overlay (in DMEM–10% FCS) above a 0.5% bottom agar layer (in DMEM–10% FCS) in 60-mm-diameter dishes as previously described (31). The plates were fed periodically with 1 ml of DMEM–10% FCS. Colonies (>0.1-mm diameter) were counted after 3 weeks.

Actin cytoskeleton staining and morphology. Actin stress fibers were visualized by staining cells with fluorescein-conjugated phalloidin (Molecular Probes). Two days after being plated on slide chambers, the cells were fixed in 3.7% paraformaldehyde, treated with 0.1% Triton X-100 solution, and then stained with phalloidin (0.005 U/μl) for 20 min at room temperature and mounted in ProLong fade-antifade (Molecular Probes).

Activated Ras, Rac, and RhoA assays. Subconfluent cells were serum starved for 18 h and then lysed with 1× Mg^{2+} lysis buffer (Ras and Rac activation assay kits; Upstate Biotechnology). Each of the cell lysates (500 μg) was affinity precipitated with 10 μl of Raf-1 Ras binding domain or PAK-1 p21 binding domain (PBD) agarose or glutathione *S*-transferase–2 C21 Sepharose conjugate (40) at 4°C overnight for the Ras, Rac-Cdc42, or RhoA activation assay, respectively. The beads were collected, washed, and resuspended in 6× Laemmli sample buffer. Western blot analysis was performed as described previously (9), using 1 μg (each) of mouse monoclonal anti-Ras, anti-Rac, and anti-RhoA (Santa Cruz Biotechnology) antibodies/ml. Horseradish peroxidase-conjugated anti-mouse immunoglobulin G (Santa Cruz Biotechnology) was used as the secondary antibody. A chemiluminescence detection system (Pierce) was used for detection of the relevant proteins. To determine the total Ras, Rac, or RhoA level, immunoblots were performed using N-ras (F155), Rac1 (C-14) or RhoA (26C4) antibodies (Santa Cruz Biotechnology) that recognize total protein.

Kinase assays. MEK, ERK, and JNK kinase assays (New England Biolabs) were performed according to the manufacturer's protocols using subconfluent cultures serum starved (0.25% FCS) for 18 h. For the MEK and ERK proteins, 500 μg of total cell lysate was immunoprecipitated with the relevant antibodies. JNK was precipitated from 250 μg of total cell lysate using the c-Jun fusion protein bead procedure (New England Biolabs). The activated MEK assay was carried out by incubating immunoprecipitated phospho-MEK with ERK protein and cold ATP (MEK1/2 kinase assay kit; New England Biolabs). The activated ERK assay was carried out by incubating immunoprecipitated phospho-ERK with Elk-1 fusion protein and cold ATP (p44/p42 ERK assay kit; New England Biolabs). The JNK assays were carried out by incubating the JNK–c-Jun fusion protein complex with cold ATP (JNK/SAPK assay kit; New England Biolabs). All of the kinase reactions were performed at 30°C for 30 min in the kinase reaction mixture of 25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, and 10 mM $MgCl_2$. The reactions were stopped by addition of 6× Laemmli sample buffer, and the proteins were separated on sodium dodecyl sulfate–8% polyacrylamide gel electrophoresis. For MEK, ERK, and JNK assays, the relevant gel was transferred onto an Immobilon membrane and Western blot analysis was performed. The blots were performed using phospho-ERK (Thr202-Tyr204) monoclonal antibody for the MEK assay, phospho-Elk-1 (Ser383) polyclonal antibody for the ERK assay, and phospho-c-Jun (Ser63) polyclonal antibody for the JNK assay. The Raf-1 assay was performed as described by Graham et al. (14). Total Raf-1 protein was immunoprecipitated from 500 μg of total cell lysate with polyclonal Raf-1 antibody. The Raf-1 assay was carried out in a coupled assay using MEK and mitogen-activated protein kinase (MAPK) substrates as intermediates and γ-^{32}P-labeled ATP (33). For the Raf-1 assay, the γ-^{32}P-labeled MAPK proteins in the gel were visualized by autoradiography. To determine the total Raf, MEK, ERK, and JNK levels, immunoblots were performed, using the respective antibodies that recognize total protein.

Dual luciferase reporter assays. To measure Elk-1 activation, a dual luciferase reporter assay kit (Promega) was used. Cells were transiently transfected using the liposome-mediated transfection technique (Lipofectin; Gibco BRL), with 2.5 μg of the 5× Gal-luciferase reporter, and 0.25 μg of the pMMLV–Gal–Elk-1 expression construct (obtained from M. Karin and R. Triesman, respectively). pRL-cytomegalovirus *Renilla* luciferase (0.02 μg) was used as the internal control reporter vector. After transfection, the cells were serum starved by incubation in DMEM containing 0.25% FCS and were lysed 18 h later in 1× cell lysis reagent (Promega). Twenty microliters of cell lysate was analyzed for luciferase activity using a Moonlight 2010 luminometer (Analytical Luminescence Laboratory).

Tumorigenicity asssays. Tumorigenicity was assessed by subcutaneous injection of 10^7 cells, resuspended in 0.2 ml of DMEM, into the flanks of 4- to 6-week-old nude athymic mice. Tumors were measured in three dimensions with linear calipers at weekly intervals.

RESULTS

Biochemical characteristics of the parental and transfectant cell lines. We have shown previously that the Raf–MEK–ERK–Elk-1 pathway, as well as JNK, is constitutively active in HT1080 and downregulated in MCH603c8 cells (32). We have expanded those studies here and shown, as expected, that high levels of constitutively activated Ras-GTP, Rac-GTP, and RhoA-GTP are found in HT1080 cells whereas only very low levels are seen in MCH603c8 cells (Fig. 1A, B, and C, respec-

