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M-Ras/R-Ras3, a Transforming Ras Protein Regulated by Sos1, GRF1, and p120 Ras GTPase-activating Protein, Interacts with the Putative Ras Effector AF6*

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M-Ras is a Ras-related protein that shares ~55% identity with K-Ras and TC21. The M-Ras message was widely expressed but was most predominant in ovary and brain. Similarly to Ha-Ras, expression of mutationally activated M-Ras in NIH 3T3 mouse fibroblasts or C2 myoblasts resulted in cellular transformation or inhibition of differentiation, respectively. M-Ras only weakly activated extracellular signal-regulated kinase 2 (ERK2), but it cooperated with Raf, Rac, and Rho to induce transforming foci in NIH 3T3 cells, suggesting that M-Ras signaled via alternate pathways to these effectors. Although the mitogen-activated protein kinase/ERK kinase inhibitor, PD98059, blocked M-Ras-induced transformation, M-Ras was more effective than an activated mitogen-activated protein kinase/ERK kinase mutant at inducing focus formation. These data indicate that multiple pathways must contribute to M-Ras-induced transformation. M-Ras interacted poorly in a yeast two-hybrid assay with multiple Ras effectors, including c-Raf-1, A-Raf, B-Raf, phosphoinositol-3 kinase δ, RalGDS, and Rin1. Although M-Ras coimmunoprecipitated with AF6, a putative regulator of cell junction formation, overexpression of AF6 did not contribute to fibroblast transformation, suggesting the possibility of novel effector proteins. The M-Ras GTP/GDP cycle was sensitive to the Ras GEFs, Sos1, and GRF1 and to p120 Ras GAP. Together, these findings suggest that while M-Ras is regulated by similar upstream stimuli to Ha-Ras, novel targets may be responsible for its effects on cellular transformation and differentiation.

The mammalian Ras superfamily is made up of over 60 GTPases that serve as molecular switches to regulate a diverse array of cellular functions. These include intracellular signal transduction for cell growth and differentiation (Ras subfamily), regulation of the actin cytoskeleton (Rho subfamily), membrane trafficking (Rab subfamily), and nuclear transport (Ran) (1–4). The Ras subfamily consists of Ha-, Ki-, and N-Ras; Krev-1/Rap1A and -1B; Rap2A and -2B; R-Ras; TC21(R-Ras2); Ral A and B; Rheb; Dex-Ras; Rin; and Rit that share several common features outside of the core GTP-binding domain (2). The classic/prototypic Ras proteins, Ha-, Ki-, and N-Ras, transduce signals for growth and differentiation from ligand-bound receptors to the nuclear transcriptional machinery and to the cytoskeleton (2, 3, 5, 6). These proteins can be constitutively activated by point mutation, contributing to the development of a broad spectrum of human malignancies (7). The introduction of equivalent activating mutations into the closely related TC21 and R-Ras proteins also results in transformation in tissue culture models (8, 9), and TC21 mutants have been identified in human tumor cell lines (10, 11). R-Ras has also been associated with apoptosis and integrin activation (12, 13). Overexpression of Rap1A/Krev-1 can induce transformation in some cells (14) but typically has been found to counter Ras-induced activities, due to competitive binding to Ras effectors (15, 16). Rheb similarly can inhibit Ras-induced transformation (17), but both the Rap and Rheb proteins may have other physiological functions; *e.g.* Rap1A contributes to the differentiation of PC12 cells into neurites via activation of B-Raf and has been linked to superoxide generation in phagocytes (18, 19). Ral acts downstream from Ras proteins in a signaling cascade that regulates the activity of phospholipase D (20).

Each of the above Ras-related proteins operates as a molecular switch that cycles between inactive GDP- and active GTP-bound states. This cycle is tightly controlled *in vivo* by two classes of regulatory proteins. Guanine nucleotide exchange factors (GEFs)[1] promote the release of GDP from inactive Ras and stabilize the apoprotein so that it can acquire the active, GTP-bound state (5). GTPase-activating proteins (GAPs) meanwhile promote rapid hydrolysis of Ras-GTP back to the inactive GDP-bound state (21), thus completing the cycle. Oncogenic mutations block GAP-stimulated GTPase activity, thus causing Ras to be locked in the active, GTP-bound, conformation. The various Ras proteins have both specific and overlapping sensitivities to GEFs and GAPs; *e.g.* the Ras GEF Sos1 can activate Ha-Ras and TC21 but not R-Ras or Rap1A (22, 23), while C3G can activate Rap1A and R-Ras but not Ha-Ras (23). Following acquisition of the active GTP-bound state, Ras can interact with a variety of cellular targets to elicit its biological effects. These include the Raf Ser/Thr protein kinases that activate the ERK cascade, phosphatidylinositol 3 kinase (PI3K) and the Ral exchange factors, RalGDS, Rgl, and Rgl2/Rlf (2). Additional Ras-interacting proteins (*e.g.* Rin1, AF6, and NORE) have been identified that may also serve as downstream effectors (24–27).

A search of the NCBI expressed sequence tag data base for Ras-related gene products revealed a novel cDNA that, upon

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[1] The abbreviations used are: GEF, guanine nucleotide exchange factor; ERK, extracellular regulated kinase; GAP, GTPase-activating protein; PI3K, phosphatidylinositol 3 kinase; MEK, mitogen-activated protein kinase/ERK kinase; HA, hemagglutinin.

complete sequencing, was found to share 55% amino acid identity with TC21 and Ki-Ras4B. This protein has since been independently described as M-Ras (28) and R-Ras3 (29). Following the introduction of an activating mutation equivalent to that known to potently enhance the oncogenic activity of Ha-Ras (30), overexpression of M-Ras(Q71L) caused morphologic and growth transformation in a manner similar to but distinct from Ha-Ras(Q61L). Similarly to Ha-Ras, overexpression of mutationally activated M-Ras also inhibited the differentiation of C2 myoblasts into myotubes. The ability of M-Ras to induce transcriptional activation and transformation was blocked by the MEK inhibitor PD98059, suggesting the importance of ERK activation to its biological activity. However, M-Ras was considerably more effective than an activated MEK mutant (MEKΔED) at inducing NIH 3T3 cellular transformation, despite being a weaker activator of Elk-1. This suggested that multiple downstream targets contribute to M-Ras-induced transformation. However, M-Ras did not interact with Ras effectors in a yeast two-hybrid interaction assay. Although M-Ras did interact strongly with AF6, a putative downstream target of the Ha-Ras protein (24, 25), AF6 overexpression did not contribute to fibroblast transformation. M-Ras was activated by the Ras-specific GEF, Sos1, and its GTPase activity was sensitive to p120 Ras GAP, suggesting that M-Ras can be regulated by similar upstream pathways to Ha-Ras. However, since M-Ras demonstrated differential Ras effector interactions as well as differential tissue distribution compared with Ha-, Ki-, or N-Ras, it may serve a unique physiological function.

