Essential Role of Raf in Ras Transformation and Deregulation of Matrix Metalloproteinase Expression in Ovarian Epithelial Cells

Aylin S. Ülkü,[1] Reinhold Schäfer,[2] and Channing J. Der[1]

[1]Department of Pharmacology, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC and [2]Institute of Pathology, Charité Humboldt University, Berlin, Germany

Abstract

Oncogenic Ras mediates its actions through activation of multiple downstream effector signaling cascades, which in turn regulate transcription factor activation and cause changes in gene expression. However, there exist striking cell type differences in effector pathways that are activated by Ras, in which effectors are sufficient or necessary to promote Ras oncogenesis, and in the gene targets of Ras transformation. Therefore, we evaluated the contribution of specific effectors in mediating H-Ras(12V) transformation of rat ovarian surface epithelial (ROSE) cells and up-regulation of matrix metalloproteinase (MMP) gene expression. First, we found that Raf activation alone was sufficient to partially reconstitute H-Ras(12V)-mediated morphological and growth transformation. However, Raf-independent signaling pathways are required for full Ras transformation of ROSE cells. Ras transformation did not cause activation of the phosphatidylinositol 3-kinase (PI3K) target, Akt, and PI3K inhibition did not reverse morphological transformation but did inhibit growth in soft agar, indicating a role for basal PI3K activity in anchorage-independent growth. Second, we determined that MMP-3 and MMP-10, but surprisingly not MMP-9, gene expression was up-regulated in Ras-transformed ROSE cells. Raf activation alone was fully sufficient and necessary for MMP transcriptional up-regulation. However, up-regulation of MMP-3 or MMP-10 gene expression alone is not critical for Ras-mediated transformation. In summary, in contrast to other epithelial cell types, Raf is a major effector for Ras transformation of ovarian epithelial cells.

Introduction

In developed countries, ovarian cancer is the 4th or 5th leading cause of cancer deaths in women (1). As the primary cause of death from gynecological malignancies, epithelial ovarian carcinomas are most commonly diagnosed as stage III or IV tumors. Despite the critical importance of understanding this disease, carcinogenesis in ovarian epithelial cells is among the least understood and studied areas in cancer research. The majority of ovarian epithelial cancers are believed to be derived from ovarian surface epithelium (OSE; 2–4). Although few models of ovarian epithelial carcinoma have been developed, immortalization of rodent and human OSE cells has provided crucial *in vitro* and *in vivo* model systems to study the molecular basis of ovarian cell oncogenesis (5).

The frequency of *ras* mutation is low (<5%) in ovarian cancer (6). However, the levels of activated Ras are elevated in human ovarian carcinoma cell lines that lack mutated Ras (7), a consequence of deregulated cell surface receptor signaling. For example, aberrant expression and activation of members of the epithelial growth factor receptor family tyrosine kinases commonly occur in ovarian cancers (8, 9). The importance of aberrant Ras activity in ovarian cell oncogenesis is supported by studies that show that mutated Ras can promote tumorigenic transformation of immortalized human and rodent OSE cells (5, 10, 11). Therefore, elucidating the specific mechanism by which aberrant Ras signal transduction causes OSE cell transformation may contribute to our understanding of the critical signaling events involved in ovarian epithelial cell carcinogenesis.

Although the role of oncogenic Ras in tumor formation and progression has been well established, Ras downstream signaling is more complex than originally believed (12, 13). First, there are more than 19 confirmed and/or potential downstream effectors of Ras function, including protein kinases, lipid kinases, and guanine nucleotide exchange factors (GEFs). The role of many of these effectors in Ras-induced transformation has not been determined. Second, much of our understanding of the contribution of specific effector function in Ras transformation comes from studies of rodent fibroblast cells. Recent studies suggest significant cell type differences in the role of specific effectors in facilitating Ras transformation of fibroblast and epithelial cells (14).

