Opposing Roles of the Extracellular Signal-Regulated Kinase and p38 Mitogen-Activated Protein Kinase Cascades in Ras-Mediated Downregulation of Tropomyosin

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We showed previously that activated Ras, but not Raf, causes transformation of RIE-1 epithelial cells, demonstrating the importance of Raf-independent pathways in mediating Ras transformation. To assess the mechanism by which Raf-independent effector signaling pathways contribute to Ras-mediated transformation, we recently utilized representational difference analysis to identify genes expressed in a deregulated fashion by activated Ras but not Raf. One gene identified in these analyses encodes for α-tropomyosin. Therefore, we evaluated the mechanism by which Ras causes the downregulation of tropomyosin expression. By using RIE-1 cells that harbor inducible expression of activated H-Ras(12V), we determined that the downregulation of tropomyosin expression correlated with the onset of morphological transformation. We found that the reversal of Ras transformation caused by inhibition of extracellular signal-regulated kinase activation corresponded to a restoration of tropomyosin expression. Inhibition of p38 activity in Raf-expressing RIE-1 cells caused both morphological transformation and loss of tropomyosin expression. Thus, a reduction in tropomyosin expression correlated strictly with morphological transformation of RIE-1 cells. However, forced overexpression of tropomyosin in Ras-transformed cells did not reverse morphological or growth transformation, a finding consistent with the possibility that multiple changes in gene expression contribute to Ras transformation. We also determined that tropomyosin expression was low in two human tumor cell lines, DLD-1 and HT1080, that harbor endogenous mutated alleles of ras, but high in transformation-impaired, derivative cell lines in which the mutant ras allele has been genetically deleted. Finally, treatment with azidothymidine restored tropomyosin expression in Ras-transformed RIE-1, HT1080, and DLD-1 cells, suggesting a role for DNA methylation in downregulating tropomyosin expression.

Changes in the expression of actin-binding proteins and in actin cytoskeletal organization are common features of malignant cancer cells (20). In particular, the downregulation of expression of nonmuscle tropomyosin proteins is commonly associated with oncogenesis. Cellular transformation caused by a variety of tumor viruses, such as Rous sarcoma virus, simian virus 40, and adenovirus, caused greatly diminished expression of tropomyosin (21, 37). In addition, rodent fibroblasts transformed with a variety of oncogenes, including erbB, fes, fms, mos, myc, c-Jun, src, raf, and ras, all show reduced expression of tropomyosin (8, 10, 25, 32, 36, 48). Ras and Raf transformation of rat liver epithelial cells also caused the downregulation of TM-2 and TM-3 (68). A decreased expression of tropomyosin has been seen in human patient-derived squamous cell, breast, ovary, and prostate carcinomas (2, 13, 67). In addition, reduced tropomyosin levels have also been correlated with metastatic potential in lung carcinoma and melanoma cells (19, 63). While the function of tropomyosins in nonmuscle cells is currently unknown, the fact that their expression is commonly suppressed in a wide spectrum of transformed cells suggests that the loss of tropomyosin contributes to the process of tumorigenesis (5, 56).

Tropomyosins are a family of cytoskeletal proteins that bind to and stabilize actin in microfilaments (44). Although the function of these proteins in muscle cell contraction is well established, their function in nonmuscle cells is less clear. Nonmuscle cells express multiple isoforms of tropomyosins that include three high-molecular-weight isoforms (TM-1, TM-2, and TM-3). Whether the different isoforms serve functionally redundant or functionally distinct cellular functions is not clear (56). The high-molecular-weight tropomyosins are found along stress fibers and are thought to play a role in stabilizing the organization of actin filaments, which in turn plays an important role in the maintenance of cell shape, cell motility, and cell-cell and cell-matrix interactions (6). Therefore, the loss of tropomyosin expression in tumor cells may prevent proper assembly of microfilaments and, consequently, contribute to the invasive and metastatic properties of cancer cells (56).

Observations from studies done in oncogene-transformed rodent fibroblasts support an important contribution of the loss of tropomyosin expression to transformation. Forced reexpression of tropomyosin was seen to consistently restore organized actin stress fibers and a flatter, more adherent cell morphology. However, the ability of tropomyosin to reverse other aspects of the transformed phenotype differed significantly. For example, forced reexpression of TM-1 or TM-2 in Ras-transformed NIH 3T3 fibroblasts restored anchorage-de-
pendent growth and impaired tumorigenic growth potential (24, 47). Additionally, forced reexpression of TM-1 in Src-transformed fibroblasts resulted in lower growth rates, a flatter morphology, reduced growth in soft agar, and improved microfilament architecture (48). In contrast, another study showed forced reexpression of TM-2 or TM-3 in Ras-transformed fibroblasts resulted in no inhibition of growth in soft agar (15). Finally, forced reexpression of TM-2 in Raf-transformed normal rat kidney (NRK) fibroblasts did cause reversion of cell morphology but did not inhibit either the growth rates of these cells or their ability to grow in soft agar (62). To date, the importance of the loss of tropomyosin expression in the transformation of epithelial cells has not been addressed.