EXPERIMENTAL PROCEDURES

cDNA Manipulation—Clone AA035915 (Genome Systems Inc.) was found to encode a full-length mouse M-*ras* cDNA. 5′ *Bam*HI and 3′ *Bam*HI and *Eco*RI sites were introduced by polymerase chain reaction-mediated DNA amplification. An activating Q71L mutation was also introduced by two-step polymerase chain reaction, and all products were sequenced prior to subcloning into the pZIP-NeoSV(X)1 (31), pCGN (32), or pGEX 2T (Amersham Pharmacia Biotech) expression vectors. Codons 4–1613 of the AF6 cDNA (provided by M. White, University of Texas Southwestern Medical Center) were subcloned as an *Eag*I fragment into the pFLAG-CMV2 vector (Eastman Kodak Co.) after frameshifting the polylinker by filling in and blunt-ligating the *Hin*dIII site. The pZIP-Ha-*ras*(Q61L), pcDNA3-cSos-CAAX (33), pRCbac-(5′-SosF) (34), pDCR-Ha-*ras*(G12V) (35) and pMLC-MEKΔED (36) plasmids have previously been described.

Northern Blot—An ~1,100-base pair fragment encoding the M-*ras* cDNA was radiolabeled using [α-^{32}P]dCTP and a random-primed DNA-labeling kit (Roche Molecular Biochemicals) and used to screen a mouse RNA Master Blot (CLONTECH), essentially as described by the manufacturers. Hybridization was detected by overnight autoradiography at −80 °C, using an intensifying screen.

NIH 3T3 Cell Culture, Transformation, and Transcription Assays—NIH 3T3 mouse fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% donor calf serum (Life Technologies, Inc.) and transfected essentially as described (37). Transforming focus forming assays and soft agar assays were performed in NIH 3T3 cells as previously outlined (37, 38). The appearance of foci was scored 16 days post-transfection. For transcriptional activation assays, NIH 3T3 cells were transfected with 0.75 μg of pZIP-*ras* together with 125 ng of Gal4-Elk and 2.5 μg of 5XGal4-Luc reporter plasmids (39) plus the indicated co-stimuli. 24 h post-transfection, cells were starved (0.5% serum), and luciferase activity was assayed the following day essentially as described (40).

C2 Cell Culture and Differentiation—C2 myoblast cells (provided by Y. Xhong) were grown in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum. One μg of pZIP plasmid encoding WT or activated M-Ras(Q71L) or Ha-Ras(Q61L) proteins were transfected into C2 cells using calcium phosphate precipitation. After 24 h, cells were selected in growth medium supplemented with 400 μg/ml G418 to establish mass populations of cells expressing the indicated Ras proteins. To induce differentiation, C2 cells were grown to 70% confluence and then fed with Dulbecco's modified Eagle's medium containing 2% horse serum and 10 μg/ml insulin (Life Technologies). After approximately 7 days, cells were scored for acquisition of differentiated characteristics.

Mitogen-activated Protein Kinase Assay—NIH 3T3 cells were co-transfected with 1 μg of pcDNA3-HA-ERK2 (encoding hemagglutinin (HA) epitope-tagged ERK2) plus 2 μg of pcDNA3 alone or encoding Ha-Ras(Q61L) or M-Ras(Q71L) using LipofectAMINE (Life Technologies). After 24 h, cells were serum-starved (0.1%) overnight and lysed in 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.27 M sucrose, 0.1% SDS, 1% Triton X-100, 0.5% deoxycholate, 1 mM Na_3VO_4 50 mM NaF, 0.1% β-mercaptoethanol, 10 mM β-glycerophosphate, 19 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. HA-ERK2 was immunoprecipitated using anti-HA monoclonal antibody (Babco) and ERK kinase activity was determined using myelin basic protein (Sigma) as substrate, essentially as described (41). c-Jun N-terminal kinase assays were performed similarly following cotransfection of NIH 3T3 cells with pCMV-FLAG-JNK and using glutathione *S*-transferase-Jun-(1–79) as substrate. To determine p38 activation levels, NIH 3T3 cells were transfected with FLAG-tagged p38α. Cells were serum-starved as above prior to lysis and Western blotting with an anti-activated p38 antibody (New England Biolabs). Equal loading of lanes was confirmed by blotting for FLAG-tagged p38 with M2 anti-FLAG antibody. Treatment with 10 μg/ml anisomycin (Calbiochem) for 10 min was used as a positive control for c-Jun N-terminal kinase or p38 activation. ERK activity in stable cell lines was determined using an anti-activated ERK polyclonal antiserum (Promega).

Yeast Two-hybrid Protein-Protein Interaction—The system used was a modification of the Fields' two-hybrid system, where one hybrid is fused between the LexA DNA binding domain (amino acids 1–211) and the Ha-Ras(G12V) or M-Ras(Q71L) protein. The second hybrid is a fusion between a nuclear localized VP16 acidic activation domain and full-length RalGDS, cRaf-1, A-Raf, B-Raf, or PI3K p110δ (provided by A. Vojtek), truncated AF6 (provided by L. van Aelst), Rin1 (provided by J. Colicelli), or c-Raf-1. Individually, these hybrids did not activate transcription. However, when co-expressed in a *Saccharomyces cerevisiae* strain that contains two integrated reporter constructs (yeast *HIS3* and the bacterial *lacZ* gene), the yeast strain expressing both hybrid proteins that are capable of forming interactions is prototrophic for histidine and contains detectable β-galactosidase activity (42). For β-galactosidase expression assays, yeasts co-expressing the indicated Ras and Ras effector plasmids were grown overnight in 5 ml of SC medium without Trp or Leu. Approximately 10^7 cells (based on A_{600}) were resuspended in 1 ml of Z buffer, lysed by the addition of two drops of 10% SDS and three drops of chloroform, and cleared by microcentrifugation. Lysates were spiked with 200 μl of *o*-nitrophenyl β-D-galactopyranoside (4 mg/ml) and incubated at 28 °C until color developed. β-Galactosidase activity was expressed as $A_{420} \times 1000/(A_{600} \times \text{time})$.

Coprecipitation of AF6 and Ras Proteins—Human embryonic kidney, 293T, cells were plated at 8×10^5 cells/90-mm dish and transfected the following day with pFLAG-CMV2-AF6 (encoding FLAG epitope-tagged AF6) and pCGN, pCGN-M-*ras*(Q71L), or pDCR-Ha-*ras*(G12V) using LipofectAMINE (Life Technologies) or calcium phosphate precipitation as described (37). Both Ras proteins were HA epitope-tagged. 48 h later, cells were washed with ice-cold phosphate-buffered saline and lysed in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.1 mM $MgCl_2$, 0.5% Nonidet P-40, 10% glycerol, 19 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation (refrigerated microcentrifuge, 10 min), the supernatant was precleared by incubation with protein A/G-agarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 10 min. The supernatant was then tumbled with 5 μg of anti-HA antibody (Babco) and 30 μl of protein A/G-agarose beads for 60 min and washed four times with lysis buffer, and proteins were separated by SDS-polyacrylamide gel electrophoresis (7% gel). Following transfer to polyvinylidene difluoride membrane (Millipore Corp.), co-precipitated AF6 was detected using M2 anti-FLAG antibody (Sigma), horseradish peroxidase-conjugated second antibody and ECL reagents (Amersham Pharmacia Biotech).