Three key Ras effector pathways have been studied extensively for their contribution to rodent cell transformation. The best characterized effectors are the Raf serine/threonine protein kinases, which on activation activate the mitogen-activated protein kinase kinase (MEK)-1/2 dual specificity protein kinases. MEK activation results in activation of the extracellular signal-regulated kinase (ERK)-1/2 mitogen-activated protein kinases (15, 16). Activated ERKs phosphorylate

and activate Ets family nuclear transcription factors and promote changes in gene expression. In rodent fibroblast cells, Raf activation is necessary and sufficient for Ras-induced transformation. In contrast, activated Raf alone is not sufficient to cause transformation of a variety of rodent and human epithelial cells (14, 17, 18). However, the recent identification of mutated B-Raf alleles in ovarian cancers suggests that perhaps Raf activation alone may be sufficient to promote the transforming actions of Ras in this cell type (19, 20).

The second best characterized Ras effectors are the class I phosphatidylinositol 3-kinases (PI3Ks; 21, 22). A major function of PI3K is the phosphorylation of phosphatidylinositol 4,5-bisphosphate to produce the short-lived second messenger phosphatidylinositol 3,4,5-trisphosphate that in turn promotes the activation of the Akt serine/threonine kinase. Ras activation correlates with phosphatidylinositol 3,4,5-trisphosphate accumulation, and expression of dominant-negative PI3K has been shown to block Ras transformation of NIH3T3 cells (22). The third best characterized effectors are the Ral GEFs (e.g., RalGDS, Rgl, and Rlf/Rgl2; 23). These GEFs stimulate activation of the Ral small GTPases, leading to the activation of proteins such as the AFX forkhead transcription factor (24). Whereas Ral GEF activation plays a limited role in Ras transformation of rodent fibroblasts (25), this effector pathway serves a more critical role for Ras transformation of human cells (14).

One common aspect of Ras effector pathway activation is the activation of transcription factors (e.g., Elk-1, nuclear factor-κB, c-Fos, and c-Jun; 15). Consequently, it is not surprising that genome-wide analyses have identified many genes for which expression is deregulated in Ras- or Raf-transformed cells (18, 26, 27). For example, we previously applied subtractive suppression hybridization, a PCR-based cDNA subtraction technique, and identified almost 300 genes with up-regulated or down-regulated expression in Ras-transformed rat fibroblasts (27). In light of differences in Ras signaling in fibroblasts and epithelial cells, these analyses have been extended to identify gene targets of Ras in rat ovarian surface epithelial (ROSE) cells. Among the genes that were up-regulated were the matrix metalloproteinases (MMP)-3 and -10 (stromelysin-1 and -2, respectively). Although MMPs are most commonly associated with promotion of tumor cell invasion and metastasis (28–30), MMP-3 has been shown to facilitate all facets of oncogenesis (31). Therefore, we have assessed the importance of Raf and other effectors in mediating Ras transformation of ROSE cells and in the up-regulation of MMP gene expression. We found that Raf activation alone was sufficient and necessary to promote Ras morphological and growth transformation and up-regulation of MMP-3/10 gene expression.

Results

Ras Effector Pathway Activation in ROSE Cells

Previous studies determined that Raf activation alone is not sufficient to facilitate Ras transformation of a variety of epithelial cells (14, 17, 18). However, the recent observations that B-Raf or Ras mutations are seen in ovarian and other human cancers suggests that Raf activation alone may be

sufficient for Ras transformation of some cell types. Oncogenic Ras has been shown to cause morphological and growth transformation of ROSE cells (10, 11). Therefore, we sought to determine whether Raf activation alone was sufficient for Ras-induced transformation of ROSE cells.