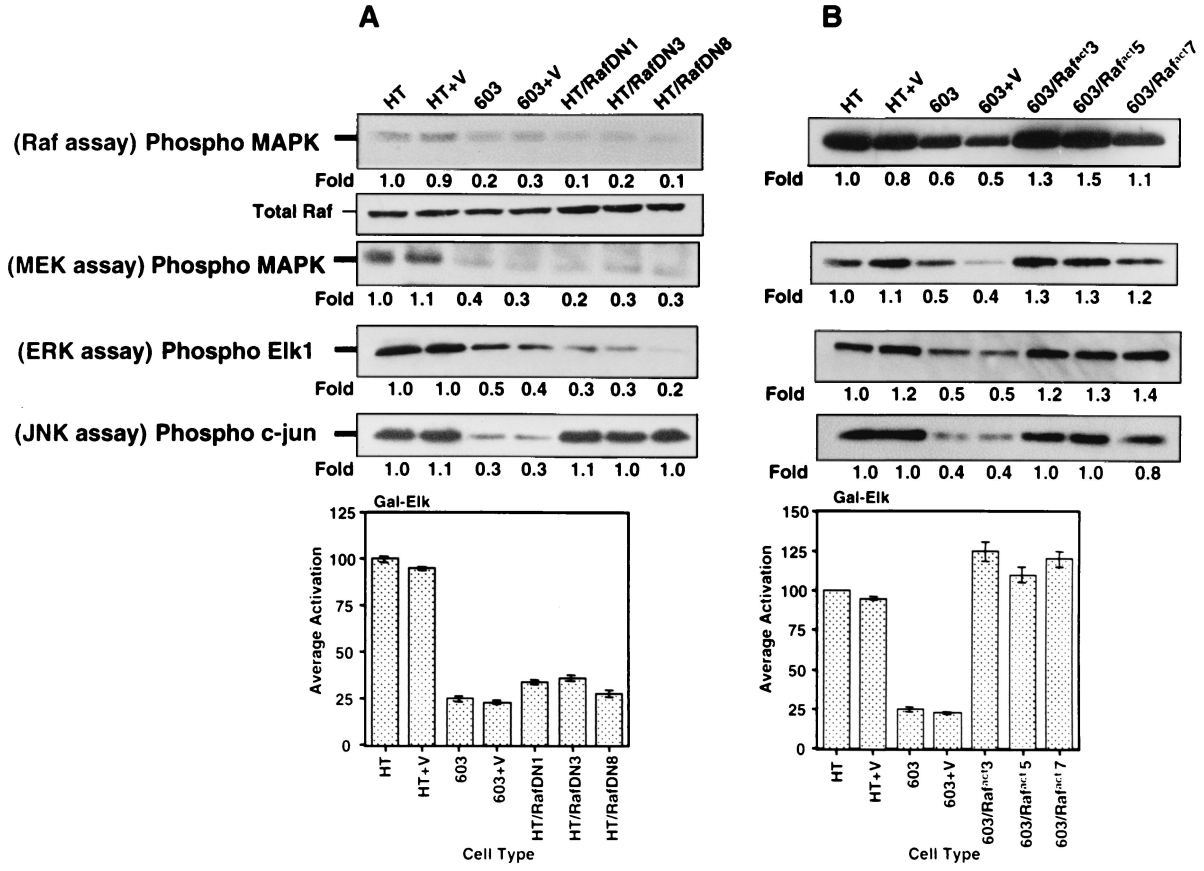

FIG. 2. In vitro Raf, MEK, ERK, and JNK kinase assays and Elk-1 activation assays performed on HT1080 (HT)-Raf DN transfectants (A) and MCH603c8 (603)-Raf^{act} transfectants (B). For the kinase assays, the fold level is relative to 1.0 for HT1080 control cells, and the Elk-1 luciferase reporter activities are expressed as percents relative to 100% for HT1080. Three independent Raf DN clones and Raf^{act} clones were analyzed. V, vector-only control. The error bars indicate standard deviations.

tively). In order to investigate the roles that these pathways play in the differential expression of the transformed and tumorigenic phenotypes in these cell types, the following studies were designed to individually downregulate or activate these pathways in HT1080 and MCH603c8 cells, respectively. In addition, we sought to discover evidence for cross talk between the Ras signaling pathways.

(i) Modulation of constitutively active Raf levels. The HT1080-Raf DN transfectants showed decreased constitutive activity of all members of the Raf-dependent pathway. The levels of activity were approximately equivalent to those seen in the MCH603c8 cells (Fig. 2A). Moreover, as shown in Fig. 2A, the Raf-1 DN transfectants showed normal levels (corresponding to that in HT1080) of endogenous total Raf-1, as well as the expressed truncated mutant RafC. There was no obvious evidence of negative-feedback cross talk between the Raf and Rac pathways. Both Rac (Fig. 1B) and JNK (Fig. 2A) retained the same levels of activity that are seen in the parental HT1080 cells. Also, there was no evidence of cross talk between the Raf and RhoA pathways (Fig. 1C). Furthermore, elevated Ras-GTP levels were maintained in the transfectants (Fig. 1A). The Raf DN protein fails to bind to Ras but forms a complex with MEK and is predicted to block MEK function (8). This is, indeed, what we observed. Unexpectedly, we also observed a decreased constitutive activity of endogenous Raf in these cells, as measured in this assay. A possible reason for this is that the Raf activity assay involves binding of immunoprecipitated total Raf protein to a MEK substrate and that the Raf DN protein has a higher affinity for the MEK substrate, possibly masking endogenous active Raf-mediated phosphorylation of the MEK substrate. Although possible, this is not the likely explanation, since the MEK substrate is in excess. Another possibility is that the Raf DN protein interferes with the activation of endogenous Raf by the endogenous mutant Ras. Further experiments are required to resolve this.

The MCH603-Raf^{act} transfectants all exhibited the predicted increase in constitutive Raf activity, as well as activation of the respective downstream members of the pathway: MEK $\rightarrow$ ERK $\rightarrow$ Elk-1 (Fig. 2B). The level of constitutive activity of each member was slightly above that seen in the HT1080 cells (approximately 1.3- versus 1.0-fold, respectively). Clear evidence of cross talk was seen, with an increase in constitutive JNK activity to a level commensurate with that seen in HT1080. The Rac and RhoA binding protein assays showed that cross talk occurred at the level of Rac as well as RhoA. The data in Fig. 1B and C show clear evidence of elevated levels of Rac- and RhoA-GTP, respectively. However, there was no elevation in Ras-GTP levels in the transfectants (Fig. 1A). Thus, the JNK activation due to cross talk seems to be Ras independent and may be Rac dependent. This pattern differs from that of the HT1080-Raf DN transfectants in that the latter did not show evidence of a diminution in levels of activated JNK, Rac-GTP, or RhoA-GTP. Thus, the constitutive activity of JNK in the latter transfectants is probably due to activation from the endogenous activated Rac.

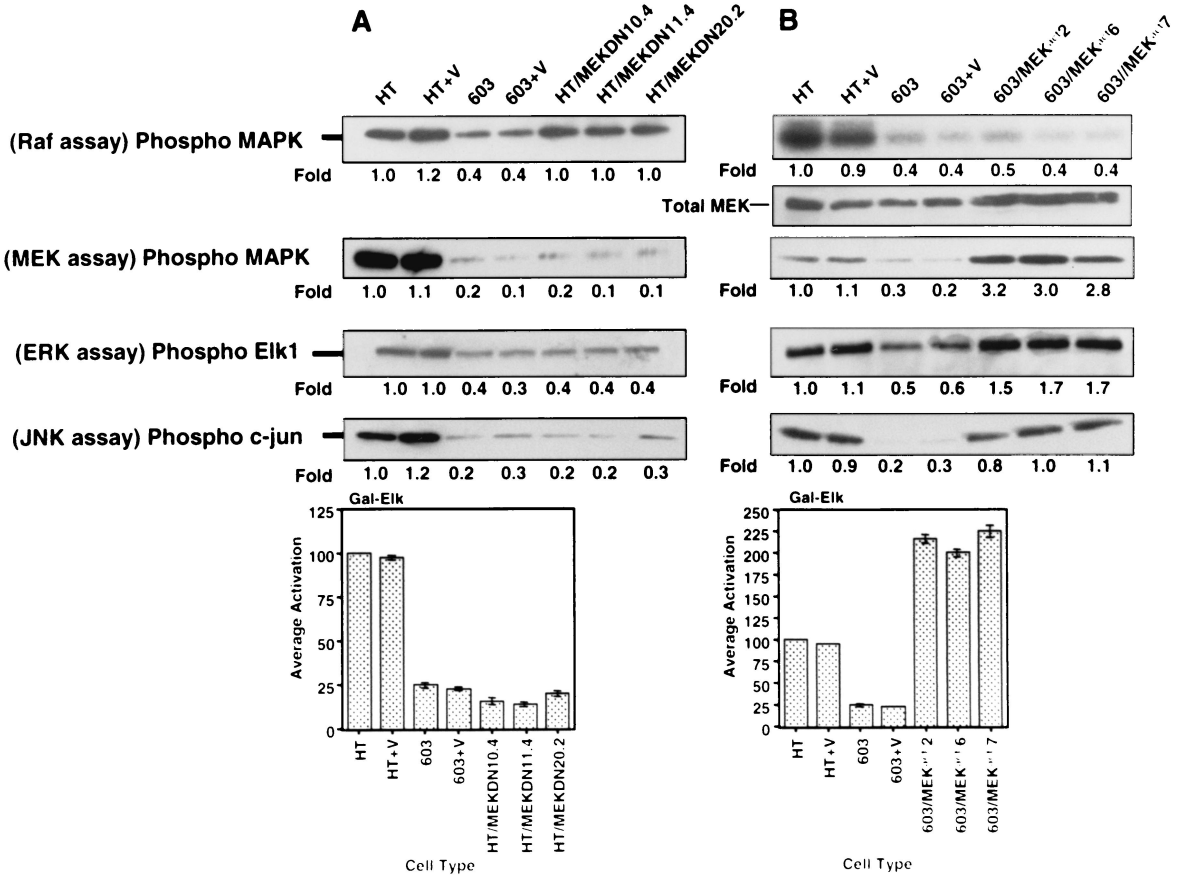

FIG. 3. In vitro Raf, MEK, ERK, and JNK kinase assays and Elk-1 activation assays performed on HT1080 (HT)-MEK DN transfectants (A) and MCH603c8 (603)-MEK^{act} transfectants (B). Fold levels were calculated as for Fig. 2. Three independent MEK DN clones and MEK^{act} clones were analyzed. V, vector-only control. The error bars indicate standard deviations.