GEF and GAP Assays—In vivo guanine nucleotide exchange assays were performed essentially as described (23). Briefly, 293T cells were transiently transfected with plasmids encoding the indicated Ras and GEF proteins. After 36 h, cells were incubated in serum and phosphate-free medium for 30 min followed by similar medium supplemented with 150 μCi of $^{32}P_i$ for an additional 4 h. Cells were washed with ice-cold phosphate-buffered saline, lysed in 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 6 mM $MgCl_2$, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.05% SDS, 19 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride, and clarified by microcentrifugation for 10 min, and Ras proteins were immunoprecipitated using anti-HA or FLAG antibody. Ras proteins were then denatured at 68 °C in 0.2% SDS, 2 mM EDTA, and guanine nucleotides were separated by thin layer chromatography on polyethyleneimine-cellulose plates using 0.75 M KH_2PO_4/HCl, pH 3.4, as solvent. Chromatograms were dried and quantified using an

FIG. 1. **Alignment of the effector-binding domains of Ras family GTPases.** Residues 30–58 of M-Ras were aligned with residues 20–48 of Ha-Ras, Ki-Ras, and Rap1 and the equivalent residues of TC21 (residues 31–59), R-Ras (residues 46–74), RalA (residues 31–59), Rit (residues 38–66), and Rheb (residues 23–51) using ClustalW. Ha-Ras residues are indicated. *Black* and *gray shading* (generated using MacBoxshade) indicates sequence identity and homology, respectively. The switch 1 domain Ha-Ras residues 32–38 are *underlined*.

AMBIS β-scanner. To measure GAP activity, glutathione *S*-transferase-M-Ras and Ha-Ras fusion proteins were expressed in *Escherichia coli* and bound to glutathione-agarose beads in the presence of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 μM GDP, 1 mM $MgCl_2$, 1% Triton X-100, 1 mM dithiothreitol, 19 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Bead-bound proteins were loaded with GTP by incubation, 10 min at 30 °C, in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mg/ml bovine serum albumin, 6 μM GTP, 5 μCi of [γ-^{32}P]GTP, 1 mM dithiothreitol. $MgCl_2$ was added to 10 mM, and free nucleotide was removed by washing beads three times with the loading buffer minus GTP. Proteins were then eluted by incubating beads with 100 μl of 100 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$ and 20 mM glutathione on ice for 20 min. 0.2 pmol of GTP-bound Ras (determined by scintillation counting) was then incubated for the indicated times with or without 5 ng of p120 Ras GAP (provided by G. Bollag, ONYX Pharmaceuticals) in a 50-μl final reaction volume containing 25 mM Tris-HCl, 1.5 mg/ml bovine serum albumin, 7.5 mM $MgCl_2$, 2 mM dithiothreitol at room temperature for the indicated times. The reaction was stopped by the addition of 4 μl of 0.5 M EDTA and 1 μl of 10% SDS and heating to 68 °C for 5 min. Guanine nucleotides were separated as above.

RESULTS

M-Ras Is Closely Related to Ras and Has a Broad Tissue Distribution—A search of the NCBI expressed sequence tag data base revealed, in addition to multiple copies of known *ras* superfamily sequences, a unique cDNA present in both mouse and human libraries. Sequencing of clone AA035915 revealed an open reading frame of 208 codons that shared ~55% identity with Ki-Ras4B and TC21. This cDNA will be referred to here as M-*ras* in accordance with Matsumoto *et al.* who recently isolated this sequence from the murine myoblast C2 cell line (28). M-Ras shares many features characteristic of the Ras subfamily of GTPases (see Refs. 28 and 29). In particular, it shares complete identity with the core effector-binding domain sequence of Ha-, Ki-, and N-Ras (residues 32–40; Fig. 1).

To address the tissue distribution of M-Ras, an [α-^{32}P]dCTP-labeled cDNA probe was generated and used to screen a mouse RNA Master Blot (CLONTECH). As shown in Fig. 2, the M-Ras message was most highly expressed in ovary and brain with lower level detection in many additional tissues including uterus, kidney, spleen, prostate, and lung. A constant level of M-Ras message was also detectable at various stages of development in the whole embryo, possibly reflecting transcription in brain. Although the M-*ras* message is widely expressed, it exhibits a different distribution pattern from that of Ha-*ras* (which is most abundant in skin, brain, and skeletal muscle), Ki-*ras* (most prevalent in gut, lung, and thymus), or N-*ras* (most abundant in thymus and testis) (43).

M-Ras Induces Cellular Transformation—To establish whether M-Ras harbors transforming ability similar to the related Ras, TC21, and R-Ras proteins, we introduced an activating mutation, Q71L, equivalent to that found in the highly transforming Ha-Ras(Q61L) (30). As seen in Fig. 3*A*, M-Ras(Q71L) had approximately 10% of the transforming potential of Ha-Ras(Q61L), generating 563 ± 29 compared with 4975 ± 296 foci/μg (mean ± S.E. for three experiments performed in quadruplicate) in NIH 3T3 cell focus forming assays. As seen in Fig. 3*B*, M-Ras-transformed foci were typically smaller than those induced by Ha-Ras, and the transformed cells were subtly different from those transformed by activated Ha-Ras and TC21,[2] being less refractile and producing more elongated extensions at the periphery. This morphology was also quite distinguishable from the dense, nonrefractile foci induced by R-Ras (9). Individual colonies were isolated from NIH 3T3 cells that had been transfected with pZIP-Ha-*ras*(Q61L), pZIP-M-*ras*(Q71L), or empty vector and stably selected on G418. These cells were then cultured in soft agar to assess anchorage-independent growth. Fig. 3*C* shows that M-Ras-transformed cells can grow in soft agar similarly to Ha-Ras-transformed cells, although the colonies from M-Ras-expressing cells were typically smaller. Like Ras-transformed cells, M-Ras-transformed cells could also survive and grow in low serum (0.5%).[2]

