Transformation is accompanied by the down-regulation of the high molecular weight isoforms of non-muscle tropomyosin. Several lines of evidence suggest that tropomyosin down-regulation may be essential for ras-induced tumorigenicity. It is unclear which of the many signaling pathways downstream of Ras are involved in tropomyosin down-regulation. Here we demonstrate that Raf activation induces tropomyosin down-regulation comparable to that induced by Ras. Expression of the effector-domain mutant Ras-G12V,Y40C, which is unable to bind Raf, induced only modest down-modulation of tropomyosin. Treatment with the MEK-specific inhibitor PD98059 had little effect on tropomyosin levels in ras- or raf-transformed cells. In contrast, a mutant form of MEK-1, MEK-1-S218A,S222A, restored tropomyosin levels in ras-transformed NIH3T3 cells almost to the levels observed in non-transformed cells. MEK-1-S218A,S222A does not inhibit MEK phosphorylation and is a poor inhibitor of ERK phosphorylation. These data suggest that this mutant form of MEK-1 interferes with a yet uncharacterized pathway controlled by Raf. We conclude that the ras-induced down-modulation of tropomyosin is predominantly Raf-mediated, but MEK-independent, and that a novel pathway exists downstream of Raf which may play an important role in regulation of the cytoskeleton.

Malignant transformation results in the down-modulation of the non-muscle high molecular weight tropomyosin isoforms TM-1, TM-2, and TM-3. These actin-binding cytoskeletal proteins are virtually undetectable in transformed cell lines generated by the induced expression of oncoproteins such as ras, src,raf, mos, fms, fos, or erbB (1–5), or by infection with tumor inducing viruses such as SV40, Rous sarcoma virus, adenovirus type 5, or MC29 (4, 6). Tumorigenic cell lines produced by the exposure of SV40-immortalized prostate cells to x-rays also have markedly lower levels of the isoforms TM-1 and TM-3 compared with those of the normal parental line (7). It is unclear how tropomyosin is down-regulated in tumor cells. Hendricks and Weintraub (4) and Cooper et al. (3) have shown that down-modulation of tropomyosin occurs both at the transcriptional as well as at the post-transcriptional level. TM down-modulation observed in tumor cells is not an artifact of prolonged cell culture or deliberate transformation, since it has also been observed in freshly isolated human prostate, breast, ovarian, and squamous cell carcinoma tissues (8–12). These data suggest that TM down-modulation is consistently associated with tumorigenesis regardless of the specific genetic alterations responsible for the transformed phenotype.

Several lines of evidence suggest that TM down-modulation is essential for transformation. TM-1 down-modulation resulting from the expression of a TM-1 antisense construct is sufficient to induce anchorage independence in immortal hamster embryo cells (13) and the reintroduction of TM-1 or TM-2 into ras-transformed cells reduces or eliminates anchorage-independent growth and tumorigenicity (5, 14–16). Analyses of multiple clones from rodent melanoma and lung carcinoma cell lines have demonstrated a strong inverse correlation between metastatic capacity and TM levels (17, 18). Collectively, these results suggest that the expression of high molecular weight tropomyosin plays an important role in maintaining the non-transformed phenotype.

Oncogenic Ras mutants activate many different downstream signaling pathways including those controlled by the activity of Ras, Rho, Cdc42, Raf, phosphatidylinositol 3-kinase and Raf (see Joneson and Bar-Sagi and Katz and McCormick, for reviews (19, 20)). To identify which pathway(ies) downstream of Ras may be involved in TM down-modulation, we analyzed the effects of activated forms of Raf, Rac, Rho, and Ras, as well as the Ras effector-domain mutant Ras-G12V,Y40C, on TM levels. We also investigated the effects of MEK and Raf inhibition in ras- and raf-transformed cells using the MEK inhibitor PD98059 and a mutant form of MEK-1, MEK-S218A, S222A. The results of our studies implicate Raf in ras-induced TM down-modulation but suggest that the critical downstream effectors may be MEK-independent. Our data provide evidence for the existence of Raf substrates in addition to MEK-1 and MEK-2 which may control the levels of actin-binding proteins including the high molecular weight TMs and regulate the phenotype of transformed cells.