We first determined the ability of oncogenic H-Ras(12V) to cause sustained activation of the Raf, PI3K, and Ral GEF effector pathways in ROSE cells. Western blot analysis was used to detect phosphorylated, active forms of ERK and Akt to determine the activation state of the Raf/MEK/ERK and PI3K/Akt effector pathways, respectively. A pull-down assay using the Ral-GTP binding fragment from the Rgl Ral GEF was used to monitor the level of activated, GTP-bound RalA. When compared with control empty vector-containing ROSE cells, H-Ras(12V)-expressing cells showed elevated levels of activated ERK and RalA-GTP (Fig. 1). However, we found no increase in activated Akt levels, indicating that H-Ras(12V) transformation of ROSE cells did not involve persistent elevation of PI3K activation. Raf and PI3K pathway activation was also verified using in vitro Raf kinase and PI3K lipid kinase assays, respectively (data not shown), with H-Ras(12V) expression increasing Raf-1, but not PI3K, kinase activity. The
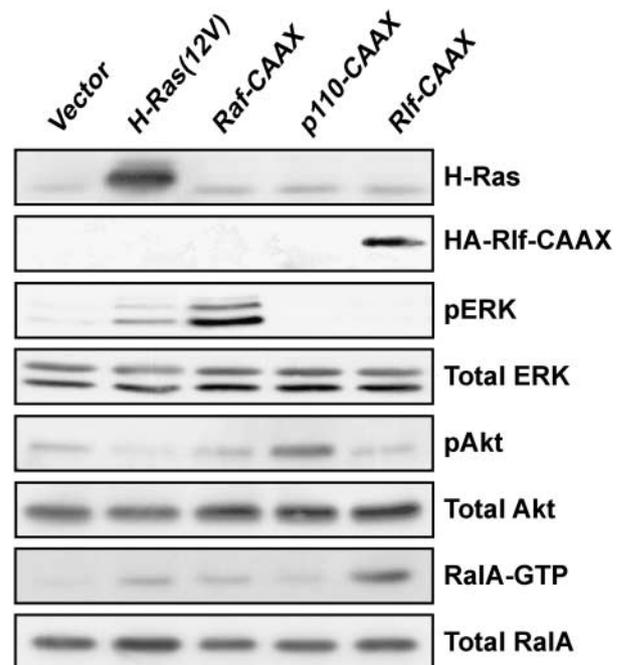

FIGURE 1. Up-regulation of Raf and Ral GEF, but not PI3K, effector pathways in Ras-transformed ROSE cells. ROSE 199 cells stably expressing pBabe-puro retroviral vectors encoding H-Ras(12V) or the plasma membrane-targeted and constitutively activated versions of Raf-1 (*Raf-CAAX*), the p110α subunit of PI3K (*p110-CAAX*), or the HA epitope-tagged Rlf (*Rlf-CAAX*) were lysed and used for Western blot analyses. Total H-Ras(12V), HA-Rlf-CAAX, and RalA protein expression was determined using anti-H-Ras, anti-HA, and anti-RalA antibodies, respectively. ERK and Akt phosphorylation and activation was determined by blot analyses with phosphospecific ERK1/2 and Akt antibodies. Replicate gels were run in parallel to probe with anti-ERK and Akt antibodies to determine total ERK1/2 and Akt protein expression. RalA activation was assayed by Ral-GTP pull-down analyses using GST-RalBD followed by immunoblotting with anti-RalA antibodies. All cell lysates were made from cultures that are serum starved for 48 h. Representative of three independent experiments.

lack of PI3K pathway activation contrasts with what has been seen with Ras transformation of NIH3T3 mouse fibroblasts (21, 32) but is similar to our PI3K pathway activation results in Ras-transformed RIE-1 rat intestinal epithelial cells (33).

Activation of Raf, but not PI3K or Rlf, Partially Reconstitutes Ras-Induced Transformation of ROSE Cells

We next determined if activation of the Raf, PI3K, or Ral GEF effector pathway alone was sufficient to cause transformation of ROSE cells. For these analyses, we established mass populations of ROSE cells stably expressing constitutively membrane-targeted and activated variants of Raf (Raf-CAAX), PI3K (p110-CAAX), and Ral GEF (Rgl-CAAX). We determined that each activated effector caused persistent activation of its downstream signaling pathway. Western blot analyses verified that Raf-CAAX- and Rgl-CAAX-expressing cells showed increased levels of activated ERK or RalA-GTP, respectively, which were comparable to or higher than the elevated levels seen in Ras-transformed cells (Fig. 1). Similarly, cells stably expressing p110-CAAX showed increased levels of activated Akt.