Previous analyses of Ras-transformed NIH 3T3 cells have provided some indication of the mechanism of tropomyosin downregulation by Ras. Ras mediates its actions by interaction with multiple downstream effectors, with the best characterized being the Raf-1 serine/threonine kinase (34, 58). Ras binds to and activates Raf, which in turn phosphorylates and activates MEK1/2, which then activate the extracellular signal-regulated kinase (ERK) mitogen-activated protein kinases (MAPKs). The second best-characterized effector of Ras is phosphatidylinositol 3-kinase (PI3K), a lipid kinase that facilitates the conversion of phosphatidylinositol 4,5-phosphate to phosphatidylinositol 3,4,5-phosphate (PIP3). PIP3 activates phosphatidylinositol 3-kinase (PI3K), a lipid kinase that facilitates the conversion of phosphatidylinositol 4,5-phosphate to phosphatidylinositol 3-kinase (PIP3), a lipid kinase that facilitates the conversion of phosphatidylinositol 4,5-phosphate to phosphatidylinositol 3,4,5-phosphate (PIP3) (50, 51). PIP3 in turn promotes the activation of the Akt/PI3K kinase pathway (35). PIP3 can also activate guanine nucleotide exchange factors (e.g., Sos and Vav) that activate the Rac small GTPase (18, 40), which in turn can activate the JNK and p38 MAPKs (11, 39). Recently, Mier and coworkers showed that Ras and Raf transformation of NIH 3T3 cells caused the same downregulation of tropomyosin expression (25). However, inhibition of ERK activation did not restore tropomyosin expression in Ras-transformed cells. Therefore, these authors concluded that, although Raf is a key effector for Ras-mediated downregulation of tropomyosin, Raf must cause this effect through an ERK-independent mechanism.

While rodent fibroblast cell lines are transformed readily by activated Ras or Raf, we and others have shown that a variety of epithelial cell types (e.g., rodent RIE-1 and IEC-6 and human MCF-10A breast and embryonic kidney epithelial cells) are transformed by Ras but not by Raf (41, 54; N. M. Hamad, J. Elconin, W. Bai, J. N. Rich, C. J. Der, R. T. Abraham, and C. M. Counter, unpublished data). Thus, the signaling cascades in fibroblasts and epithelial cells, while similar, exhibit important differences in biological outcome (58). As one approach to delineate the Raf-independent mechanisms important for Ras transformation of epithelial cells, we recently utilized representation analysis (22, 31) to identify genes in which expression was deregulated by Ras but not Raf (57, 59).

This study identified genes that were transcriptionally either upregulated by Ras but not Raf, or downregulated by Ras but not Raf. Among the latter group of genes was the rat gene encoding α-tropomyosin. Therefore, we initiated studies to determine the mechanism by which Ras caused downregulation of tropomyosin and to assess whether this loss of expression contributed to Ras transformation of these cells. We found that activation of ERK and inactivation of p38 MAPKs were important in downregulation of tropomyosin. Although the loss of tropomyosin correlated strongly with Ras-induced transformation, we found that restoration of tropomyosin expression alone was not sufficient to reverse any aspect of Ras transformation. Thus, both the mechanism and the role of tropomyosin downregulation in Ras transformation shows striking cell type differences.

**MATERIALS AND METHODS**

**Cell lines.** RIE-1 rat intestinal epithelial cells are spontaneously immortalized, nontransformed, diploid, epithelial growth factor-responsive cell line (provided by Robert J. Cooley, Jr. [Vanderbilt University], and were maintained in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum (FCS). The DLD-1 human colon adenocarcinoma cell line containing one endogenous mutant K-ras(61K) allele and the derivative cell line of DLD-1 lacking the mutant K-ras allele (DKO-3) (provided by R. Cooley) were maintained in RPMI supplemented with 10% FCS. The reduced ability of the DKO-3 variant to form colonies in soft agar and tumors in athymic nude mice has been described previously (45, 60). The HT1080 human fibrosarcoma cell line containing one endogenous mutant N-ras(61K) allele and the derivative cell line of HT1080 lacking the mutant ras allele (MCH603C8) (provided by E. Stanbridge, University of California at Irvine) were maintained in Dulbecco modified Eagle medium supplemented with 10% FCS, 25 mM HEPES, and 1 mM sodium pyruvate; for the 603C8 cells, 1× HAT (hypoxanthine-aminopterin-thymidine; Sigma) was also added.