MATERIALS AND METHODS

Cell Lines and Tissue Culture—NIH3T3 and ras-transformed NIH3T3 cell lines were generous gifts of L. Feig, Tufts University (16). All cell lines were grown in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA) containing 10% calf serum and 50 μg/ml gentamicin (Life Technologies, Inc., Gaithersburg, MD).

Monoclonal Antibodies—TM311, a monoclonal antibody which recognizes a common epitope in the first exon of TM-1, TM-2, and TM-3 (21) was purchased from Sigma. Anti-Raf (clone C-12) and anti-ERK-1 (clone K-23) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-MEK-1 (clone 25) was purchased from Transduction Laboratories (Lexington, KY). Anti-phospho-MEK and anti-phospho-ERK antibodies were purchased from Santa Cruz Biotechnology. Anti-Ras (clone LA069) was purchased from Quality Biotech (Camden, NJ). Secondary antibodies (goat anti-mouse IgG and goat anti-rabbit IgG) were purchased from Jackson ImmunoResearch Laboratories.
anti-rabbit IgG) conjugated to horseradish peroxidase were purchased from Bio-Rad.

Treatment with PD98059—A total of 200,000 cells per well were plated in 6-well plates in Dulbecco's modified Eagle's medium with 10% serum. The following day, the medium was replaced by fresh Dulbecco's modified Eagle's medium with 5% serum including the indicated concentration of PD98059 (New England Biolabs) in MeSO or an equal volume of MeSO (0.1% v/v). Cells were incubated for the times indicated.

Expression Vectors and Stable Transfections—The expression vectors v-ras-G23V in pJ41 (22, 23), v-raf in pBR322 (24), and ras-20A in pJ41 (25) were generous gifts of L. Feig, Tufts University. pBR322 was obtained from D. Roth, Beth Israel Deaconess Medical Center. Rhod-Q63L in pZipneo (26) was provided by C. Der, University of North Carolina. pSRa and flag-tagged onco-1bc (a rho-specific GEF) (27) in pSRa were obtained from D. Toksoz, Tufts University. H-Ras-G12V and the effector-domain mutant H-Ras-G12V,Y40C in pSRa (28, 29) were generous gifts of M. White, University of Southwestern Texas. e-Myc-tagged Ras-Q61L (26) in pRC/CMVneo was obtained from B. Schaffhausen, Tufts University. The dominant-negative form of MEK-1, MEK-1-S218A,S222A, in pAUCTneo (30), was a generous gift of M. Symons, Onyx Pharmaceuticals and contains a neomycin resistance gene (neo<sup>+</sup>), the tetracycline transactivator under control of a cytomegalovirus promoter, and MEK-1-S218A,S222A under the control of a minimal cytomegalovirus promoter fused to the tetracycline promoter.

Fibroblasts grown to 50% confluency in a 6-well plate in medium without serum were stably transfected with the above expression vectors (0.5–2 µg of DNA) using 10 µl of LipofectAMINE (Life Technologies, Inc.). Expression vectors were co-transfected with pSV<sup>neo</sup> in a molar ratio of 10:1, except for rho-Q63L in pZipneo and MEK-1-S218A,S222A in pAUCTneo, which contain the neo<sup>+</sup> gene, as indicated. Clones were selected using cloning cylinders and grown in medium with 10% serum and 500 µg/ml G418 (Mediatech). Transfections with MEK-1-S218A,S222A in pAUCTneo were performed in the presence of 1 µg/ml tetracycline and cells were kept in tetracycline-containing medium unless otherwise indicated.