We next evaluated whether persistent activation of any one Ras effector signaling pathway was sufficient to cause transformation of ROSE cells. Similar to fibroblasts and other epithelial cells, Ras-transformed ROSE cells exhibited a dramatically altered cellular morphology (Fig. 2). When compared with the control empty vector-containing cells, which were indistinguishable in appearance from the parental ROSE

199 cells, the Ras-transformed cells lost the epithelial cell morphology and cobblestone-like growth pattern but exhibited a highly refractile, fibroblastic morphology and growth in a disorganized fashion with few cell-cell interactions. Surprisingly, expression of constitutively activated Raf-1 alone resulted in a similar morphological transformation for ~50% of the Raf-CAAX-infected mass population, whereas PI3K or Rlf activation alone did not alter cellular morphology.

These observations support the conclusion that activation of the Raf/MEK/ERK, but not PI3K or Rlf, signaling pathway alone may be sufficient for Ras-induced morphological transformation of ROSE cells. To further evaluate this possibility, we determined if activated MEK alone is sufficient to cause morphological transformation of ROSE cells. Mass population of cells stably expressing a constitutively activated mutant of MEK1 (MEK1ΔED) also underwent morphological transformation, although the degree and extent was significantly lower than that seen for the Raf-CAAX-expressing cells (Fig. 2). Only a small fraction of stably expressing cells showed a transformed morphology, and colony forming efficiency was much reduced when compared with Raf-expressing cells. However, because MEK1ΔED-expressing cells showed significantly lower levels of ERK activation when compared with Raf-CAAX-expressing cells (data not shown), the weaker transforming activity of activated MEK may be due, in part, to a less robust activation of ERK. Consistent with this possibility, we found that clonal isolates of MEK1ΔED-expressing cells that exhibited greater levels of ERK activation also showed greater morphological and growth transformation (data not shown).
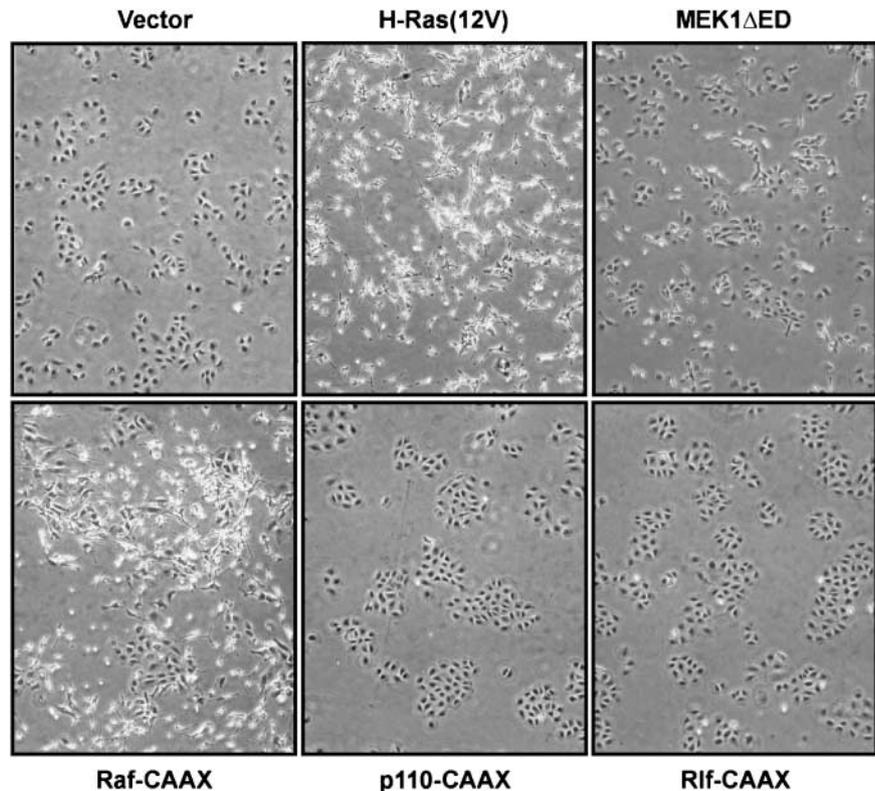

FIGURE 2. Only activated Raf and MEK1 cause morphological transformation of ROSE cells. ROSE 199 cells stably expressing the indicated proteins were plated at subconfluent and equivalent densities in growth medium and photographed for cell morphology at 24 h.