Mass populations of RIE-1 cell lines stably expressing activated forms of K-Ras4B [K-Ras(12V)] and Raf1 [Raf(21W)] were established by transfection with pZIPNeoSV(x)1 retrovirus expression vectors, where expression of the inserted gene is regulated by a Moloney long terminal repeat (LTR) promoter, and have been described previously (41). Mass populations of RIE-1 cells stably expressing constitutively activated forms of Ras1 and Ras1(61L) (29) were generated in a similar fashion. The retroviral vector pCPTV3, in which expression is controlled by the 5′ Moloney murine leukemia virus LTR, was used to generate mass populations of RIE-1 cell lines stably expressing N-terminal-deleted, constitutively activated forms of mouse Vav (pCTV3-HΔN-186-vav) and Dbl (pCTV3-HΔN-186-vav) (1) similarly to those generated by retrovirus infection with the pZIP-NcoSv(x)1 construct. Stable expression of Ras1(63L), Rac1(61L), and DblHA1 have previously been shown to cause tumorigenic transformation of NIH 3T3 cells (29). Mass and clonal populations of mass-transformed RIE-1 cell lines stably expressing nonmuscle, high-molecular-weight tropomyosins TM1, TM2, or TM3 were established by co-transfection of pGEM expression vectors containing full-length cDNAs for TM1 to TM3, for which expression is controlled by the cytomegalovirus promoter (provided by Richard Janssen, National Cancer Institute), along with a retroviral expression vector (pBabe-puro), and selected with puromycin. RIE-1-K-Ras4B-transformed RIE-1 cell lines stably expressing a constitutively activated form of MKK6b(EE), a specific upstream activator of p38, were established as described elsewhere (K. Pruitt, J. K. Westmoreland, and C. J. Der, unpublished data). DNA methylation analyses. To assess the role of DNA methylation in Ras regulation of tropomyosin expression, 5-aza-2′-deoxycytidine (Sigma) was used at 1 μM in growth medium for 3 or 6 days. Azadeoxycytidine is an inhibitor of cytosine methyltransferase which methylates CpG islands in genomic DNA (27). RNA isolation and Northern blot analyses. Total RNA from cultured cells and tumors was isolated by the guanidine thiocyanate, acid-phenol method (7). For Northern blot analyses, 25 μg of total RNA was size fractionated over 1.4% formaldehyde gels, transferred to Hybond-N nylon membrane (Amersham), and hybridized to 32P-labeled DNA probes. The tropomyosin probe used was a fragment corresponding to the nucleotide coding sequence from positions 115 to 350 of the rat α-tropomyosin 2 (GenBank M60666 and M60667, respectively, and rat brain α-tropomyosin [TMBr-1] gene [GenBank M34335.1], which are identical in this region). Verification of equivalent loading of RNA was done by hybridization with a GAPDH (glyceraldehyde-3-phosphate dehydrogenase) DNA probe. Each hybridization was done by incubation with 2 × 106 cpm per ml in a solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50% formamide, 5% Denhardt’s, 0.5% sodium dodecyl sulfate (SDS), 0.1% NP-40, 50 mM sodium phosphate (pH 7.0), and 0.05% sodium pyrophosphate at 42°C for 24 to 48 h and washed in 1× SSC with 0.1% SDS at 50°C and then in 0.2× SSC with 0.1% SDS at 55°C. Western blot analyses. Subconfluent, exponentially growing cells of cultures were harvested in a buffer containing 50 mM sodium fluoride, 1 mM dithiothreitol, 1 mM phenylmethyisulfonyl fluoride, and 25 μg each of aprotinin and leupeptin/ml. Protein lysates (30 μg) were resolved by SDS–12.5% polyacryl-
amide gel electrophoresis (PAGE), transferred to Immobilon-P (Millipore) membranes, incubated with a monoclonal antibody to tropomyosin (clone TM311; Sigma), and detected with an anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham) by enhanced chemiluminescence (ECL; Amersham).

**Indirect immunofluorescence analyses.** Subconfluent, exponentially growing cultures of cells grown on glass coverslips were fixed for 10 min in 3% formaldehyde in 1 × phosphate-buffered saline (PBS), permeabilized in 0.1% Triton X-100 in 1 × PBS for 5 min, stained with fluorescein isothiocyanate-labeled phalloidin (Sigma P-5282) at 1:250 or with antibody to tropomyosin (1:400), and visualized with an anti-mouse rhodamine-labeled secondary antibody at 1:40 (Jackson Laboratories).

**RESULTS**

K-Ras(12V), but not Raf(22W), causes downregulation of tropomyosin in RIE-1 cells. While both activated Ras and Raf can readily transform NIH 3T3 fibroblasts, only Ras can transform the RIE-1 rat intestinal epithelial cell line. Instead, we have found that both Raf-dependent and Raf-independent pathways play a crucial role in transformation of RIE-1 and other epithelial cells (41). To gain further insight into how Ras contributes to the acquisition of a transformed phenotype, we recently utilized representational difference analysis to identify genes that were aberrantly expressed by the sustained activation of Ras, but not Raf, in RIE-1 cells (57). Our analyses identified 10 genes that were strongly upregulated in Ras-transformed RIE-1 cells but undetectable in activated Raf-22W-expressing or control (empty-vector-transfected) RIE-1 cells by Northern blot analysis. In addition, we also identified 12 genes that were expressed at high levels in vector only and Raf-expressing RIE-1 cells but absent in Ras-transformed cells. One such gene found to be strongly downregulated by Ras, but not Raf, encodes for the actin-binding protein tropomyosin. Based on GenBank analysis, our tropomyosin gene fragment (235 bp) showed the highest DNA sequence identity (99%) to the rat α-tropomyosin 2 and 3 genes (GenBank accession no. M60666 and M60667, respectively), and the rat brain tropomyosin 1 (TMBr-1) (GenBank accession no. M60667) gene. Our gene fragment corresponds with nucleotide coding sequences from residues 115 to 350 of the tropomyosin 2 and 3 genes and with residues 102 to 337 of the TMBr-1 gene, which are all identical at these DNA sequence positions.

As shown in Fig. 1, we found that, relative to control RIE-1 cells stably transfected with the empty pZIP-NeoSV(x)1 vector, a tropomyosin transcript of ca. 1.7 kb, corresponding to the α-tropomyosin gene, was strongly downregulated in RIE-1 cells stably expressing activated K-Ras(12V) but not Raf-22W. The RIE-1 results contrast with what we observed in NIH 3T3 cells, in which the same size transcript was downregulated by both Ras and Raf. These results are, however, consistent with previous studies by others (25) showing that high-molecular-weight TM-1, TM-2, and TM-3 proteins were downregulated by both Ras and Raf in NIH 3T3 cells.