M-Ras Suppresses the Differentiation of C2 Myoblasts—M-Ras was so named due to its isolation from a C2 myoblast cDNA library. Further, it was found that the M-Ras message level decreased upon differentiation of C2 cells into myofibers (28). This suggested that M-Ras might be responsible for restraining cellular differentiation. The differentiation of cultured myoblasts into myotubes is normally kept in check by the inhibitory effects of serum mitogens on the transcriptional activity of muscle regulatory factors such as MyoD. However, upon serum deprivation, cells differentiate and then fuse to form myotubes. Oncogenic Ras can inhibit this process and, in the presence of serum, can induce myoblast transformation (44). As shown in Fig. 4, while C2 myoblasts expressing wild type M-Ras still differentiated and fused into myotubes upon fetal bovine serum withdrawal, cells overexpressing activated Ha- or M-Ras did not. Both Ha-Ras(Q61L) and M-Ras(Q71L) caused morphological transformation of C2 cells in the presence of serum.[3]

M-Ras Weakly Activates ERK2—Since M-Ras could induce similar biological effects to Ha-Ras, we next wished to address whether this was due to M-Ras activating the same downstream effectors utilized by Ha-Ras. Ras family GTPases can induce the activation of several mitogen-activated protein kinase cascades. Therefore, we compared the ability of Ha- and M-Ras to activate the ERK, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinases. As shown in Fig. 5*A*, following transient coexpression of M-Ras or Ha-Ras with HA-tagged ERK2 in NIH 3T3 cells, the kinase activity of immunoprecipitated ERK2 was only moderately elevated (~2-fold) by M-Ras(Q71L) compared with a 50-fold elevation by Ha-Ras(Q61L). Although TC21 did not induce ERK activation following transient transfection (8), stably transformed cells possess elevated ERK activity. Therefore, we used an anti-activated ERK antibody that only recognizes ERKs that have been doubly phosphorylated on Thr and Tyr to assess the activation state of ERKs in stably transformed cells. As shown in Fig. 5*B*, while there was considerably more ERK activation in Ha-Ras(Q61L)-transformed cells, there was a modest elevation of active, phosphorylated, ERKs in M-Ras-expressing *versus* control cells that may contribute to their transformed state. There was little detectable c-Jun N-terminal kinase activation by Ha- or M-Ras, as determined by an immunocomplex kinase assay using glutathione *S*-transferase-Jun-(1–79) as substrate.[2] Similarly, following co-transfection of NIH 3T3 cells with activated Ha- or

[2] L. A. Quilliam, A. F. Castro, and C. Bi, unpublished observation.
[3] C. B. Martin and C. J. Der, unpublished observation.

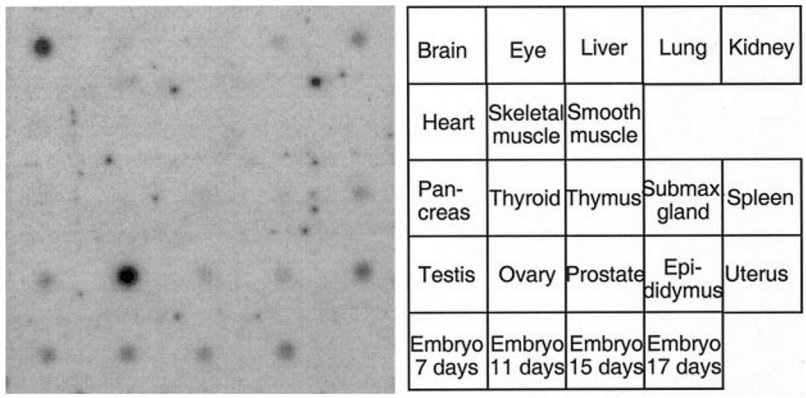

FIG. 2. **The M-Ras message is widely expressed.** A mouse mRNA Master Blot (CLONTECH) was hybridized with a ^{32}P-labeled probe generated from the 1.1-kilobase pair mouse M-Ras cDNA.

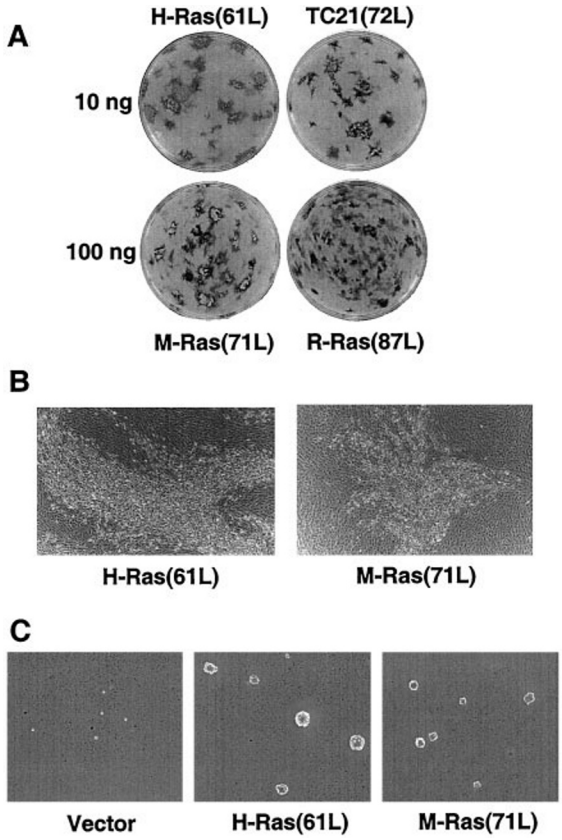

FIG. 3. **M-Ras induces morphologic transformation of NIH 3T3 cells.** *A*, NIH 3T3 cells transfected with plasmids encoding the indicated proteins were grown in regular culture medium for 12–18 days prior to staining with crystal violet. Data are representative of four experiments performed in quadruplicate. *B*, individual foci from *A* were photographed at × 4 magnification. *C*, NIH 3T3 cells stably expressing the indicated Ras proteins were grown in soft agar for 2 weeks prior to photographing colonies at × 4 magnification.

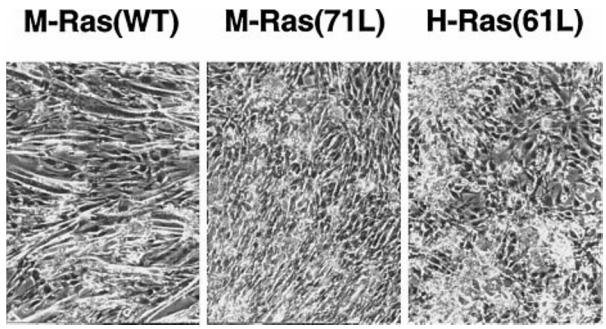

FIG. 4. **Activated M-Ras attenuated the fusion of C2 myoblasts.** C2 myoblasts were transfected with pZIP plasmid constructs encoding the indicated Ras proteins. Following stable selection on G418, >50 colonies were pooled and replated. Cells were shifted from growth medium containing 15% FBS to 2% horse serum, 10 μg/ml insulin to induce muscle differentiation. The *left panel* shows that, similarly to control cells, cells expressing wild type M-Ras differentiate and fuse to form myotubes. In contrast, cells expressing mutationally activated M-Ras(Q71L) or Ha-Ras(Q61L) fail to form myotubes. Instead, these cells became transformed and overcame density arrest. Results show representative fields from several independent experiments.