Furthermore, because Raf does exhibit MEK-independent functions (34), perhaps these activities also account for the more potent transforming activity of Raf when compared with MEK.

To determine the effectors important for oncogenic Ras induction of growth transformation, we next examined the ability of specific Ras effectors to mediate anchorage-independent growth. Whereas empty vector-infected ROSE cells failed to form colonies in soft agar, H-Ras(12V)-expressing ROSE cells formed large colonies, indicative of the loss of anchorage dependence for cell growth and proliferation (Fig. 3). Similar to our observations with morphological transformation, activation of PI3K or Ral GEF signaling pathway alone did not result in colony formation. In contrast, expression of activated Raf alone resulted in significant colony formation, although the frequency was ~30% of that seen with Ras-transformed cells. In addition to a reduced efficiency in colony formation, Raf-CAAX-expressing cells also formed smaller colonies, suggesting that Raf activation alone is not sufficient to mimic completely Ras-induced oncogenic anchorage-independent growth. Cells expressing activated MEK also formed colonies, although MEK1ΔED-induced colonies were smaller and fewer than those formed by cells with activated Raf (data not shown). We conclude that Ras activation of Raf effector signaling alone is sufficient to cause both morphological and growth transformation of ROSE cells.

MEK and PI3K Activation Are Required for Ras-Induced Anchorage-Independent Growth but not for Morphological Transformation