(ii) Modulation of constitutively active MEK levels. The MEK DN protein used in these studies functions by binding the endogenous activated Raf in HT1080 cells, thereby preventing its activation of endogenous MEK. Thus, the MEK DN protein functions as a RAF inhibitor whereas the previously described Raf DN protein is an inhibitor of activated MEK. The HT1080-MEK DN transfectants all showed significant decreases in constitutive MEK, ERK, and Elk-1 activities, approximating the levels seen in MCH603c8 cultures (Fig. 3A). No decrease in Raf activity was noted, indicating that "back talk" from MEK did not occur. There was, however, a significant decrease in JNK activity. This result was somewhat unexpected because Rac-GTP levels remained elevated (Fig. 1B) and previous studies have shown that Ras activation of JNK is dependent on the presence of activated Rac (23). The reason why Rac-GTP levels remain high in the HT1080-MEK DN cells is presumably because the mutant Ras constitutively activates Rac. We also observed that decreased constitutive activity of endogenous MEK in HT1080-MEK DN cells had only a modest effect on the constitutive levels of RhoA-GTP (Fig. 1C). This again is likely due to the continued expression of mutant N-ras in these cells, activating endogenous RhoA.

The MCH603c8-MEK^{act} transfectants showed normal levels of endogenous total MEK protein in addition to the exogenous truncated mutant MEK^{act} protein (Fig. 3B). The MCH603c8-MEK^{act} transfectants all had a significantly increased level of constitutive MEK activity that was approximately threefold higher than that seen in HT1080 cells (and 10-fold higher than that in MCH603c8 cells). Modestly elevated levels of constitutive ERK and Elk-1 activities were also noted—approximately 1.5- to 2.25-fold higher than that seen in HT1080 cells (Fig. 3B). Again, cross talk was observed, with activation of both Rac and JNK (Fig. 1B and 3B). The levels of constitutive Rac and JNK activities were approximately the same as those seen in HT1080 cells.

The fact that HT1080-Raf DN transfectants (presumably still containing endogenous activated Raf) do not inhibit JNK activity whereas the HT1080-MEK DN transfectants do might indicate that Raf rather than MEK activation is required for JNK activation. However, in the case of the MCH603-MEK^{act} transfectants, where Raf is not activated and there is no mutant N-ras, there is clear evidence of JNK activation (Fig. 3B). Taken together, the data indicate that cross talk is possible at the level of MEK or involving its downstream partner(s), resulting in the enhancement of JNK activation.

(iii) Modulation of constitutively active Rac levels. The HT1080-Rac DN transfectants possessed a reduced level of constitutive JNK activity (Fig. 4A). There was also a corresponding reduction in the level of constitutive Raf activity, to a level approximating that seen in MCH603c8 cells, suggestive of negative cross talk between Rac and Raf. Unexpectedly, there was no corresponding reduction in the constitutive levels of activity of MEK, ERK, or Elk-1 (Fig. 4). Thus, although there is clear evidence for some interaction between Rac and Raf, the fact that N-ras remains constitutively active (Fig. 1A) leaves open the possibility that Ras may activate MEK via a

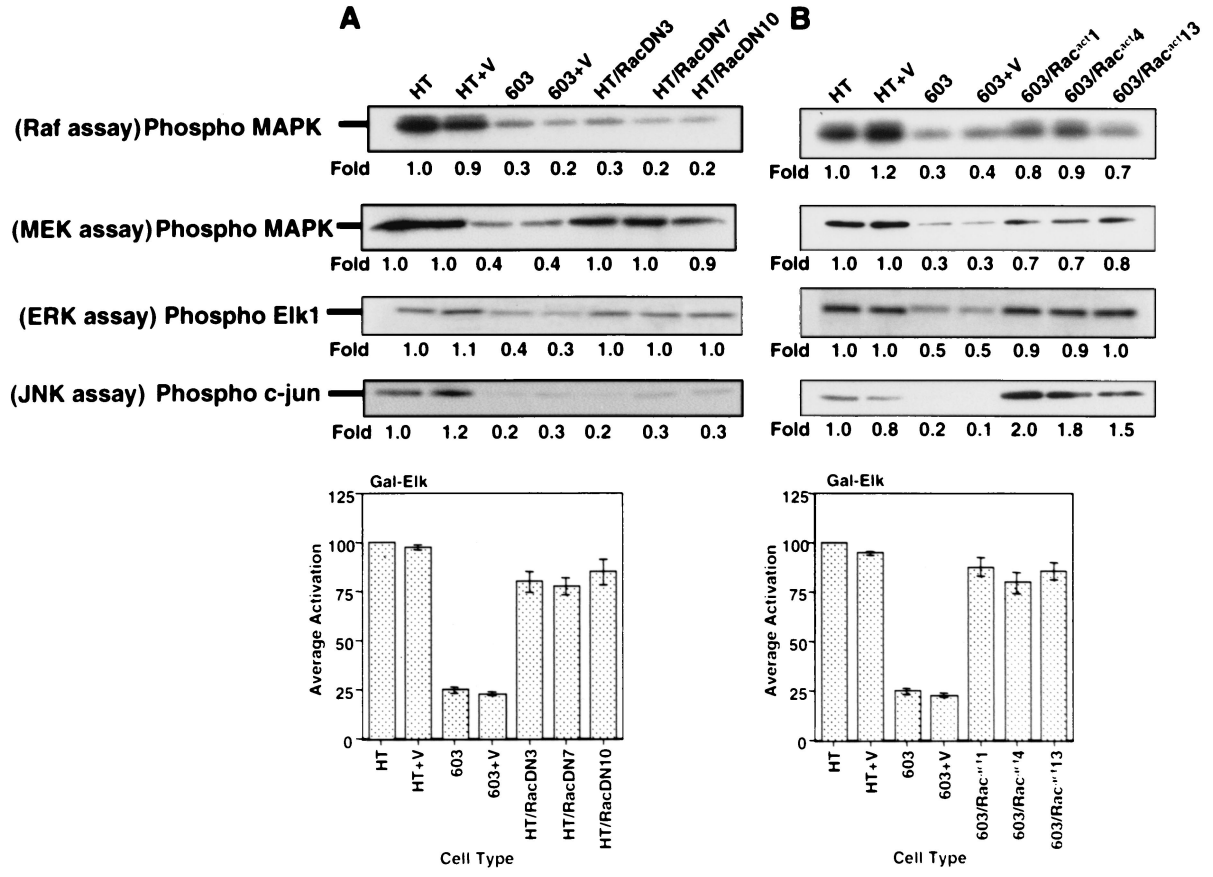

FIG. 4. In vitro Raf, MEK, ERK, and JNK kinase assays and Elk-1 activation assays performed on HT1080(HT)-Rac DN transfectants (A) and MCH603c8 (603)-Rac^{act} transfectants (B). Fold levels were calculated as for Fig. 2. Three independent Rac DN clones and Rac^{act} clones were analyzed. V, vector-only control. The error bars indicate standard deviations.