Whole Cell Extracts and Western Blotting—Nearly confluent monolayers of fibroblast cultures in 6-well plates were washed twice with cold phosphate-buffered saline. Cells were lysed with 250 µl of lysis buffer (1% SDS in 62.5 mM Tris, pH 6.8) containing protease inhibitors (1 µg/ml aprotinin, 1 µg/ml leupeptin, and 100 µg/ml phenylmethylsulfonyl fluoride) and phosphatase inhibitors (1 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM sodium fluoride, 10 mM β-glycerophosphate, and 1 mM sodium pyrophosphate). Samples were boiled to reduce the viscosity and protein concentrations were determined using the bicinchoninic acid protein assay from Pierce (Rockford, IL). Protein samples were mixed 4 to 1 (v/v) with 5 × loading buffer (6% SDS, 62.5 mM Tris, pH 6.8, 25% β-mercaptoethanol, 50% glycerol, and 0.02% bromphenol blue). Equal amounts of protein per lane were electrophoresed on an SDS-polyacrylamide gel and subsequently analyzed by Western blotting. Densitometric analysis was performed using the Bio-Rad Imaging Densitometer GS-700 in combination with Molecular Analyst Software. ERK In-gel Kinase Assay and MEK and ERK-Phosphorylation Assays—The ERK in-gel kinase assay was performed as described previously (16). In vivo Raf and MEK activities were determined by assaying the levels of phosphorylation of their substrates MEK-1, and ERK-1 and ERK-2, respectively, using the anti-phospho-MEK and anti-phospho-ERK antibodies from New England Biolabs. ERK phosphorylation levels were normalized to the total ERK levels as determined by Western blotting using an anti-ERK-1 antibody from Santa Cruz Biotechnology.

RESULTS

Role of Raf in ras-mediated Down-modulation of Tropomyosin—We previously demonstrated that v-Ki-ras-transformed NIH3T3 cells have substantially reduced levels of the high molecular weight isoforms of tropomyosin (TM-1, TM-2, and TM-3; Ref. 16). Ras is a molecular switch controlling many different signaling pathways involved in tumorigenesis. In order to determine which signaling pathway(s) downstream of Ras might be involved in TM down-regulation, NIH3T3 cell lines stably expressing the activated forms of Raf, Rac, Rho, Ras, or Lbc were generated and the levels of high molecular weight isoforms of tropomyosin were determined by Western blotting. Cell lines expressing the activated forms of Rae, Rho, Ras, or Lbc did not have reduced TM levels relative to non-transfected NIH3T3 cells (not shown). In contrast, cell lines transfected with constitutively active forms of Raf (v-raf and ras-20A) had markedly decreased TM-1 and TM-2 levels and a complete disappearance of TM-3, identical to the changes induced by v-Ki-ras (Fig. 1). These results indicate that Raf activity is sufficient to down-regulate TM expression.