Multiple isoforms of high-molecular-weight tropomyosins exist, and cell type variation in what isoforms are expressed has been described (44). An accurate characterization of the isoforms expressed requires two-dimensional gel separation. Our one-dimensional banding profiles were similar to previously published data (25); thus, we followed this nomenclature for our analyses and designated three main tropomyosin bands as TM-1, TM-2, and TM-3. Our Western blot analyses of RIE-1 cells stably transfected with cDNA expression vectors encoding these three tropomyosin isoforms was consistent with our use of this nomenclature (see Fig. 3).

**Downregulation of tropomyosin is a late event in Ras-mediated transformation of RIE-1 cells.** We next wanted to determine whether the downregulation of tropomyosin expression corresponded with Ras activation or, alternatively, with Ras-mediated transformation. To address this question, we utilized an RIE-1 cell line that harbors an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible expression vector encoding activated H-Ras(12V) (26). In the absence of IPTG, these cells exhibited the flat, epithelial cell cobblestone-like morphology and anchorage-dependent growth properties (Fig. 2A) seen with the untransformed parental RIE-1 cells (not shown). After 4 days of exposure to IPTG, the morphology of these cells became highly refractile and elongated in appearance and formed rapidly proliferating colonies in soft agar (Fig. 2A).

To compare the kinetics of H-Ras(12V) protein expression with the induction of transformation in the IPTG-treated cells, we performed Western blot analyses on cell lysates from cell populations exposed to IPTG for 0 to 96 h. As shown in Fig. 2B, while the induction of H-Ras(12V) protein was detected as early as 6 h after the addition of IPTG, the cells retained a normal untransformed morphology for up to 24 h of continuous IPTG treatment. Partial morphological transformation was not seen until 48 h and was not complete until after 96 h of treatment. A significant reduction in the α-tropomyosin mRNA was observed at 48 h of treatment and nearly absent by 96 h. A decrease in the tropomyosin protein (TM-1) was detectable at 72 h of treatment. Loss of TM-2 and TM-3 was not observed at any time point up to 96 h but was detectable in RIE-1/Ras stable cell line analyses (see Fig. 3A). Thus, loss of TM-2 and TM-3 requires longer than 96 h in response to Ras-mediated transformation. The loss of the α-tropomyosin
transcript and TM-1 protein coincided with the approximate time point when these cells became morphologically transformed. Since we observed a lag in time between oncogenic Ras expression and loss of tropomyosin mRNA and protein, these results suggest that downregulation of the α-tropomyosin gene does not appear to be caused directly by Ras activation and, instead, may be a secondary consequence of Ras-mediated transformation. However, because of the possibility that tropomyosin mRNA and protein possess prolonged half-life stability, our data do not completely preclude the possibility that loss of tropomyosin expression is directly a consequence of Ras activation.

**Forced overexpression of tropomyosin fails to revert transformation of Ras-expressing RIE-1 cells.** It has been shown previously that forced overexpression of various isoforms of tropomyosin protein and RNA expression is delayed significantly after onset of H-Ras(12V) protein expression. H-Ras(12V)-inducible RIE-1 cells were incubated in the presence of 1 mM IPTG for the times indicated and refed with growth medium supplemented with fresh IPTG at 48 h, and duplicate cultures at each time point were used for protein lysates or for isolation of total RNA. Equal amounts of protein lysates (30 μg) were resolved by separation on 15% (Ras) or 12.5% (tropomyosin) SDS-PAGE gels, transferred to Immobilon-P, and blotted with anti-H-Ras serum (no. 146; Quality Biotechnologies) or anti-tropomyosin serum (clone TM311; Sigma), followed by HRP-conjugated secondary antibody and visualized by ECL. Total RNA (25 μg) was size fractionated over formaldehyde-agarose gels, transferred to Hybond-N (Amersham), and hybridized to 32P-labeled cDNA probes of α-tropomyosin or GAPDH, with the latter used as a control for loading.
tropomyosin (TM-1, TM-2, or TM-3) in Ras-transformed fibroblasts were capable of morphologically reverting the transformed phenotype of these cells. The tropomyosin-overexpressing cells regained the flat, nonrefractile morphology and actin stress fibers characteristic of untransformed cells (15, 24, 47). However, while some of the studies also showed a parallel reduction in growth in soft agar and tumor formation in nude mice (24, 47), one study showed no inhibition of growth in soft agar (15). In addition, in a separate study looking at TM-2 overexpression in Raf-transformed NRK fibroblasts, while a flat morphology and increased stress fiber formation were observed, TM-2 did not inhibit the growth rates of these cells or their ability to grow in soft agar (62). Thus, we sought to determine whether tropomyosin could revert the transformed phenotype of Ras-expressing RIE-1 cells.

For these analyses, we transfected RIE-1 cells with expression vectors encoding TM-1, TM-2, or TM-3 and isolated clonal populations (three clones for each isoform) of drug-resistant, stably transfected cells. Overexpression of the introduced tropomyosin gene was verified by Western blot analyses and found to be comparable to or greater than the protein levels seen in untransformed RIE-1 cells (Fig. 3A). When these results were compared to control Ras-expressing cells transfected with the empty vector, we saw no significant change in cell morphology (Fig. 3B), in the ability to grow in soft agar (Fig. 3C and D), or in the ability to restore actin stress fiber formation (Fig. 3E). Essentially identical results were seen with mass populations of Ras-transformed cells stably expressing ectopic TM-1, TM-2, or TM-3 (data not shown). Thus, while other studies showed that forced reexpression of tropomyosin can revert the Ras-transformed phenotype of fibroblasts, we found that it did not cause any detectable reversion of Ras transformation of RIE-1 epithelial cells.