To determine whether activation of a specific signaling pathway is necessary for Ras-mediated ROSE cell morphological transformation and anchorage-independent growth, we performed the assays described above in the presence of pharmacological inhibitors of MEK and PI3K. First, we treated equal numbers of stably infected cells with the UO126 MEK or LY294002 PI3K inhibitor for 24 h and then examined the consequences on the morphology of Ras-transformed cells. Complete inhibition of ERK and Akt activation by treatment with each inhibitor was confirmed by Western blot analysis for the phosphorylated, active forms of ERK and Akt (data not shown). In the presence of 15 μM UO126, H-Ras(12V)-expressing cells underwent a complete reversion of morphological transformation (Fig. 4), with reversion observed as early as after 8 h of UO126 treatment. Treatment with 50 μM of another MEK1/2 inhibitor, PD98059, also caused morphological reversion (data not shown), suggesting that nonselective inhibition of other pathways by UO126 did not contribute to reversion of the H-Ras(12V) morphological phenotype. Consistent with the lack of PI3K activation in Ras-transformed cells, we found that treatment with 10 μM (Fig. 4) or 20 μM (data not shown) LY294002 to inhibit the PI3K signaling pathway did not alter the morphology of Ras-transformed ROSE cells. These results suggest that Ras activation of the Raf/MEK/ERK pathway is
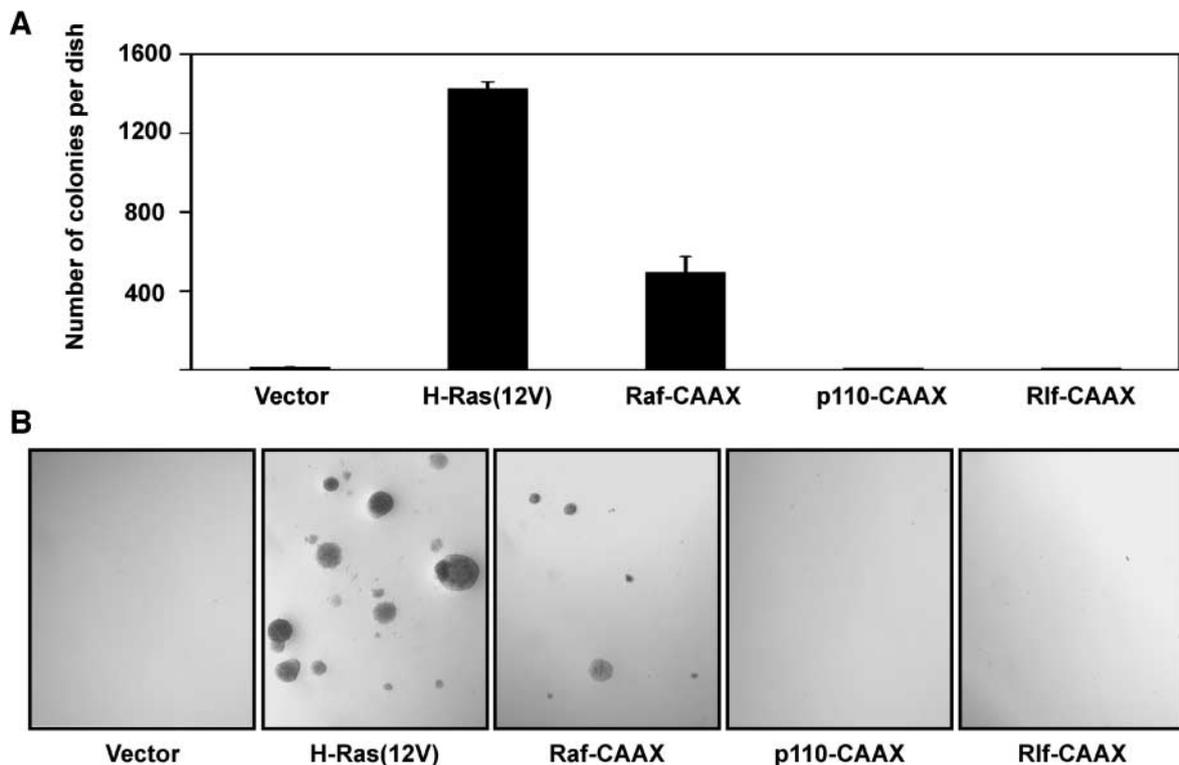