M-Ras plasmids and FLAG epitope-tagged p38, neither GTPase was found to significantly stimulate p38 activity, as determined by anti-active p38 antibody blotting (Fig. 5*C*).

M-Ras-induced Elk-1 Activation and Cellular Transformation Require the MEK/ERK Pathway—Ha-Ras can induce gene expression via the transcription factor Elk-1, which lies downstream of the Raf/ERK cascade. Similarly, M-Ras(Q71L) induced Elk-driven luciferase expression but, as in the transformation studies, was less potent than Ha-Ras(Q61L) (Fig. 6*A*). To establish whether activation of Elk-1 by M-Ras was due, at least in part, to the modest elevation of ERK activity reported above, we incubated cells with the MEK inhibitor PD98059 for 16 h prior to harvesting. Elk-1 has also been reported to be sensitive to p38 activation in some cell types (reviewed in Ref. 45). Therefore, we also pretreated cells with the p38 inhibitor SB203580. As shown in Fig. 6*A*, PD98059 almost completely blocked the ability of M-Ras or activated MEK to induce luciferase activity in the Gal-Elk reporter assay, suggesting that the MEK/ERK pathway was probably the sole upstream mediator of Elk-1 phosphorylation. In contrast, SB203580 modestly stimulated M-Ras-induced luciferase activity, suggesting that p38 could negatively regulate Elk-1 activation.

To determine whether the MEK/ERK pathway was also essential for M-Ras-induced transformation, NIH 3T3 focus forming assays were performed in the presence of 50 μM PD98059 or 10 μM SB203580. While the MEK inhibitor had only a modest effect on the focus forming ability of Ha-Ras(Q61L), it completely blocked M-Ras(Q71L)-induced transformation (Fig. 6*B*). At a concentration of 10 μM PD98059 or using another MEK inhibitor, U0126 (46), there was still a loss (U0126) or considerable reduction (10 μM PD98059) of M-Ras-induced foci, but only the morphology of Ha-Ras-transformed cells was altered.[2] In contrast, the presence of SB203580 potentiated M-Ras-induced transformation. These findings suggested that M-Ras was acting via MEK to induce transformation. However, since the strongly activating MEKΔED mutant that potently stimulated Elk-1 activation did not induce focus formation (Fig. 6*C*), it seemed likely that M-Ras must also signal via pathways in addition to Raf/MEK/ERKs to invoke its biological effects.

M-Ras Cooperates with Raf, Rho, and Rac to Induce NIH 3T3 Cell Transformation—Co-transfection of NIH 3T3 cells with two or more Ha-Ras effectors (*e.g.* Rho plus Raf or Raf plus RalGDS) results in their cooperation to induce Ras-like foci (47, 48). Therefore, we speculated that M-Ras might synergize with

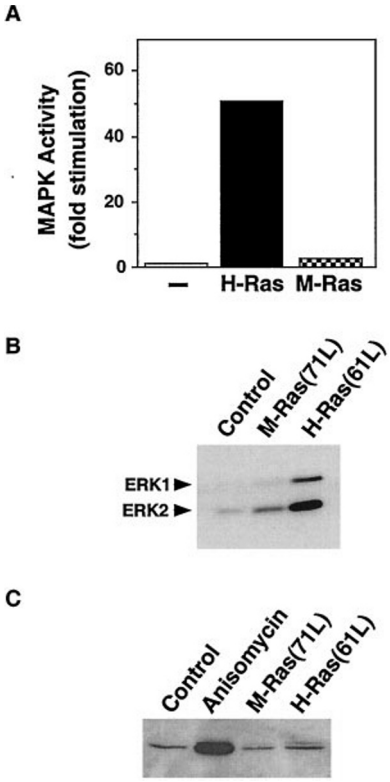

FIG. 5. **Ha- but not M-Ras can activate ERK2.** *A*, NIH 3T3 cells were transfected with 1 μg of empty pZIP or vector encoding Ha-Ras(Q61L) or M-Ras(Q71L) along with 1 μg of pcDNA3 HA-ERK2. Following serum starvation, cells were lysed, HA-ERK2 was immunoprecipitated, and kinase activity was determined using myelin basic protein as substrate. Data are representative of three experiments performed in duplicate. *B*, a pooled population of G418-selected NIH 3T3 cells harboring pZIP or vector encoding M-Ras(Q71L) or Ha-Ras(Q61L) were serum-starved, and cell lysates were blotted with anti-activated ERK antibody. Data are representative of two independent experiments. *C*, NIH 3T3 cells were transfected with pZIP plasmids as in *A*, except they were cotransfected with pFLAG CMV-p38α. Following serum starvation, p38 activity in cell lysates was determined using an anti-activated p38 antibody. Anisomycin stimulation was for 10 min. Data are representative of four independent experiments. *MAPK*, mitogen-activated protein kinase.