To determine if Raf activity is essential for ras-induced TM down-modulation, we tested the effects of the Ras effector-domain mutant, Ras-G12V,Y40C, which is unable to bind Raf, in non-transformed NIH3T3 cells. NIH3T3 cell lines were stably transfected with vector only or with vectors containing H-ras-G12V,Y40C or H-ras-G12V. Expression of the transfected genes was validated by Western blot analysis (Fig. 2A) and examination of cellular morphology. NIH3T3 cells transfected with vector alone were flat and grew as a monolayer. The H-ras-G12V-transfected cells showed a spindle-shaped morphology and grew in multiple layers. The H-ras-G12V,Y40C-transfected cells showed an intermediate phenotype (not shown). To confirm that the Ras-G12V,Y40C mutant was indeed unable to interact with Raf, we determined the levels of ERK-phosphorylation in these cell lines. To exclude the influence of growth factors, the cells were serum-starved for 24 h. Fig. 2B shows that the levels of ERK-phosphorylation in the ras-G12V,Y40C-transfected cells (normalized to total ERK protein levels) were comparable to those in normal NIH3T3 cells transfected with vector only. In contrast, the levels of ERK-1 and ERK-2 phosphorylation were increased 4.0- and 2.5-fold, respectively, in ras-G12V-transfected cells relative to normal NIH3T3 cells. Stable expression of H-ras-G12V induced a 98% decrease in the combined levels of TM-2 and TM-3 as determined by densitometry and a 90% reduction in those of TM-1 (Fig. 2C). Conversely, stable expression of H-ras-G12V,Y40C induced only a 40% decline in the TM levels. This difference in TM down-regulation was not due to differences in transgene expression, since the levels of H-ras-G12V,Y40C present in the transfectants used in this study were actually higher than those of H-ras-G12V (Fig. 2A). This result suggests that Raf activity is necessary for the complete down-regulation of tropomyosin induced by the ras oncogene, but that Raf-independent pathways may contribute to TM down-regulation.
Role of MEK in ras- and raf-mediated Down-modulation of Tropomyosin—In addition to the Ras-G12V,Y40C study, we tested the effects of PD98059, a well characterized inhibitor of Raf-mediated MEK activation (31), on TM levels in ras- and raf-transformed NIH3T3 cells. Ras-transformed cells were treated with various concentrations of PD98059 in Me$_2$SO or with Me$_2$SO alone for 5, 24, and 48 h and ERK-1 and ERK-2 activities were assayed using the in-gel kinase assay. ERK activities in ras-transformed cells were almost completely inhibited when the cells were treated with the MEK inhibitor PD98059 for 24 or 48 h (Fig. 3A). Treatment with PD98059 for 24 h at concentrations of 25, 50, and 100 $\mu$M decreased ERK-1 phosphorylation (normalized) by 83, 94, and 97%, respectively, and ERK-2 phosphorylation by 80, 90, and 98%, respectively. Treatment at the same concentrations of PD98059 for 48 h decreased the level of ERK-1 phosphorylation by 78, 65, and 55%, and that of ERK-2 by 87, 62, and 58%. The drug had only a modest effect on TM-2 levels and almost no effect on TM-1 levels (Fig. 3B). In fact, the TM-1 levels actually decreased below baseline level when cells were incubated with PD98059 at a concentration of 100 $\mu$M (Fig. 3B). Furthermore, PD98059 failed to restore TM-3 expression. The results shown in Fig. 3 have been corroborated in a subsequent study in which the duration of exposure to PD98059, and suppression of ERK activity, were extended to 72 h. In this study, TM levels remained depressed to the same extent as those observed at 48 h of exposure (not shown).

Results similar to those reported above were obtained with raf-transformed cells. In these studies, both Raf-20A, a constitutively active form of Raf-1 with 319 amino acids deleted from the NH$_2$ terminus and containing only the kinase domain, and v-Raf, which contains all effector domains of Raf-1, were used. Both forms of Raf preferentially phosphorylate ERK-2. Levels of phosphorylated ERK-2 in raf-transformed cells were comparable to those in v-Ki-ras-transformed cells, whereas levels of phosphorylated ERK-1 in raf-transformed cells were approximately 70% lower than those in ras-transformed cells (Fig. 4A). Treatment of raf-20A-transformed cells with the MEK inhibitor at 50 $\mu$M for 48 h decreased the normalized levels of phosphorylated ERK-1 and ERK-2 by 97 and 90%, respectively. Treatment of v-raf-transformed cells with the inhibitor resulted in virtually undetectable ERK-1 phosphorylation and a more than 95% decrease of ERK-2 phosphorylation (Fig. 4A). As with the ras-transformed cells, MEK inhibition in raf-transformed cells resulted only in a minor increase in TM-2 levels and no change in those of TM-1 or TM-3 (Fig. 4B).