pathway bypassing Raf. The continued significant levels of Rac-GTP in the HT1080-Rac DN transfectants may be due to the constitutive activation of endogenous Rac by the endogenous mutant N-ras protein and the existence of multiple Rho guanine nucleotide exchange factors (GEFs) which are not all completely inhibited by interaction with the Rac DN protein. Also, the endogenous mutant N-ras protein may be able to activate endogenous Rac via a mechanism that does not involve the GEFs that form unproductive interactions with the Rac DN protein. It is known that the size of the Rho-GEF family far exceeds that of Rho-GTPases, thus raising the possibility of redundancy of GEF function (42). The transfected Rac DN protein presumably does not interfere with the pull-down of the endogenous Rac-GTP, since it does not bind to PAK PBD. The mechanism by which the Rac DN protein interferes with JNK activation, even though there is evidence of continued levels of endogenous active Rac-GTP, is not known but may well occur in a PAK-independent fashion. A possible explanation is that, in the presence of Rac DN protein, one or more GEFs that are capable of activating endogenous Rac may do so in a fashion that precludes activation of downstream JNK. One such candidate is the GEF Tiam-1, which is a potent Rac GEF that stimulates PAK-1 but is a poor inducer of JNK activation (51). There was no evidence of negative cross talk between the Rac and RhoA pathways (Fig. 1C). This, again, could be due to the persisting levels of endogenous Rac-GTP in these transfectants

The MCH603-Rac^{act} transfectants, as expected, showed increased Rac (Fig. 1B) and JNK (Fig. 4B) activities but no increased Ras activity (Fig. 1A). Additionally, there was clear evidence of cross talk with the Raf-dependent pathway. In this case, all members showed increased levels of activity, approximating those seen in HT1080 cells. Thus, activated Rac effectively cross activates Raf, possibly via PAK-1 (2, 33), in these cells. In contrast, although HT1080-Rac DN transfectants had decreased levels of activated Raf, this inhibition did not extend to the downstream members, MEK, ERK, and Elk-1 (Fig. 4). Furthermore, commensurate with the increase in Rac activity, there was a corresponding increase in RhoA (Fig. 1C).

(iv) Modulation of constitutively active RhoA levels. The HT1080-RhoA DN transfectants resulted in a reduced level of RhoA-GTP (Fig. 1C). Other than that, there was no corresponding reduction in the constitutive levels of activity of Ras, Rac, JNK, Raf, MEK, ERK, or Elk-1 (data not shown), suggesting a lack of negative-feedback cross talk. The MCH603-RhoA^{act} transfectants, as expected, showed increased RhoA-GTP levels (Fig. 1C), but no increased Ras, JNK, Raf, MEK, ERK, or Elk-1 levels were observed (data not shown). However, there was clear evidence of a corresponding increase of Rac-GTP levels in the MCH603-RhoA^{act} transfectants (Fig. 1B), indicating cross talk between RhoA and Rac.

Cell shape and cytoskeletal architecture alterations. We have previously reported (31) that HT1080 cells (mutant N-ras) are rounded, with a lack of actin stress fibers in the cytoplasm and accumulation at the cell margins. The cells also show features of membrane ruffling. Conversely, MCH603c8 cells (wild-type N-ras only) are flat with well-organized cytoplasmic actin stress fibers and little evidence of membrane

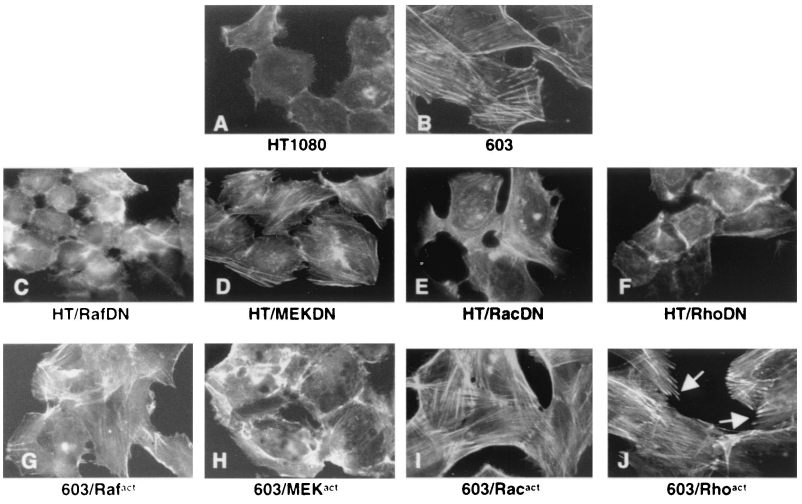

FIG. 5. Actin stress fiber organization in parental HT1080 (HT) and MCH603c8 (603) cells and their respective DN and constitutively active mutant transfectants. The cells were stained with fluorescein-conjugated phalloidin. The arrows indicate the enhancement of adhesion plaques. Magnification, ×200

ruffling. Examples of these phenotypes are shown in Fig. 5A and B.

It has been reported that transient expression of activated Ras, Rac, or RhoA in cells results in formation of actin stress fibers (15, 17, 39). However, chronic stimulation of these pathways, as seen in Ras-transformed cells, results in inhibition of stress fiber formation (2). Thus, chronic Ras-mediated activation of Raf-dependent and/or Raf-independent pathways may promote the loss of stress fibers and induce cell rounding. It may then be expected that inhibition of Ras-mediated chronic stimulation of one or more of these pathways may restore organized actin stress fibers. Consistent with this possibility, we found that expression of the MEK DN protein restored actin stress fibers in HT1080 cells (Fig. 5D). However, although DN Raf blocked MEK and ERK activation in HT1080 cells, it did not restore stress fibers (Fig. 5C). Furthermore, activated Raf expression in MCH603c8 cells did not cause the same profound loss of stress fibers in MCH603c8 cells as was seen with activated MEK (Fig. 5G and H, respectively). Thus, Ras activation of the MEK-ERK pathway in HT1080 cells may be necessary, but not sufficient, to promote Ras-mediated loss of stress fibers.

Since expression of constitutively active Raf and MEK in MCH603c8 cells also caused activation of the Rac-JNK pathway, this pathway may also contribute to Ras-induced stress fiber loss. We did find that expression of the Rac DN protein in HT1080 cells caused a partial restoration of stress fibers and a flatter shape (Fig. 5E). However, introduction of activated Rac alone into MCH603c8 cells did not cause any loss of stress fibers (Fig. 5I). Thus, Ras activation of the Rac-JNK pathway may also contribute to stress fiber loss, but its activation alone is also not sufficient. Finally, expression of DN RhoA did not cause a restoration of stress fibers in HT1080 transfectants, nor did expression of activated RhoA cause a loss of stress fibers in MCH603c8 cells (Fig. 5F and J, respectively), though there was evidence of an enhancement of adhesion plaques (Fig. 5J). Thus, in these fibrosarcoma cells, Ras-induced loss of stress fibers seems to be mediated primarily, but not exclusively, through chronic MEK and JNK activation. It is formally possible that some of the effects we observed may be due to autocrine effects, wherein secreted factors may influence this phenotype. However, conditioned medium from HT1080 cells did not influence the organization of actin stress fibers in MCH603c8 cells or its transfectants and, correspondingly, conditioned medium from MCH603c8 cells had no effect on the organization of stress fibers in HT1080 or its transfectants (data not shown).