Tropomyosin is not downregulated by constitutive activation of Rho family small GTPases. We next evaluated the mechanism by which Ras causes downregulation of tropomyosin expression. Oncogenic Ras transformation of rodent fibroblasts has been shown to require the function of Rho family GTPases, such as RhoA, Rac1, and Cdc42 (71). Since Rho family GTPases are regulators of actin cytoskeletal changes (4, 17), we sought to determine whether activated RhoA, Rac1, or Cdc42 could also downregulate tropomyosin expression. For these analyses, we established mass populations of RIE-1 stably transfected with expression vectors encoding the constitutively activated RhoA(63L) or Rac1(61L) mutant proteins, as well as with constitutively activated versions of Dbl family proteins, which function as activators of Rho family GTPases. These activated Rho GTPases or Dbl family proteins have been shown previously to alter the actin organization and cause tumorigenic transformation of rodent fibroblasts (28). Interestingly, in contrast to what has been described for rodent fibroblasts, activation of Rho GTPases also did not cause any detectable morphological or growth transformation of RIE-1 cells (data not shown). As shown in Fig. 4, relative to vector-only-transfected RIE-1 cells which expressed TM-1, TM-2, and TM-3, tropomyosin expression (TM-1 and TM-3) was decreased by Ras, but not Raf-22W, RhoA(61L), Rac1(61L), Vav, or Dbl. Thus, the mechanism by which Ras downregulates tropomyosin expression in RIE-1 cells probably does not in-
volve the downstream activation of the small GTPases Rho, Rac, or Cdc42.

The ERK and p38 MAPK cascades show opposing roles in regulation of tropomyosin expression. We next evaluated the involvement of various signaling pathways activated by Ras in causing the downregulation of tropomyosin expression. In addition to the Raf/ERK pathway, the PI3K/Akt effector pathway has also been shown to be important for Ras transformation of rodent fibroblasts (51). Therefore, we utilized pharmacologic inhibitors to evaluate the importance of these Ras effector pathways in Ras-mediated downregulation of tropomyosin expression.

We showed previously that Ras activation of the Raf/ERK pathway was necessary, but not sufficient, for Ras transformation of RIE-1 cells (41). Here, we found that inhibition of ERK activation in the Ras-expressing RIE-1 cells by treatment with the U0126 or PD98059 MEK inhibitors did partially restore TM-1, TM-2, and TM-3 expression (Fig. 5). Thus, ERK activation contributes to the downregulation of tropomyosin expression. In contrast, we found that the PI3K inhibitor, LY294002, had no effect on expression of tropomyosin in the Ras-expressing RIE-1 cells. These results are consistent with our observation that the PI3K/Akt pathway is not involved in Ras transformation of RIE-1 cells and that LY294002 treatment does not reverse Ras transformation of these epithelial cells (38).

We also showed previously that oncogenic Ras stimulates an epidermal growth factor receptor (EGFR)-mediated autocrine pathway in RIE-1 cells and that inhibition of the EGFR can partially revert the morphological and growth transformation of Ras-transformed RIE-1 cells (14, 42). Here, we found that treatment of Ras-transformed RIE-1 cells with the PD153035 EGFR inhibitor also partially restored TM-1, TM-2, and TM-3 expression (Fig. 5). We also found that the treatment of untransformed RIE-1 cells with exogenous transforming growth factor α (TGF-α) caused a downregulation of TM-1, TM-2, and TM-3 expression (data not shown). This downregulation is
consistent with our previous determination that exogenous TGF-α caused morphological and growth transformation of RIE-1 cells (41). Thus, EGFR activation also contributes to Ras-mediated downregulation of TM-1, -2, and -3 expression.

Recently, we determined that treatment of Raf-expressing RIE-1 cells with the SB203580 p38 MAPK inhibitor alone was sufficient to cause the cells to exhibit similar morphological and growth transformation characteristics seen with Ras-transformed RIE-1 cells (Pruitt et al., unpublished). Consistent with this observation, we found that p38 activity was downregulated by Ras and not Raf in RIE-1 cells. Therefore, we determined whether inhibition of p38 would also promote the loss of tropomyosin expression in Raf-expressing RIE-1 cells. As shown in Fig. 6A, Raf-expressing cells treated with SB203580 showed greatly diminished levels of TM-1, TM-2, and TM-3 compared to Raf-expressing cells treated with dimethyl sulfoxide (DMSO) vehicle only. These data show that inhibition of the p38 pathway alone is not sufficient to downregulate tropomyosin since the control RIE-1 cells treated with the p38 inhibitor, SB203580, did not show a decrease in tropomyosin expression. Interestingly, Ras-transformed RIE-1 cells treated with SB203580 showed further reductions in TM-2 and TM-3 expression relative to the DMSO vehicle only or untreated Ras-expressing cells.

Our observation with SB203580 strongly suggests that p38 inactivation is involved with tropomyosin downregulation. If so, p38 activation should restore expression of tropomyosins in Ras-transformed cells. To address this possibility, we utilized
Ras-transformed RIE-1 cells, into which we introduced a constitutively active form of MKK6b/EE which specifically phosphorylates and activates p38. p38 activity was increased to levels seen in untransformed RIE-1 cells, and morphological and growth transformation was reversed in these cells (Pruitt et al., unpublished). As shown in Fig. 6B, forced reexpression of MKK6b/EE in Ras cells partially restored expression of TM-1 and TM-2 to levels seen in cells transfected with vector only.