MEK-1-S218A,S222A Inhibits ras-induced Down-modulation of Tropomyosin—Expression of the MEK-1 mutants MEK-1-S217A and MEK-1-S221A in ras-transformed NIH3T3 cells induces reversion to a non-transformed phenotype with flattening of the cells and restitution of actin cables (32). This observation suggests that expression of mutants of MEK-1 in which the serine phosphorylation sites of Raf have been mutated may affect cytoskeletal organization, possibly by restoring the expression of various actin-binding proteins known to be down-modulated in transformed cells. To test this hypothesis, we evaluated the effects of the MEK-1 mutant MEK-1-S218A,S222A on tropomyosin expression in v-Ki-ras-transformed NIH3T3 cells. In this study, v-Ki-ras-transformed cells were stably transfected with tetracycline-regulable MEK-1-S218A,S222A. In the absence of tetracycline, the level of MEK-1-S218A,S222A increased 9-fold over background within 48 h (Fig. 5A). At this point, the levels of phosphorylated ERK-1 and ERK-2 (normalized) were decreased by 60 and 63%, respectively, compared with those of cells grown in the presence of tetracycline (Fig. 5B), indicating that MEK-1-S218A,S222A was functionally active. Addition of tetracycline to cell cultures of untransformed v-Ki-ras-transformed cells had no effect on ERK phosphorylation levels (not shown). The degree of MEK inhibition induced by MEK-1-S218A,S222A (Fig. 5B) was much less than that induced by PD98059 (Fig. 3A). In addition, MEK-S218A,S222A expression did not inhibit MEK-1 phospho-
Evidence of a Novel Signaling Pathway Downstream of Raf

The aggressive behavior of ras-transformed cells is a result of its ability to activate multiple signaling pathways capable of cooperating in the promotion of anchorage-independent growth, motility, invasiveness, and metastasis. Of the various signaling molecules downstream of Ras, the serine/threonine kinase Raf is perhaps the most vital to the transforming activity of the GTPase. Constitutively activeraf mutants are able to directly transform NIH3T3 cells (33, 34) and inhibition of Raf suppresses ras-induced transformation of NIH3T3 cells (32). Active forms of Rac, Ral, and Rho on the other hand, are either non-transforming, weakly transforming, or functional only when introduced in combinations (26, 35, 36). In the studies reported herein, we assessed the ability of active mutants ofraf, rac, rho, ral, and lbc to down-modulate the high molecular weight TM isoforms and observed thatraf was the only one capable of inducing TM down-modulation when introduced as a single oncogene. This parallel between the ability of an oncogene to down-regulate TM and to transform immortalized fibroblasts not only singles out Raf as the downstream Ras target most essential for this cytoskeletal modification but also reinforces the notion that TM down-modulation is a critical step in the acquisition of the malignant phenotype.

The fact that Raf activity induces the down-modulation of TM does not necessarily indicate that it plays an essential role in ras-mediated cytoskeletal modifications. To determine if Raf activity was necessary for ras-induced TM down-modulation, we assessed the effects of the effector-domain mutant Ras-G12V, Y40C, which is unable to interact with Raf. The expression of this Ras mutant does indeed reduce TM levels, indicating that signaling pathways other than those activated through Raf may contribute to the cytoskeletal modifications observed in ras-transformants. However, this reduction is only modest and does not approach the nearly complete down-modulation induced by Ras-G12V or oncogenic Raf. These data suggest that Raf activation is essential for the nearly complete disappearance of the high molecular weight TM isoforms observed in ras-transformed cells.

So far, the only well characterized signaling pathway downstream of Raf is the MEK/ERK pathway. Since Raf activity appears to be essential for the ras-induced TM down-modulation, it was expected that inhibition of MEK in ras- andraf-transformed cells would lead to TM restoration. Surprisingly, the MEK inhibitor PD98059 had only a modest effect on ras- orraf-induced down-regulation of TM levels. In the presence of PD98059, only the TM-2 isoform was partially up-regulated while TM-1 and TM-3 isoforms were virtually unaffected. These data indicate that MEK activity is not necessary for complete ras-orraf-induced down-regulation of TM.