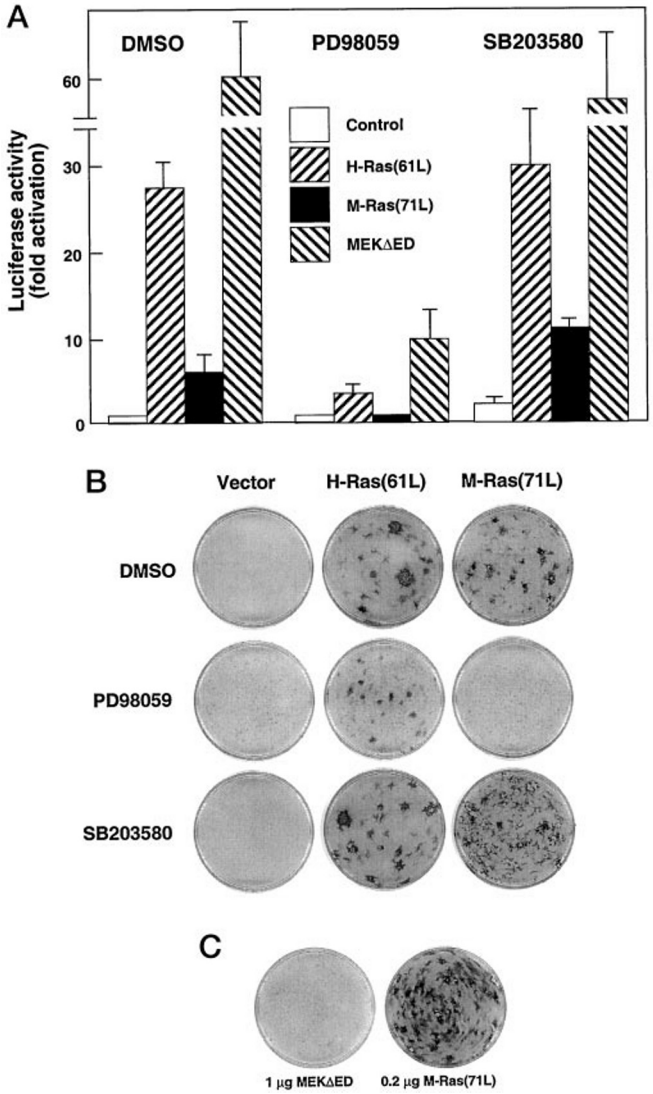

FIG. 6. **Ha-Ras, M-Ras, and MEK induce Elk-1 activation.** *A*, NIH 3T3 cells were transfected with plasmids encoding the indicated proteins, along with Gal4-Elk and 5XGal-Luc reporter plasmids. The ability of Ras and MEK proteins to induce luciferase expression was determined following serum starvation. Where indicated, Me_2SO (*DMSO*) vehicle (0.1%, v/v), 50 μM PD98059, or 10 μM SB203580 was added 16–18 h prior to cell lysis. Results (mean ± S.D.) are representative of at least two experiments performed in duplicate. *B*, NIH 3T3 cells were transfected with the indicated pZIP-*ras* plasmids. After 3 days, 50 μM PD98059, 10 μM SB203580, or 0.1% Me_2SO vehicle was added to the growth medium. Fresh medium plus drug was added every 2 days, and transforming foci were visualized after 14 days culture. Data are representative of two experiments performed in triplicate. *C*, NIH 3T3 cells were transfected with 1 μg of pMCL-MEKΔED or 200 ng of pZIP M-*ras*(Q71L), and transforming foci were visualized after 14 days of culture. Data are representative of two experiments performed in triplicate.

Ha-Ras effectors not already efficiently activated by M-Ras. As shown in Fig. 7*A*, M-Ras cooperated with activated mutants of Raf, Rho, and Rac to induce transforming foci, suggesting that it signaled via alternative effector proteins.

M-Ras Interacts with AF6 but Not with Other Putative Ras Effectors—The data described above suggested that M-Ras relied on a downstream target(s) in addition to ERKs to induce morphologic transformation. Since Ras signals via multiple effectors to elicit its biological responses, it was possible that Ha-Ras targets also contributed to M-Ras-induced transformation. Alternatively, since M-Ras diverges from Ras in sequences flanking the core effector-binding region (spanning residues 35–40 and 50–55; Fig. 1), and these flanking sequences are important for Ras effector binding (2), it was possible that M-Ras might have only limited interaction with known Ras effectors. To determine which Ras effectors M-Ras might signal through, we examined the ability of M-Ras(Q71L) to interact with various known or putative Ras target proteins in a yeast two-hybrid interaction assay. Ha-Ras bound strongly to c-Raf-1, A-Raf, B-Raf, RalGDS, PI3K, and the Ras-binding domains of Rin1 and AF6. However, while M-Ras interacted with the Ras-binding domains of c-Raf-1 or AF6, it did not significantly associate with full-length Raf proteins or the Ras binding domains of various other Ha-Ras effectors (Table I). This suggested that direct activation of Raf, PI3K, or Ral GEFs might not be required for M-Ras-induced morphologic transformation. To determine whether M-Ras interacts with AF6 *in vivo*, a FLAG-tagged full-length AF6 was co-expressed in 293T human embryonic kidney cells with HA-tagged M-Ras(Q71L) or Ha-Ras(G12V). Following immunoprecipitation of HA-tagged Ras proteins, co-precipitated AF6 could be detected by blotting with M2 anti-FLAG antibody (Fig. 8). M-Ras(Q71L) was found to be more effective than Ha-Ras(G12V) in precipitating AF6. Co-transfection of Ha-or M-Ras constructs with pCMV2-FLAG-AF6 did not result in increased focus forming activity (Fig. 7*B*),[2] suggesting either that the endogenous AF6

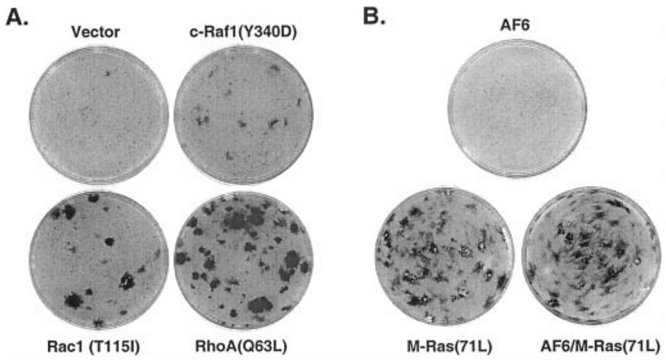

FIG. 7. **M-Ras cooperates with Ras effectors to induce morphological transformation.** *A*, NIH 3T3 cells were transfected with 20 ng/dish pZIP-M-*ras*(Q71L) along with 2 μg of plasmids expressing activated Raf, Rac, Rho, or empty vector. Transforming foci were visualized after 14 days culture. Representative of four experiments performed in at least triplicate. *B*, NIH 3T3 cells were cotransfected with plasmids encoding AF6 and/or M-Ras(Q71L) as indicated. Data are representative of three experiments performed in triplicate.

TABLE I

M-Ras preferentially interacts with the Ras-binding domain of AF6

pBTM116-Ha-*ras*(G12V) or pBTM116-M-*ras*(Q71L) LexA DNA binding domain fusion constructs were expressed in yeast-containing plasmids encoding VP16 transactivation domain fusions of c-Raf-1, A-Raf, B-Raf, PI3K p110δ, and Ral GDS or the Ras-binding domains of c-Raf-1, AF6, and Rin1 (42). The ability to induce β-galactosidase activity was then determined using *o*-nitrophenyl β-D-galactopyranoside as substrate. Data are representative of three experiments performed in duplicate on random colonies. Similar results were obtained using yeast patches on nitrocellulose filters and correlated with the ability of yeast to grow on His^- plates (++, strong growth; +, partial growth; −, no growth on YC medium minus Trp/His/Ura/Leu/Lys plates).