In vitro growth kinetics. Growth curves of HT1080, MCH603c8, and their various transfectants are shown in Fig. 6. The HT1080-Raf DN, Rac DN, and RhoA DN clones had growth rates intermediate between those of HT1080 and MCH603c8 cells (Fig. 6A). The MEK DN cells are the slowest growing of the transfectants, although they grow more rapidly than MCH603c8 cells (Fig. 6A). All of the constitutively active MCH603c8 transfectants, Raf^{act}, MEK^{act}, Rac^{act}, and

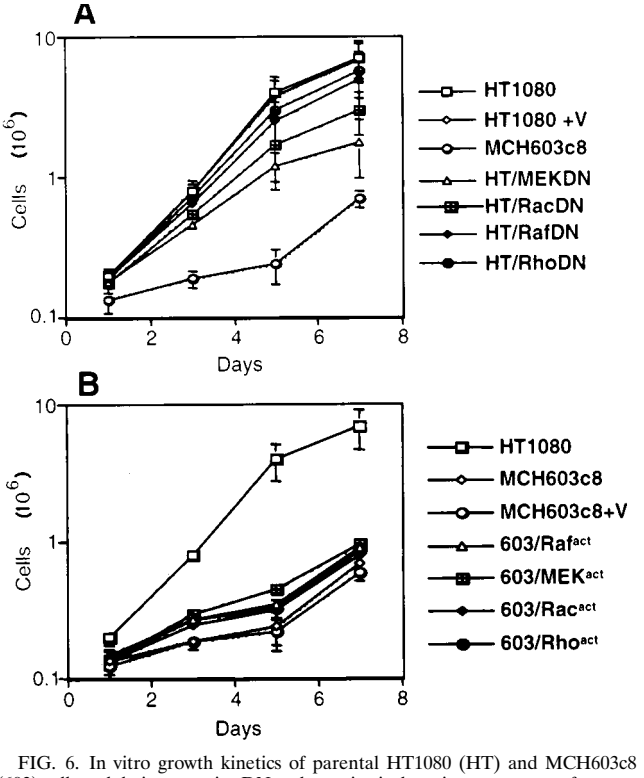

FIG. 6. In vitro growth kinetics of parental HT1080 (HT) and MCH603c8 (603) cells and their respective DN and constitutively active mutant transfectants. The error bars indicate standard deviation.

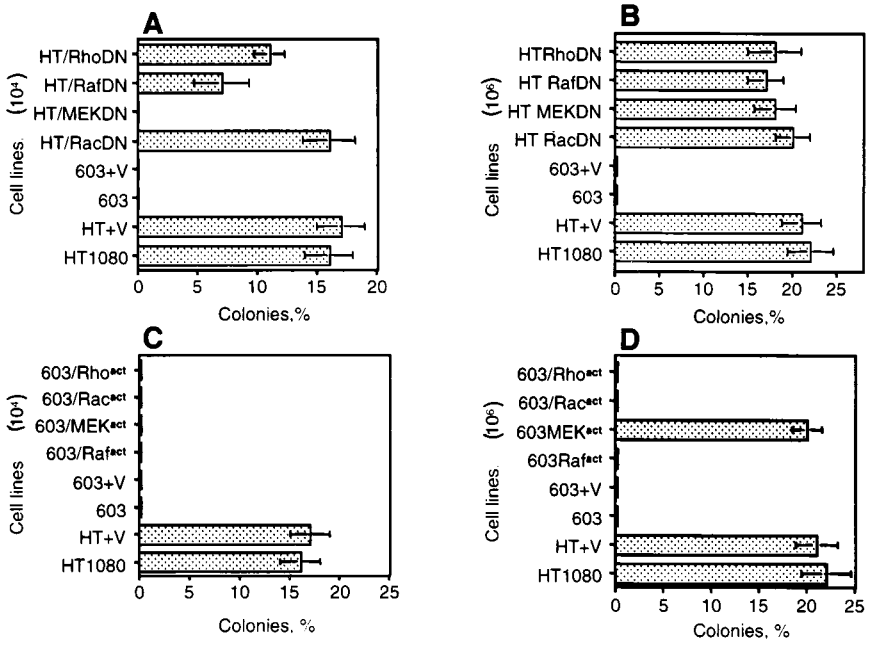

FIG. 7. Anchorage-independent assays. A total of 10^4 (A and C) and 10^6 (B and D) cells were plated per 60-mm-diameter petri dish in soft agar. Colonies (>0.1-mm diameter) were counted after incubation for 3 weeks at 37°C, with periodic refeeding with fresh growth medium. HT, HT1080 cells; 603, MCH603c8 cells; V, vector-only control. The error bars indicate standard deviations.

$RhoA^{act}$, had growth rates identical to that of the parental MCH603c8 cells (Fig. 6B). Similar growth curves were carried out under low-serum conditions (0.25% FCS). None of the parental or transfectant cell lines grew appreciably under these conditions (data not shown).

Growth in soft agar. We showed previously that HT1080 cells formed colonies in soft agar at low (10^4 cells) and high (10^6 cells) densities of plating in soft agar in 60-mm-diameter petri dishes. Conversely, MCH603c8 did not grow under any of these conditions (31). In the case of the HT1080 DN transfectants, the MEK DN cells failed to form colonies when plated at 10^4 per 60-mm-diameter dish (Fig. 7A). The Raf DN and RhoA DN cells formed colonies, but to a lesser degree than the HT1080 parental cells. Rac DN transfectants formed colonies as well as HT1080 did. When plated at the higher density of 10^6 cells per dish, all of the transfectants formed colonies in soft agar at an efficiency comparable to that seen with the HT1080 cells (Fig. 7B). None of the MCH603c8 transfectants expressing activated Raf, MEK, Rac, or RhoA formed colonies in soft agar when plated at 10^4 cells per dish (Fig. 7C). However, MEK^{act} transfectants did form colonies when plated at the higher density of 10^6 cells per dish, whereas none of the other MCH603c8 transfectants did so (Fig. 7D). Thus, the ability of MCH603-MEK^{act} cells to form colonies in soft agar at the higher density is consistent with the aggressive tumorigenic phenotype (see below). As was noted with the actin stress fiber experiments, there was no effect of conditioned medium from HT1080 or MCH603c8 on the anchorage-independent growth of any of the transfectants (data not shown).

In vivo tumor formation. As previously reported (31) and illustrated in Fig. 8, HT1080 and MCH603c8 are both tumorigenic in athymic nude mice. However, the kinetics of tumor expansion differ significantly. HT1080 is aggressively tumorigenic, with large tumors ($\geq$600 mm^3) forming within 20 days. The MCH603c8 tumors follow a more indolent course, with tumor sizes of $\geq$400 mm^3 within 70 days. None of the HT1080 DN transfectants had altered tumor kinetics, and they retained their aggressive tumorigenic phenotype (Fig. 8A). Similarly, none of the MCH603c8 activating transfectants showed altered tumor kinetics, with the singular exception of the MCH603c8-MEK^{act} cells. These cells not only converted to an aggressive tumorigenic phenotype but were significantly more aggressive than the HT1080 cells (Fig. 8B). Representative tumor reconstitutes were established in cell culture and reimplanted into nude mice. They retained their original phenotype of weak or aggressive tumor formation (data not shown).

DISCUSSION

N-, H-, and K-*ras* genes are commonly mutated in human cancers. The mutant Ras proteins constitutively orchestrate signal transduction, involving multiple branched pathways and culminating in metabolic signals that influence the malignant phenotype of cancer cells. Despite intensive scrutiny, it is still unclear which elements of these multiple signaling pathways are critical for neoplastic behavior. Experimental models have often consisted of overexpression of transduced mutant *ras* genes in rodent cells, a paradigm that does not necessarily emulate physiological conditions of endogenous mutant Ras expression and activation in human cancer cells. In this study we have attempted to manipulate the activation of endogenous components of the Raf, Rac, and RhoA pathways in the HT1080 human fibrosarcoma cell line, which contains an endogenous mutant allele of N-*ras*, and its derivative MCH603c8 cell line, which lacks the mutant allele.

Downregulation of Ras signaling pathways in HT1080 cells. DN mutants of Raf, MEK, and Rac all showed evidence of downregulation of activity of downstream members of the linear pathway involved (Fig. 2 to 4 and 9). No evidence was found for downregulation of upstream members of the respective linear pathways. Thus, a unidirectional flow of activation is implied from these results. This is confirmed with the activation studies in MCH603c8 (see below).