The inability of PD98059 to restore TM levels contrasts sharply with the effects of MEK-1-S218A, S222A and PD98059 on TM down-modulation. The different effects of MEK-1-S218A, S222A and PD98059 on TM down-modulation are not due to differences in their respective potency. In fact, the MEK inhibitor suppresses ERK phosphorylation far better than does MEK-1-S218A, S222A.

The mutant MEK-1-S218A, S222A used in our studies lacks the serine phosphorylation sites at positions 218 and 222, thereby preventing it from being activated by Raf. In a yeast two-hybrid
screening, this mutant was found to bind tightly to all Raf isoforms (38). Furthermore, of 29 clones isolated, Raf proteins were the only ones identified as interacting with this mutant MEK. The related triple mutant MEK-1-K97A,S218A,S222A, which has an additional defect in ATP binding, co-immunoprecipitates with Raf and ERK (39). These MEK mutants and others have been shown to block chemotaxis toward fibronectin and to suppress the transformed phenotype of ras-transformed cells (30, 32). Because of these properties, these MEK mutants are regarded as dominant negative forms of MEK-1. However, despite this designation, few of these mutants have actually been shown to inhibit the Raf/MEK/ERK pathway. In fact, numerous studies, including those in the present report, have shown that MEK-1 mutants in which one or more of the amino acids at positions Lys-97, Ser-218, and Ser-222 have been mutated to alanine are very poor inhibitors of MEK and/or ERK activation (32, 39, 40). Our results suggest that MEK-1-S218A,S222A interferes with a yet uncharacterized pathway distinct from the MEK/ERK cascade leading to the down-regulation of tropomyosin.

Although Raf isoforms were the only MEK partners identified in earlier two-hybrid experiments (38), MEK has recently been shown to bind to the adaptor protein Grb10 and to the carboxyl-terminal region of kinase suppressor of Ras (41–43). Thus, the ability of MEK-1-S218A,S222A to normalize TM levels in ras-transformed cells may be due to an interaction between the mutant form of MEK-1 and one of these proteins or others yet to be identified.

MEK is not the only downstream effector of Raf. In some cells, Raf activates the p70 S6 kinase (44) and NF-κB (45). Whether these factors play a role in ras-induced down-regulation of TM remains to be determined. The activation of NF-κB by Raf is probably mediated by JNK (45). Since the expression of a constitutively active Rac, which activates JNK, does not down-modulate TM (not shown), it seems unlikely that NF-κB activation would be responsible for the TM down-modulation observed in ras-transformed cells. We observed that rapamycin, an inhibitor of p70 S6 kinase, had no effect on TM levels in ras-transformed cells (not shown). These negative data suggest that the non-MEK Raf target responsible for TM down-regulation may be novel.

Our results on the regulation of TM are reminiscent of those reported by Ramocki et al. (29) on the regulation of MyoD in transfected fibroblasts. Although MyoD expression has recently been shown to be ERK-dependent in C2C12 myoblasts (46), Ramocki et al. (29) reported that the ras- and rac-CAAX-mediated down-regulation of MyoD transcriptional activity in C3H10T1/2 fibroblasts co-transformed with MyoD and a muscle-specific luciferase reporter gene was not reversed by PD98059. These results and ours emphasize the notion that not all Raf-induced phenomena are dependent on the activity of MEK. Ramocki et al. (29) also observed that the Ras-G12V,Y40C effector domain partially inhibited MyoD transcriptional activity, a finding comparable to our observation that the Ras-G12V,Y40C mutant has a modest down-modulating effect on TM levels. Collectively, our data and those of Ramocki et al. (29) indicate that Raf activation is the dominant means by which Ras reduces TM levels and suppresses MyoD transcriptional activity in transformed fibroblasts. Our results support the notion that MEK-independent signaling pathways exist downstream of Ras and Raf that regulate cellular transformation and morphology (46).