FIGURE 3.   Activated Raf alone causes partial growth in soft agar. **A.** ROSE 199 cells stably expressing the indicated protein were assayed for anchorage-independent growth by colony formation in soft agar. Cells were suspended in growth medium containing 0.6% agar and replenished with fresh growth medium for 14–16 days at which time the number of colonies ≥1 mm diameter was quantitated. *Bars,* SE resulting from three experiments performed in duplicate. **B.** Representative of colony size and colony forming efficiency for each cell line.
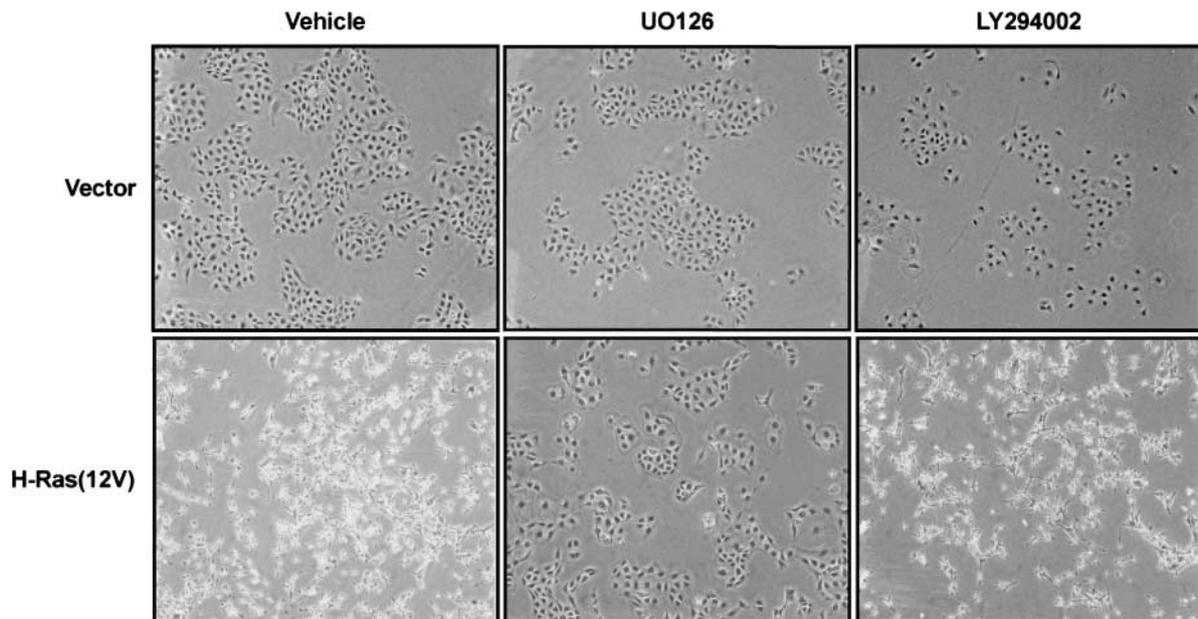
FIGURE 4. ERK, but not PI3K, activity is required for Ras-induced morphological transformation of ROSE cells. ROSE 199 cells stably expressing the indicated protein were plated at equivalent density in growth medium. After 24 h, cells were replenished with medium supplemented with DMSO (*vehicle*), 15 µM UO126 MEK1/2 inhibitor, or 10 µM LY294002 PI3K inhibitor, and the cells were photographed to monitor changes in cell morphology after 24 h.

necessary and sufficient for Ras-mediated morphological transformation. Interestingly, when we treated Raf-transformed ROSE cells with LY294002, a significant suppression of growth was seen, with cell rounding and detachment from the dish (data not shown). This different response may reflect the fact that oncogenic Ras can activate Raf-independent pathways with prosurvival functions.

We next assessed the contribution of MEK and PI3K activation to oncogenic Ras-induced anchorage-independent growth. Treatment of cells expressing H-Ras(12V) with 15 µM UO126 completely blocked soft agar colony formation as compared with H-Ras(12V)-expressing cells treated with vehicle alone (Fig. 5). Unexpectedly, although inhibition of PI3K did not alter the morphology of cells expressing oncogenic Ras, treatment with 10 µM LY294002 dramatically decreased colony formation. To confirm that the lack of colony formation was not the result of cell death, we stained soft agar colonies with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and found that UO126- and LY294002-treated cells were viable (data not shown). Because PI3K activity is not elevated in Ras-transformed ROSE cells, perhaps basal PI3K activity is necessary for oncogenic Ras induction of anchorage-independent growth. However, because LY294002 treatment did not greatly reduce Akt activity below basal levels, another possibility is that the inhibition seen may reflect other inhibitory activities of LY294002 (35).

Activation of the Raf/MEK/ERK Signaling Pathway Is Necessary and Sufficient to Mediate Oncogenic Ras Up-Regulation of MMP-3 and MMP-10 Gene Expression

Oncogenic Ras causes transformation, in part, by deregulation of gene expression. However, the contribution of specific genes to Ras transformation remains poorly understood. Recently, we employed a differential expression gene cloning approach to identify genes deregulated in Ras-transformed ROSE cells. From this analysis, MMP-3 and MMP-10 gene expression was found to be up-regulated in Ras-transformed ROSE cells. We sought to examine which Ras effector signaling pathway contributed to MMP-3 and MMP-10 gene transcription in ROSE cells.