	Ha-Ras		M-Ras	
	Growth without His	β-Galactosidase activity	Growth without His	β-Galactosidase activity
AF6	++	292.9	++	417.9
c-Raf-1 N-term	++	15	+	5.1
c-Raf-1	+	5.7	−	1.3
A-Raf	++	8.9	−	1.1
B-Raf	++	23.6	−	1.1
PI3K	++	73.6	−	1.4
Ra1GDS	++	8.6	−	1.6
Rin1	++	9.6	−	1.6

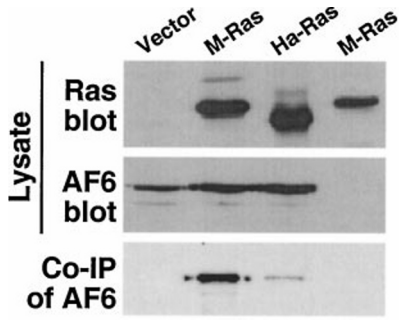

FIG. 8. **AF6 coimmunoprecipitates with Ha- and M-Ras.** 293T cells were transfected with empty vectors, HA-tagged Ha- or M-Ras, or FLAG-tagged AF6 as indicated. Protein expression was confirmed by immunoblotting cell lysates for Ras proteins using anti-HA antibody (*upper panel*) or for AF6 using anti-FLAG antibody (*middle panel*). Ras proteins were immunoprecipitated using anti-HA antibody, and coprecipitation of AF6 was determined by immunoblotting with M2 anti-FLAG antibody (*lower panel*). As seen in *lanes 2* and *3* (*lower panel*), both Ha- and M-Ras could coimmunoprecipitate AF6. Data are representative of four similar experiments.

is sufficient to support M-Ras induced transformation or that it plays no role in this process.

M-Ras Is Activated by Sos1 and GRF1—It has previously been demonstrated that residues within switch 1, switch 2, and α-helix 3 of Ras contribute to its interaction with GEFs (5, 49). Since M-Ras shares considerable sequence homology with Ha-, Ki-, and N-Ras within these regions, we wished to determine if M-Ras too was sensitive to Ras GEFs. Co-expression of wild type M-Ras with the membrane-targeted catalytic domains of Sos1 or GRF1 resulted in elevation of the M-Ras GTP/GDP ratio (Fig. 9). Consistent with this result, Sos1 cooperated with M-Ras to induce luciferase expression from a Gal/Elk luciferase reporter plasmid, although GRF1 was less effective.[2]

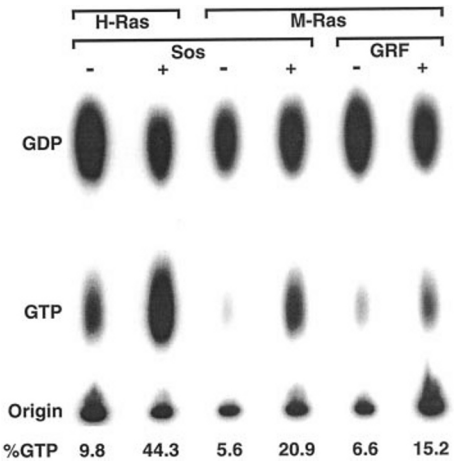

FIG. 9. **M-Ras is activated by Ras GEFs.** 293T cells were transfected with plasmids encoding epitope-tagged wild type M-Ras or Ha-Ras along with pRCbac, pRCbac-(5′-SosF) (34), pcDNA3, or pcDNA3 c-CDC25-CAAX (33) as indicated. After 36 h, the guanine nucleotide pool was metabolically labeled in the absence of serum using $^{32}P_i$. Cells were then lysed, and Ras-bound GTP and GDP were determined by immunoprecipitation and thin layer chromatography. Sos1 and GRF1/CDC25 elevated the GTP levels of both Ha-Ras and M-Ras. *%GTP* indicates the average (GTP cpm/(1.5 × GDP) cpm + GTP cpm) × 100 ratio from two (Sos) or three (GRF) independent experiments.

M-Ras Is Regulated by the p120 Ras GAP—Residues within switch 1 and the proximal region of switch 2 of Ha-Ras are responsible for its interaction with p120 Ras GAP. These domains are conserved between Ha- and M-Ras. To determine whether M-Ras GTPase activity was sensitive to regulation by p120 GAP, we expressed Ha-Ras and M-Ras as glutathione *S*-transferase fusion proteins in *E. coli* and compared their ability to hydrolyze bound [α-^{32}P]GTP in the presence of the purified GAP. As shown in Fig. 10, Ha- and M-Ras had similar basal GTPase activities that were equally augmented by exposure to p120 GAP. Therefore, it appears that M-Ras can be regulated by some of the same GEFs and GAPs as Ha-Ras.

DISCUSSION

Ha-, Ki-, and N-Ras are expressed in a wide variety of tissues and couple upstream stimuli from a diverse array of ligand-activated receptors to multiple downstream effector proteins (2, 5, 6). Recent data suggest that these prototypic Ras proteins have different affinities for downstream targets and may elicit different as well as common biological outcomes (50–55). Ras-related proteins are typically activated by a limited repertoire of upstream GEFs and interact with only select downstream Ras effectors to provide an additional level of signaling specificity. For example, R-Ras and Rap1A respond differently to Ras GEFs, activate some but not all Ras effectors, and have significantly different biological effects to Ras, at least in part due to association with unique downstream targets (13, 18, 23, 56–58). We report here the characterization of an additional Ras family member, M-Ras. M-Ras shares slightly higher similarity with TC21/R-Ras2 than other members of the Ras subfamily of GTPases and so has also been referred to as R-Ras3

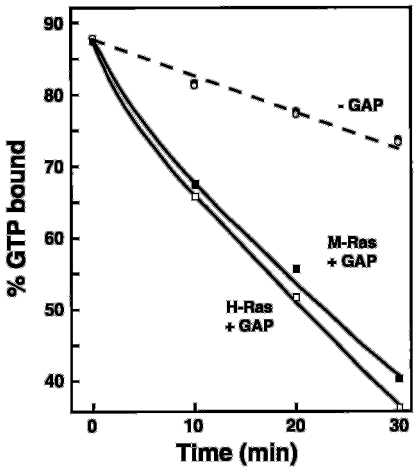

FIG. 10. **M-Ras GTPase activity is stimulated by p120 Ras GAP.** 0.2 pmol of [α-^{32}P]GTP-bound Ha- or M-Ras were incubated in the absence (*circles*) or presence (*squares*) of recombinant p120 Ras GAP for the indicated times. Hydrolysis of GTP was then determined following separation of GTP and GDP by thin layer chromatography. Data are representative of two experiments performed in duplicate. *Open symbols* indicate Ha-Ras, and *closed symbols* indicate M-Ras.

(29). Alignment of Ras-related proteins revealed that while the switch I domain of M-Ras is completely conserved with that of Ras and TC21, there is considerable divergence in the flanking residues (28, 29) that could significantly influence the ability of M-Ras to bind and/or activate Ras effectors.

The M-*ras* message level was reduced upon differentiation of C2 myoblasts into myofibers (28). Since M-Ras overexpression inhibited the differentiation of C2 myoblasts in the current study, it is possible that its expression is linked to the suppression of muscle cell development. Studies on C3H10T1/2 cells suggest that a novel Ras-activated signaling pathway is important for the inhibition of myoblast differentiation (44, 59). Due to its ability to inhibit myogenesis despite limited interaction with Ha-Ras targets, M-Ras may provide a useful tool to identify this novel pathway.