Thus, we conclude that Ras downregulation of TM-1, TM-2, and TM-3 in RIE-1 cells requires both the activation of the ERK and the inactivation of the p38 MAPK pathways. Taken together, these results suggest that Ras utilizes both the Raf/ERK pathway and the p38 MAPK pathway to modulate tropomyosin expression.

Treatment of Ras-transformed cells with azadeoxycytidine, a DNA methylation inhibitor, restores the expression of tropomyosin mRNA. Downregulation of tropomyosin is known to occur at both the transcriptional and posttranscriptional level (25). Given that transformations caused by a variety of oncogenes and tumor viruses result in the downregulation of tropomyosin (25), we reasoned that they may all share an activity...
in common that results in repression of tropomyosin expression. One common mechanism for suppression of gene expression in human carcinomas involves DNA promoter methylation of genes to block transcription (55, 65). For example, the expression of various tumor suppressor genes, including BRCA1, E-cad, hMLH1, p16, VHL, and Rb-1, are inactivated by hypermethylation in human cancers (66). Therefore, we assessed the possibility that methylation is involved in Ras suppression of tropomyosin expression.

To determine whether the loss of tropomyosin expression was due to promoter methylation, we treated Ras-expressing RIE-1 cells with the demethylating agent azadeoxycytidine and then determined whether tropomyosin expression was restored. Azadeoxycytidine is a specific inhibitor of DNA methyltransferase (49, 70) and has been used widely to assess the role of methylation in regulation of gene expression. As shown in Fig. 7A, α-tropomyosin mRNA and TM-1, TM-2, and TM-3 protein expression was restored after 6 days of treatment with azadeoxycytidine compared to untreated Ras-expressing cells. Thus, these results demonstrate that the mechanism by which tropomyosin expression is downregulated at the transcriptional level in Ras-transformed RIE-1 cells is by methylation of the promoter. Interestingly, azadeoxycytidine treatment also caused a partial reversion of the transformed phenotype of Ras-transformed cells (Fig. 7B).

Azadeoxycytidine restores tropomyosin expression in transformed Raf-expressing cells treated with the p38 MAPK inhibitor SB203580. Our analyses in RIE-1 cells suggested that the mechanism by which Ras downregulates TM-1, -2, and -3 expression was through the activation of the ERK MAPK pathway and the inhibition of the p38 MAPK pathway. Since TM-1, -2, and -3 expression was restored in the Ras-expressing cells upon treatment with azadeoxycytidine, we wanted to determine whether the Raf-expressing cells treated with the p38 inhibitor also lost tropomyosin expression due to DNA methylation.

To determine this, we evaluated mRNA expression levels in Raf-expressing RIE-1 cells treated with SB203580 in the presence or absence of azadeoxycytidine or with vehicle only (DMSO). As shown in Fig. 7C, as observed in Ras-expressing RIE-1 cells, Raf-expressing cells treated with SB203580 in the presence of azadeoxycytidine showed α-tropomyosin mRNA levels comparable to those of untreated parental cells. In addition, similar to what we had observed for Ras-transformed cells, treatment of the SB203580-treated Raf-expressing cells with azadeoxycytidine also caused a partial reversion of the transformed morphology of these cells (Fig. 7D). Thus, these results demonstrate that the mechanism by which tropomyosin transcription is repressed in the Ras-expressing and the SB203580-treated, Raf-expressing cells is by DNA methylation.

Tropomyosin expression is suppressed by oncogenic Ras transformation of human tumor cells and increased with azadeoxycytidine treatment. Our analyses in RIE-1 cells indicated that tropomyosin downregulation was a consequence of oncogenic Ras-mediated transformation. To extend these analyses, we sought to determine whether the downregulation of tropomyosin expression is also associated with Ras-mediated transformation of human tumor cells. For these analyses, we evaluated the expression of tropomyosin in two human
FIG. 7—Continued.
tumor cell systems where it has been demonstrated previously that oncogenic Ras is critical for tumorigenic transformation. Shirasawa and colleagues used homologous recombination to knock out the mutated K-ras(13D) allele present in DLD-1 human colon carcinoma cells, and they determined that the loss of oncogenic Ras function resulted in a drastic impairment in the transformed phenotype of DLD-1 cells in vitro and in vivo (60). Similarly, Stanbridge and colleagues identified a genetic variant of the HT1080 human fibrosarcoma cell line that had lost the mutated N-ras(61K) allele. This loss coincided with impaired growth in soft agar and tumor formation in nude mice (45). Using these cell lines, we determined whether the expression of tropomyosin correlated with the ras mutation status of DLD-1 and HT1080 cells.

We performed Northern blot analyses to compare the level of α-tropomyosin gene expression in the parental DLD-1 cell line [K-ras(13D)-positive] and a variant that had lost the mutated K-ras(13D) allele (DKO-3) and in the parental HT1080 cell line [N-ras(61K)-positive] and the MCH603c8 variant that had lost the mutated N-ras(61K) allele. α-Tropomyosin expression was downregulated in parental DLD-1 and HT1080 cell lines but greatly elevated in their ras mutation-deficient counterparts (Fig. 8). These results show that loss of α-tropomyosin expression in the HT1080 and DLD-1 human tumor cell lines is Ras dependent and correlates with oncogenic Ras-mediated transformation of human tumor cells. Finally, similar to what we found with RIE-1 cells, α-tropomyosin mRNA levels were also increased when the DLD-1 and HT1080 cells were treated with azadeoxycytidine (Fig. 9). Thus, promoter methylation may be a general mechanism of Ras-mediated downregulation of tropomyosin expression.