There were two unexpected observations in the experiments

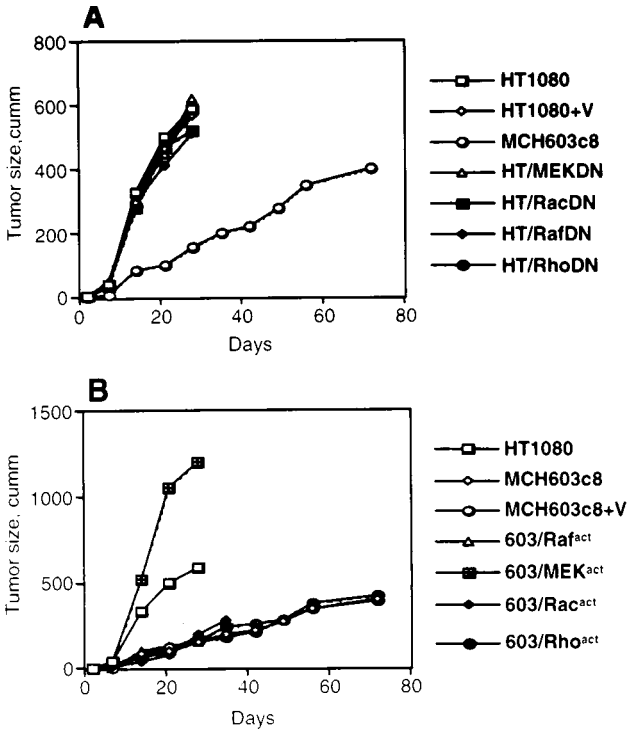

FIG. 8. Tumorigenicity assays of HT1080 (HT) and HT1080-DN transfectants (A) and MCH603c8 (603) and MCH603c8-activating mutant transfectants (B). Each point is the average of the tumor sizes of all sites inoculated (a total of 6 for parental cells and 18 for the transfectants, combining three independent clones). cumm, cubic millimeters.

with the DN proteins. We found that endogenous activated Raf levels decreased in the HT1080-Raf DN transfectants. Since the Raf DN protein functions to inhibit MEK activation (8) and our Raf assay is a coupled reaction that involves immunoprecitation of total Raf, one would expect to measure the activity of the endogenous Raf activated by the endogenous mutant N-ras protein. We do not know why this level of activity is not seen. Possibly, the Raf DN protein interferes with endogenous activation of the MEK substrate. This is unlikely, given that the MEK substrate is in excess. Another possibility is that the kinetics of interaction of the Raf kinase inhibitor with Raf-1 may be altered, providing a greater suppression of activation of the Raf-MEK-ERK pathway. It is known that the Raf kinase inhibitor coimmunoprecipitated with Raf-1, using Raf-1 antibodies (50).

Since Rac DN protein functions by unproductively interacting with members of the family of Rho-GEF proteins, it might be expected that levels of endogenous Rac-GTP would be decreased in the HT1080-Rac DN transfectants. This was not the case (Fig. 1B). Given that there are multiple GEFs capable of interacting with Rac protein, it is possible that Rac DN protein did not sequester all of the GEFs capable of interacting with the endogenous Rac protein. Further experimentation is necessary to resolve these unexpected findings.

Each of the DN mutants caused downregulation of the activities of its downstream members to levels approximating that seen in MCH603c8 cells, which lack the mutant N-ras allele. Thus, each of the DN mutants is potent in the sense of reducing the activities of downstream members in the respective linear pathway. However, the expression of the DN mutants did not have a significant effect on the levels of expression of total protein of each member of the respective pathway (data not shown).

Activation of Ras signaling pathways in MCH603c8 cells. Expression of each of the activating mutants of Raf, MEK, and Rac resulted in potent activation (Fig. 5 to 7) of the relevant protein and respective downstream members. Activation of upstream members was not observed. Thus, back talk within a linear pathway was never observed whether the originating signal was inhibitory (HT1080-DNs) or activating (MCH603c8-Acts). Most of the constitutively active mutants examined contained levels of activated protein that approximated that seen in HT1080 cells. This was also true of the activated levels of endogenous downstream members within the respective linear pathway. Since the derivative cell lines are stable transfectants, it is not known whether the absence of clones expressing significantly higher levels of activated protein indicates that such elevated levels are toxic to the cells. Such a phenomenon has been noted with overexpression of oncogenic Ras and Raf mutants, which may result in apoptosis or differentiation rather than neoplastic transformation (26, 43). A significant exception to this observation in our studies was the MCH603c8-MEK^{act} clones. Each clone had an approximately 3-fold-higher level of activated-MEK expression than that seen in HT1080 cells (and a 10-fold higher level than in MCH603c8 cells). Levels of activated ERK and Elk-1 were also higher (1.5- to 2-fold) than the levels seen in HT1080 cells. As discussed below, this elevated level of activated MEK was associated with dramatic effects on the in vitro and in vivo phenotypes of the stable transfectants.

Cross talk between the pathways is bidirectional. Clear evidence for cross talk (direct or indirect) was seen in both the DN and activating mutant transfected clones. In the case of the HT1080-Raf DN clones, there was no indication of negative-feedback cross talk with the Rac-JNK pathway or RhoA (Fig. 1B, 2, and 1C, respectively). However, the MEK DN clones showed decreased activity of JNK (Fig. 3A) but, again, not of Rac and RhoA (Fig. 1B and C). The persisting levels of Rac-GTP and RhoA-GTP can be explained by the fact that the HT1080-MEK DN clones continue to express mutant N-ras, thereby mediating continued activation of endogenous Rac and RhoA. Thus, negative-feedback cross talk with JNK presumably originated from MEK or a downstream member and occurred at the level of JNK directly or below Rac in the Rac pathway. It is somewhat surprising that the Raf DN transfectants did not show evidence of negative cross talk with JNK, since the level of endogenous MEK activity decreased to a level only slightly above that seen in the MEK DN transfectants (compare Fig. 2A with Fig. 3A). In the case of the MCH603c8 transfectants, both the Raf^{act} and the MEK^{act} clones exhibited positive cross talk that involved both Rac and RhoA and induced activation of JNK. It is, of course, possible that subtle differences in the levels of activation of relevant members of the respective pathways may have significant effects on the ability to cross talk, either positively or negatively. Interestingly, the RhoA^{act} mutants also showed activation of Rac (Fig. 1B) whereas the HT1080-RhoA DN clones showed no evidence of negative cross talk with the Rac (Fig. 1B) or Raf-MEK-ERK pathway (data not shown). In the HT1080-RhoA DN clones, Rac GTP and Raf-MEK-ERK activities were observed to be at levels similar to that seen in HT1080 cells. This may reflect unidirectional cross talk or may be a reflection of subtle differences in levels of activation or downregulation not revealed in these studies. It is well established that twofold or even smaller increases or decreases in the level of a signaling protein may have profound effects on the ultimate target(s) (16). In our studies we find that activation of Rac induces activation of RhoA and vice versa. This is consistent with earlier observations (15). More recently, it has been