Since Ras, TC21, and (less potently) R-Ras can induce morphological transformation of cultured cells, we examined the transforming potential of M-Ras harboring a strongly activating mutation. Although M-Ras was not as effective as Ha-Ras in these assays, it had equivalent potency to R-Ras. A similar observation has been made by Kimmelman *et al.* (29). Since Ras is mutationally activated in ~30% of human tumors and TC21 has also been found to be overexpressed or mutated in several human tumor cell lines (10, 11, 60), it will be important to establish whether M-Ras too can contribute to the development of human malignancies.

It was proposed previously that the weaker transforming ability of M-Ras *versus* Ha-Ras in NIH 3T3 focus forming assays was due to its less effective activation of the Raf/ERK cascade (29). This conclusion was based on the ability of M-Ras/R-Ras3 to cooperate with Raf to induce ERK activation and focus formation. Alternatively, the increase in Raf-induced transformation may have been due to M-Ras-induced activation of a distinct, complementary pathway or pathways to Raf/MEK/ERK. Indeed, it has been documented that Raf can cooperate with other Ras effectors, including PI3K, RalGDS, and the Rho family GTPases, Rho, Rac, and CDC42, to induce transformation (48, 61–63). Consistent with this notion, we have seen a significant effect of Rho, Rac, or Raf on M-Ras-induced transformation. Since M-Ras could activate Elk-1, despite its weak activation of ERK2, it was possible that it utilized alternate pathways to Raf/MEK/ERK to elicit gene expression. However, since MEK inhibitors almost completely attenuated M-Ras-induced Elk-1 activation, gene expression is presumably mediated by MEK. The ability of PD98059 to also block M-Ras-induced focus formation suggested that MEK is a key mediator of M-Ras-induced transformation. However, since M-Ras was considerably more potent than the MEKΔED mutant (that strongly activated Elk-induced luciferase activity) at inducing transformation (activated MEK is only transforming in secondary focus assays (36, 64)), other downstream targets must also contribute to M-Ras action. Although p38 has been reported to activate Elk-1 in NIH 3T3 cells (65), we found that the p38 inhibitor SB203580 potentiates M-Ras-induced transactivation. We are currently investigating the mechanism whereby inhibition of p38 leads to increased basal and M-Ras-induced Elk-1 activation.

Although TC21 and R-Ras associated with the isolated Ras-binding domain of c-Raf-1, they did not interact with full-length Raf (8, 56). A similar observation was made here with M-Ras and is consistent with the inability of M-Ras to strongly activate ERKs or induce gene expression. It appeared that PI3Kδ, Rin, or RalGDS also may not contribute significantly to M-Ras-induced transformation, based on their lack of interaction with M-Ras(Q71L) in the two-hybrid assay. It must be noted, however, that there are multiple isoforms of these effectors that might be differentially regulated by M-Ras (29). Indeed, we have found that the PI3K inhibitor, LY2940022, can block M-Ras-induced transformation.[2]

Two-hybrid protein interaction data indicated that the only putative Ras effector tested that bound with significant affinity to M-Ras was AF6. AF6 was originally identified as a chimeric translocation partner of ALL-1 in certain human leukemias (66). It has also been shown to interact with the C terminus of Eph family receptor tyrosine kinases, via its PDZ motif (67), and to the tight junction protein ZO-1. This latter interaction may be responsible for the localization of AF6 to tight junctions in epithelial cells and to sites of cell-cell contact in other cell types (68, 69). Little is currently known about how AF6 might mediate Ras function. However, some clues have come from studies in *Drosophila*, where the AF6 homologue, Canoe, that is involved in compound eye development, is genetically linked to Ras. Further, since the Ras and ZO-1 binding sites on AF6 overlap, it has been proposed that Ras might compete with ZO-1 to disrupt tight junction integrity (68). Consistent with this notion, expression of activated Ras in MCF-10A breast epithelial cells resulted in their acquiring a fibroblastic morphology with a loss of adherens and tight junctions (70). Since M-Ras could coprecipitate full-length AF6 from 293 cell lysates, this interaction may be physiologically relevant and merits further investigation. This is also the first demonstration of Ha-Ras interaction with AF6 *in vivo*. Determination of whether the higher affinity of AF6 for M-Ras(Q71L) *versus* Ha-Ras(G12V) represents higher affinity toward M-Ras or lower affinity for a G12V *versus* Q61L Ras mutant will require further investigation. Regardless, the inability of AF6 to cooperate with M-Ras to induce NIH 3T3 cell foci suggests that either the endogenous AF6 is sufficient to support M-Ras induced transformation or plays no role in this process.

Sequences within switch 1 and 2 are known to influence the ability of p120 Ras GAP to activate the GTPase activity of Ras. Since both switch domains of M-Ras are highly conserved with those of Ha-Ras (the Y13–259 monoclonal antibody that recognizes the secondary structure of Ras switch 2 can efficiently immunoprecipitate M-Ras),[2] it was not surprising that M-Ras was sensitive to p120 GAP regulation. Several Ras-related proteins, including the R-Ras family are sensitive to p120 Ras GAP. Therefore, it is likely that divergent residues flanking residues 32–40 of M-Ras are not critical for dictating GAP

specificity. Since both Sos1 and GRF1 can activate M-Ras, it appears that the extended effector-binding domain may not contribute to GEF selectivity either. However, M-Ras did appear less responsive to GRF1 in luciferase assays.[2] Regulation by Sos1 suggests that M-Ras in addition to the prototypic Ha-, Ki-, or N-Ras might mediate the growth-stimulatory signal initiated by receptor tyrosine kinases. Further, since M-Ras and GRF1 are both abundantly expressed in brain, M-Ras may mediate signals associated with long term memory acquisition (71).

Ha-Ras T35S, E37G, and Y40C mutants that are defective in their ability to activate Raf, PI3K, or RalGDS have been used extensively over the past 3 years to establish a role for these downstream effectors in Ras function (35, 63, 72, 73). One outcome of these studies is the notion that additional Ras effectors may exist. For example, Ramocki *et al.* reported that although Ras(G12V/T35S), Ras(G12V/E37G), and Ras(G12V/Y40C) can mimic the ability of Ras(G12V) to inhibit the differentiation of C3H10T1/2 cells into a skeletal muscle lineage, this effect cannot be mimicked by Rac and Rho nor blocked by the MEK inhibitor PD98059 (44, 59). Since M-Ras does not interact strongly with Raf, RalGDS, PI3K, or Rin and cooperates with Rho to induce NIH 3T3 cell transformation, it will be important to determine whether it induces its biological effects via targets other than AF6 and, if so, whether these too contribute to Ras-induced transformation. We are currently screening for novel M-Ras targets that might mediate the biological effects of it and other Ras family proteins.

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