DISCUSSION

Activated Ras and Raf can cause transformation of NIH 3T3 mouse fibroblasts, whereas only Ras causes transformation of RIE-1 epithelial cells (41). Thus, to evaluate the Raf-independent mechanisms by which Ras causes transformation of RIE-1 cells, we used representational difference analysis to identify genes in which expression was deregulated by sustained activation of Ras but not Raf in RIE-1 cells. These analyses identified tropomyosin as a gene downregulated in activated Ras-expressing cells but not in Raf-expressing cells. Since these results contrasted with observations made in studies with NIH 3T3 and NRK rodent fibroblasts, where both Ras and Raf caused downregulation of tropomyosin (25, 62), we evaluated the mechanism by which Ras caused downregulation of tropomyosin in RIE-1 epithelial cells. Our observations support the opposing actions of the ERK and p38 MAPK cascades in the regulation of DNA methylation resulting in the transcriptional silencing of tropomyosin expression. We also found that loss of tropomyosin expression correlated strongly with morphological transformation of RIE-1 cells, supporting an important contribution of the loss of tropomyosin function to Ras transformation. However, in contrast to observations made with Ras-transformed rodent fibroblasts, we found that restoration of TM-1, TM-2, or TM-3 alone was not sufficient to reverse morphological or growth transformation of Ras-transformed RIE-1 cells. Thus, the loss of tropomyosins may play a more complex role in Ras transformation of epithelial cells.

FIG. 8. Oncogenic Ras-dependent downregulation of α-tropomyosin in human tumor cells. Total RNA (25 μg) from DLD-1, DKO-3 (a derivative of DLD-1 with the mutant K-ras allele deleted), HT1080, and MCH 603c8 (a derivative of HT1080 with the mutant N-ras allele deleted) was size fractionated over formaldehyde-agarose gels and analyzed by Northern blotting with 32P-labeled DNA probes for tropomyosin or GAPDH.

In agreement with previous studies, we confirmed that the sustained activation of Raf alone is capable of causing a downregulation of tropomyosin in NIH 3T3 mouse fibroblasts. Thus, Ras utilizes primarily the Raf effector pathway to inhibit tropomyosin expression in this cell type (25, 32, 62). In contrast, we showed by both Northern and Western blot analyses that Raf activation alone cannot induce downregulation of tropomyosin in RIE-1 cells. However, we found that inhibition of ERK activation with the U0126 MEK inhibitor restored expression of tropomyosin in Ras-transformed RIE-1 cells. This result also contrasts with observations with Ras-transformed

FIG. 9. α-Tropomyosin expression is increased in human tumor cell lines treated with the DNA demethylating agent azadeoxycytidine. DLD-1 and HT1080 cells (containing activating mutations in Ras) were maintained in growth medium supplemented with DMSO vehicle only or with 1 μM azadeoxycytidine for 6 days. Total RNA (25 μg) was size fractionated over formaldehyde-agarose gels and analyzed by Northern blotting with 32P-labeled DNA probes of tropomyosin or GAPDH. The data shown are representative of two independent experiments.
NIH 3T3 cells, where ERK inactivation did not restore tropomyosin expression (25). Thus, while Raf activation of ERK-independent signaling is sufficient to cause downregulation of tropomyosin expression in NIH 3T3 cells, Raf activation of ERK is necessary, but not sufficient, to cause downregulation of tropomyosin in RIE-1 cells. These results emphasize the striking cell type differences in how oncogenic Ras regulates tropomyosin gene expression.

We also found that treatment of Raf-expressing RIE-1 cells with the SB203580 p38 MAPK inhibitor caused a loss of tropomyosin expression. This treatment also caused morphological and growth transformation of these cells, providing further support that loss of tropomyosin expression is important for Ras transformation of RIE-1 cells. Thus, the ERK and p38 MAPK cascades serve opposing roles in the regulation of tropomyosin gene expression. Both MAPK cascades stimulate the activities of a variety of transcription factors. Further assessment of how ERK and p38 regulate the expression of tropomyosin will require the isolation and analysis of promoter sequences of the high-molecular-weight tropomyosins.

To determine the importance of the downregulation of tropomyosin transcription and protein expression in Ras-induced transformation, we utilized three approaches. First, we used RIE-1 cells harboring an inducible H-Ras(12V) expression plasmid to determine whether the loss of tropomyosin expression correlated with Ras activation or Ras-induced transformation. We found that, whereas H-Ras(61L) protein expression was detected by 6 h, the downregulation of tropomyosin mRNA and protein levels did not decrease until 48 and 72 h, respectively, and correlated with the onset of morphological transformation. This contrasts with the downregulation of a second gene, transgelin, that we identified in our representative difference analysis study. Transgelin, also an actin-binding protein, showed a downregulation in expression which correlated directly with the induction of H-Ras(12V) protein expression (59). Thus, our data suggest that the downregulation of tropomyosin expression is not a direct consequence of Ras activation and, instead, may be due to secondary events or to induction of the transformed state. Alternatively, it is possible that tropomyosin RNA and protein both have prolonged half-lives, so it still remains possible that Ras has a more direct effect on tropomyosin expression. However, given the coordinated downregulation of tropomyosin transcript followed by protein observed in our experiments, we believe our data would support the former. A second approach that we employed to evaluate a role for tropomyosin downregulation in Ras transformation involved the analyses of two ras mutation-positive human tumor cell lines. Previous studies showed that the function of oncogenic Ras is critical for the transformed and tumorigenic growth properties of DLD-1 human colon carcinoma cells and HT1080 human fibrosarcoma cells (45, 60). We found that the downregulation of tropomyosin is also dependent on oncogenic Ras function in these cells. Thus, these results show that the downregulation of tropomyosin in these human tumor cells is dependent on Ras activation and correlated with growth transformation.