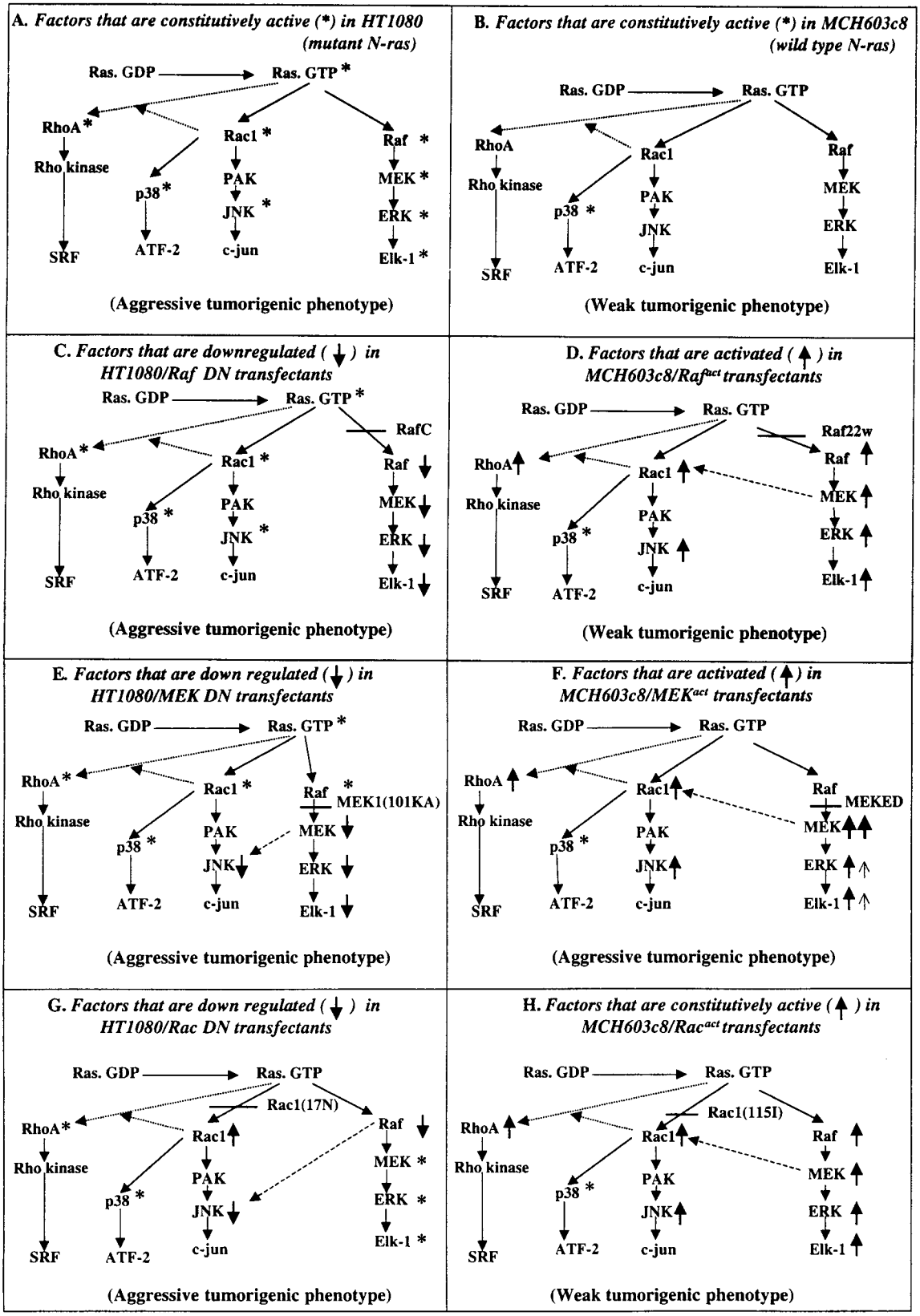

FIG. 9. Schematic diagrams of the downregulated and activated status of the downstream members of the Ras-mediated signaling pathways in the transfectants compared to those of the parental HT1080 and MCH603c8 cells. The down and up arrows indicate downregulation and activation of kinase activity, respectively, of those proteins that has occurred as a consequence of expression of transfected DN (HT1080-Raf DN, HT1080-MEK DN, and HT1080-Rac DN) and activated (MCH603c8-Raf^{act}, MCH603c8-MEK^{act}, and MCH603c8-Rac^{act}) mutants, respectively. The horizontal and diagonal arrows indicate probable cross talk between the Raf and Rac1 pathways. The positive or negative cross talk may be direct or indirect. Also, the specific level at which the cross talk takes place is not clear in every case.

shown that Rac signaling antagonizes RhoA activity in mouse NIH 3T3 cells (40). Our results are at variance with these observations. As with many other Ras-related phenomena, there may be multiple explanations, including differences in cell lineages, Rac, and RhoA mutants studied and experimental conditions.

A limited degree of negative cross talk between Rac and the Raf-dependent pathway was seen in the HT1080-Rac DN clones. Raf activity was reduced to a level seen in MCH603c8 cells, but levels of activated MEK, ERK, and Elk-1 were unaltered (Fig. 4A). There are several possible explanations for the above finding: (i) there may be subtle threshold level effects on activation; (ii) Ras may be able to activate MEK in a Raf-independent fashion; and (iii) since we have not assessed the activity of all the relevant Raf isoforms, there is a possibility that continued activation of a Raf isoform other than Raf-1 is responsible for the observed MEK–ERK–Elk-1 activity. In the MCH603c8-Rac^{act} clones, elevated levels of activated Raf, MEK, ERK, Elk-1, and RhoA were seen, indicating active cross talk (Fig. 4B). In no case was Ras activity affected (Fig. 1A), indicating that cross talk in either direction occurs below this master signal transducer. The differences in potency between the positive cross talk to the Raf-dependent pathway in MCH603c8-Rac^{act} cells and negative cross talk from the HT1080-Rac DN cells may again reflect the fact that subtle differences in threshold levels of activation may influence downstream signaling (16, 48). It must be noted, however, that in the HT1080 cells the DN effects of each mutant are superimposed upon the constitutive signaling of the endogenous mutant N-ras protein. The various levels of cross talking that we have observed in these DN and constitutively active transfectant cell lines are clearly complex. The mechanisms of cross talk among the Ras signaling pathways, both positive and negative, remain obscure. Although the specificity of signaling and interactions between partners involved in such signaling may be facilitated by scaffold proteins (41), these possibilities remain untested. It is likely, however, that the transfectant cell lines described in this report will prove to be useful experimental models for future studies. The changes in levels of activated protein, up or down, in all of the experiments were not due to alterations in rates of transcription or translation of the relevant proteins. No significant alterations were noted in the total levels of each protein, which were assayed in each experiment (data not shown).

Dissociation of in vitro-transformed phenotypic traits from in vivo tumorigenic phenotypes. Several unexpected effects on cytoskeletal architecture and in vitro growth characteristics were seen in the various DN and constitutively active mutant transfectants. We expected that the levels of activated Rac and RhoA would have significant effects on the distribution of actin microfilaments, based upon the observations of Hall and others (2, 15). However, there was no effect on the well-organized actin fibers in the MCH603c8-Rac^{act} clones (Fig. 5I). Although there was a small degree of restoration of organized actin stress fibers in the HT1080-Rac DN clones, it did not come close to the extensive stress fiber organization seen in MCH603c8 cells (Fig. 8B and E). The most dramatic effects were seen in the MEK transfectants. The HT1080-MEK DN clones acquired distinct organized actin microfilament cytoskeletons (Fig. 5D). Conversely, the MCH603c8-MEK^{act} clones had a completely disorganized pattern of actin staining (Fig. 5H). The lack of an effect of activated or DN Rac and RhoA transfectants on the organization of actin stress fibers was unexpected. However, it should be noted that most studies of RhoA and Rac-Cdc42 effects on stress fiber induction are short term, including microinjection of relevant proteins (2, 15, 34). Chronic stimulation, as seen in Ras transformation assays and in cancer cells with endogenous mutant Ras alleles, is most often accompanied by inhibition of stress fiber formation (2). The effects are also likely to be cell type specific. The signaling circuitry associated with N-ras-mediated control of stress fiber formation (or lack of it) in HT1080 cells is clearly complex. However, MEK or its downstream partner(s) appears to be a critical component of that circuitry. It is interesting in this context to note that earlier we had observed that treatment of HT1080 cells with the MEK inhibitor PD098059 resulted in the restoration of organized actin stress fibers (32). More recently, we have transfected MCH603c8-MEK^{act} cells (disorganized actin stress fibers) with MKP-1, the phosphatase that dephosphorylates and inactivates ERK1 and -2 (46). We found that levels of active ERK decrease to the levels observed in MCH603c8, accompanied by a dramatic restoration of actin stress fibers (S. Gupta et al., unpublished data). The levels of constitutively active MEK remain high in these double transfectants. Thus, a critical component of the regulation of actin stress fiber formation in these cells is the activated status of ERK. Furthermore, the MCH603c8–MEK^{act}–MKP−1 double transfectants retained their aggressive tumorigenic phenotype. Thus, as with the MEK DN transfectants, the restoration of organized actin stress fibers had no effect on the aggressive tumorigenic phenotype.