Third, we evaluated the consequences of forced reexpression of tropomyosin on the transformed properties of Ras-transformed RIE-1 cells. Surprisingly, we found that forced reexpression of tropomyosin isoform TM-1, TM-2, or TM-3 alone failed to cause any detectable reversal of the transformed properties of K-Ras(12V)-transformed cells. Despite expressing tropomyosin at levels higher than those seen in untransformed cells, no reversal of the transformed morphology or growth in soft agar was seen. These results contrast with previous studies wherein forced reexpression of TM-1 (47), TM-2 (15, 24, 64), or TM-3 (15) caused reversion of morphological transformation and, in some cases, also reversion of growth transformation (47).

Our inability to reverse two aspects of Ras transformation of RIE-1 cells suggests that, unlike the situation with rodent fibroblast cell lines, the forced reexpression of one tropomyosin isoform alone may not have been sufficient to restore tropomyosin function. Consistent with this possibility, studies have found that forced expression of tropomyosin protein may not assemble properly into the actin cytoskeleton (46). While several studies have shown that forced reexpression of a single isoform was sufficient to reverse the transformed properties of Ras-transformed fibroblasts, one study also showed that forced coexpression of TM-1 along with TM-2 in Ras-transformed fibroblasts resulted in the formation of well-organized microfilaments and the suppression of tumorigenicity, demonstrating a cooperative effect between tropomyosin proteins (56). This study supports earlier in vivo work showing that tropomyosins exist as dimers and in vitro experiments, suggesting heterodimeric interactions among the tropomyosins (23, 30). Thus, perhaps forced coexpression of two or more tropomyosins will be required to reverse transformation of Ras-transformed RIE-1 cells. Finally, it is possible that the concurrent loss of other actin-binding proteins (e.g., transgelin) prevents reexpression of tropomyosin alone to restore proper actin microfilament structure. A better understanding of the function of tropomyosin in nonmuscle cells will be required before we can determine whether the restoration of the expression of a single tropomyosin isoform alone is sufficient to restore tropomyosin function in Ras-transformed RIE-1 cells.

A common mechanism for gene silencing is by DNA methylation (53). Therefore, we investigated whether Ras caused the downregulation of tropomyosin transcription through cytosine methylation of the promoter. We showed that treatment with azadeoxycytidine, a known inhibitor of CpG methylation (27), restored tropomyosin mRNA expression not only in RIE-1 cells overexpressing Ras but also in the ras mutation-positive DLD-1 and HT1080 human tumor cell lines. Thus, our data strongly suggest that the mechanism for loss of tropomyosin expression in Ras-expressing cells involves promoter silencing through aberrant cytosine methylation. In addition, the restoration of tropomyosin expression with azadeoxycytidine treatment appears to be specific since the expression of several other genes we identified that were downregulated by Ras (e.g., transgelin) in RIE-1 cells were not restored upon azadeoxycytidine treatment (data not shown). Finally, we also observed that DNA demethylation also caused partial reversion of the transformed morphology of both the Ras-expressing RIE-1 cells and the Raf-expressing RIE-1 cells treated with the p38 MAPK inhibitor SB203580. Thus, methylation and silencing of gene expression may be an important mechanism of transformation of RIE-1 cells.

How might Ras promote the methylation and inactivation of tropomyosin promoter activity? Several studies indicate that
Ras activation causes upregulation of transcription and expression of the dnmt1 DNA methyltransferase gene. Overexpression of Ras in mouse embryonal P19 cells has been shown to induce the rate of transcription of the DNA methyltransferase gene 10- to 20-fold, resulting in genome-wide demethylation (52, 61). In another study, inhibition of Ras activity in the Y1 mouse adrenocortical tumor cell line, which exhibited a 30-fold overexpression of wild-type K-Ras, resulted in a 50-fold decrease in both enzyme activity and transcription of DNA methyltransferase (33). Additionally, injection of dnmt1 antisense oligodeoxynucleotides inhibited the growth of Y1 tumors in mice (49). In a separate study, Ras transformation of IEC-18 rat intestinal epithelial cells showed increased DNA methyltransferase activity, methylation of the p16 tumor suppressor gene, and decreased expression of DNA methyltransferase. The methyltransferase activity, methylation of the p16 gene, and decreased expression of DNA methyltransferase are all reduced in promoting transformation, it will be important to delineate whether this mechanism will also be seen in other cell types (3, 12, 33).

In summary, our analyses of Ras-mediated downregulation of tropomyosin support a model where oncogenic Ras activation causes reexpression of p16. Oncogenic Ras-mediated downregulation of transcription of genes encoding lysyl oxidase and Fas ligand has also been reported to involve DNA methylation (9, 43). Thus, experiments to determine whether any of the DNA methyltransferase genes, dnmt1, -3a, and -3b (69), are upregulated by activated Ras and not Raf, and whether its expression is dependent on ERK activation and p38 inactivation will be important to analyze in the RIE-1 cells and are currently being assessed. Support for this possibility is suggested by several studies which showed that activation of e-Jun, a Raf-independent signaling pathway of Ras, may be important for the stimulation of dnmt1 gene expression (3, 12, 33).

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