The kinetics of in vitro growth also had no influence on the aggressive nature of the tumorigenic phenotype. The HT1080 Raf and MEK DN clones had in vitro growth rates intermediate between those of HT1080 and MCH603c8 (Fig. 6). However, all of them retained the aggressive in vivo tumorigenic phenotype (Fig. 8). All of the MCH603c8 activating mutant clones retained the same in vitro growth kinetics as the parental MCH603c8 cells (Fig. 6). However, the MCH603c8-MEK^{act} clones acquired a highly aggressive tumorigenic phenotype (Fig. 8). Thus, in vitro growth kinetics were dissociable from in vivo tumor growth kinetics.

The one in vitro trait that did show some correlation with in vivo tumor kinetics was anchorage-independent growth (Fig. 7). The only MCH603c8 transfectant cells to form colonies in soft agar were the MEK^{act} clones. However, colonies formed only when cells were plated at high density and not at low density (10^4/dish). Interestingly, the same phenomenon was seen with the HT1080-MEK DN clones. Thus, partial restoration of the ability of MCH603c8-MEK^{act} clones to grow in soft agar was associated with the conversion to an aggressive tumorigenic phenotype. However, the partial loss of anchorage independence in the HT1080-MEK DN clones had no effect on the kinetics of tumor growth. Although it is possible that autocrine effects influence growth in soft agar or actin stress fiber formation, we found no evidence for this in conditioned-medium experiments (data not shown).

A summary of the in vitro and in vivo phenotypic traits of the parental cells and their stable transfectants is presented in Table 2. Although the dissociation of in vitro traits of transformation from the ability to form tumors may seem surprising at first glance, given the preponderance of reports identifying such correlations, many exceptions have also been reported (44, 45). It is likely that such correlations are dependent on cell type, host species, and the nature of the genetic mutations that have occurred in the genesis of any given cancer.

No single Ras signaling pathway is associated with the aggressive tumorigenic phenotype. The only manipulation of HT1080 or MCH603c8 cells that produced a change in the respective tumorigenic phenotype was the overexpression of activated MEK in MCH603c8-MEK^{act} transfectants. This resulted in the acquisition of an aggressive tumorigenic pheno-

TABLE 2. In vitro and in vivo phenotypes of parental cells and transfectant clones

Cell line[a]	Actin stress fiber organization	Anchorage-independent growth[c]	Tumor growth[d]
HT1080	D	+	A
MCH603c8	O	−	W
HT1080-Raf DN	D	+	A
HT1080-MEK DN	O	−[e]	A
HT1080-Rac DN	D (partial restoration)	+	A
HT1080-Rho DN	D	+	A
603-Raf^{act}	O	−	W
603-MEK^{act}	D	−[e]	A
603-Rac^{act}	O	−	W
603-Rho^{act}	O	−	W

[a] 603, MCH603c8.
[b] O, organized; D, disorganized.
[c] In soft agar (10^4 cells/dish). +, growth; −, no growth.
[d] A, aggressive; W, weak.
[e] Colonies formed when cells were plated at 10^6 per dish.

type that was even more aggressive than that of HT1080 cells (Fig. 8). Activation of MEK has been shown to be both necessary and sufficient for neoplastic transformation of mouse NIH 3T3 cells (11). However, a careful perusal of the results in our study show that activation of MEK and its downstream partners alone cannot explain our result. In the case of the HT1080 transfectants, both Raf DN and MEK DN clones show evidence of decreased activity of MEK and downstream members and, in the case of MEK DN clones, decreased activity of JNK. However, there is no effect on the aggressive tumorigenic phenotype. It should be noted that, with the exception of activated Ras and Rac, the Raf DN clones have the same biochemical profile of activated members of the pathways as MCH603c8.

A summary of the activated status of each member of the Ras-mediated signaling pathways in these transfectants compared to the parental MCH603c8 cells, and the HT1080 (mutant N-ras) cells, is presented in Fig. 9. The Raf^{act}, MEK^{act}, and Rac^{act} transfectants of MCH603c8 are particularly informative regarding the role of activated MEK in conversion to an aggressive tumorigenic phenotype. Both the Raf^{act} and Rac^{act} clones had levels of activated Raf, MEK, ERK, and Elk-1 approximately equal to that seen in HT1080 cells (Fig. 3 and 4). Thus, the Raf^{act} and Rac^{act} clones show essentially the same profiles of activated members of the signaling pathways as HT1080, with the exception that Ras is not activated. However, neither the MCH603c8-Raf^{act} nor the Rac^{act} clones acquired the aggressive tumorigenic phenotype. The MCH603c8-MEK^{act} transfectants had levels of activated MEK that were approximately 10-fold higher than those seen in MCH603c8 cells and 3-fold higher than those in HT1080 cells (Fig. 3B). All of these transfectants acquired the aggressive tumorigenic phenotype. The MCH603c8-MEK^{act} transfectants also had levels of activated ERK and Elk-1 above that seen in HT1080 cells. In related studies (Gupta et al., unpublished), we have created MCH603c8-MEK^{act}–MKP-1 double transfectants in which the high levels of activated MEK are retained but levels of activated ERK drop to those seen in the parental MCH603c8 cells. These double transfectants retain their aggressive tumorigenic phenotype. Thus, the elevated levels of activated MEK do, indeed, seem to be a critical factor for aggressive tumorigenic growth.

Evidence for a novel Ras-dependent pathway. Taken together, these data do not support the contention that activation of MEK alone is the critical event for acquisition of the aggressive tumorigenic phenotype. Neither do the data support the notion that activation of downstream members and cross activation of the Rac and RhoA pathways are sufficient or necessary (see the data for the HT1080 DN mutants) for the aggressive tumorigenic phenotype. These results lead us to conclude that overexpression of activated MEK in MCH603c8-MEK^{act} transfectants is the key event in converting these cells to an aggressive tumorigenic phenotype. We further speculate that this overexpression of activated MEK results in a "spill-over" effect that activates an as-yet-unidentified pathway that is critical for this conversion. An interesting possibility is that overexpression of activated MEK may perturb the protein scaffolding that contributes to the specificity of MAPK signaling (41, 48) and allow "forbidden" partners to interact and become activated. We speculate that this pathway may also be activated by mutant Ras in a MEK-independent manner. We base this conclusion on the fact that HT1080-MEK DN transfectants have significantly lower levels of endogenous activated MEK but retain their activated-Ras levels (Fig. 1A) and their aggressive tumorigenic phenotype.

It is possible that the key Ras-dependent pathway may be an already-identified one not examined in this study. It may be equally possible that a hitherto-undiscovered pathway is involved. We are actively pursuing these possible scenarios. We do know that activation of the PI3K pathway is not the critical event (Gupta et al., unpublished data). PI3K is constitutively active in both HT1080 and MCH603c8 cells due to constitutive PDGF production and consequent activation of the cognate PDGF receptor.

Therapeutic possibilities. Modulation of activated Ras activity and/or its downstream effectors is being actively pursued by pharmacologic intervention strategies (29). These include Ras and MEK inhibitors. Our studies with a human fibrosarcoma cell line possessing a mutant N-*ras* allele predict that Ras inhibitors have a potential benefit but that MEK inhibitors do not. If a critical novel pathway is involved in the control of the aggressive tumorigenic phenotype, it will provide an additional therapeutic target for treatment of cancers expressing oncogenic Ras mutant proteins. It remains to be seen if such a putative novel pathway is common to N-, H-, and K-ras signaling.

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