To determine whether activation of a specific Ras effector signaling pathway was capable of increasing MMP-3 and MMP-10 transcription in ROSE cells, we first performed Northern blot analysis on RNA collected from stably infected cells using MMP-3- or MMP-10-specific cDNA probes. H-Ras(12V)-expressing cells showed striking increases in MMP-3 and MMP-10 transcript levels (Fig. 6). Raf activation alone also up-regulated MMP-3 and MMP-10 transcripts to levels comparable with that seen in Ras-transformed cells. In contrast, activation of the PI3K and Rlf effector pathways alone did not cause any up-regulation of MMP-3 and MMP-10 expression.

We next sought to establish whether Raf/MEK/ERK or PI3K effector pathway activation was required for Ras-mediated up-regulation of MMP-3 and MMP-10 gene expression. We treated H-Ras(12V)-expressing cells with 15 µM UO126 or 10 µM LY294002 for 24 h prior to RNA collection and Northern blot analysis. Inhibition of MEK activity with UO126 treatment completely reversed Ras-induced up-regulation of MMP-3 and MMP-10 transcript levels, lowering expression levels to that found in empty vector-infected cells (Fig. 7). In contrast, treatment with LY294002 appeared to cause a modest increase in MMP-3 and MMP-10 expression levels.
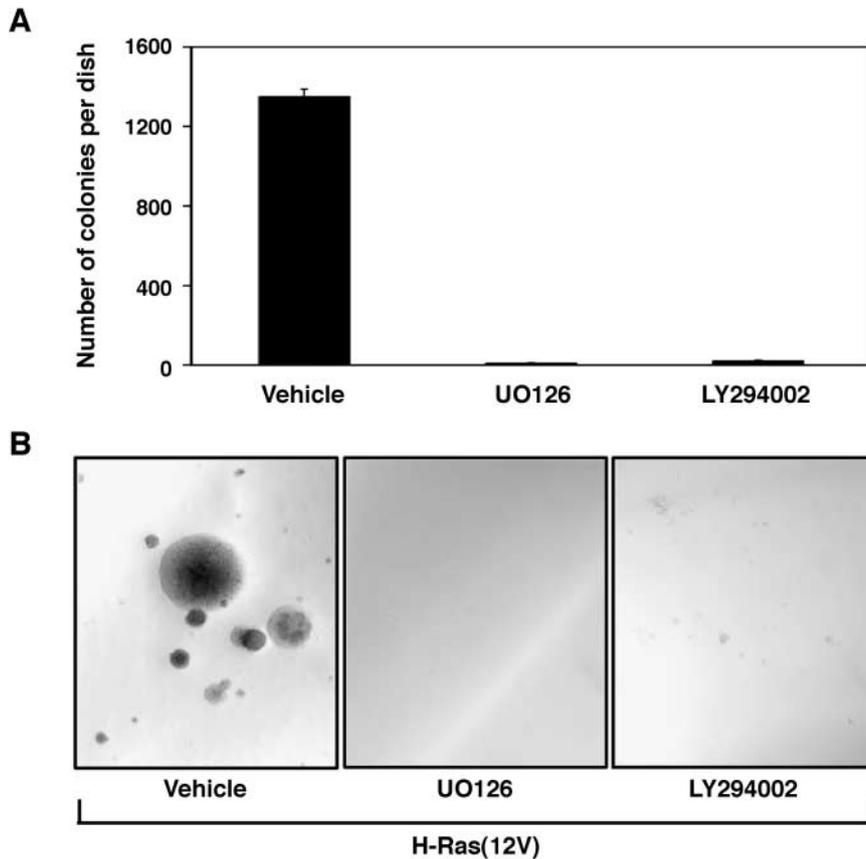
FIGURE 5. ERK and PI3K activities are required for Ras-induced anchorage-independent growth. **A.** H-Ras(12V)-transformed ROSE 199 cells were assayed for anchorage-independent growth in the presence of pharmacological inhibitors of MEK or PI3K. Cells were suspended in growth media containing 0.6% agar and DMSO (*vehicle*), 15 μM UO126, or 10 μM LY294002 and maintained for 14–16 days at which time colonies ≥1 mm were counted. *Bars,* SE resulting from two experiments performed in duplicate. **B.** Representative of colony size.

Activated Ras and Raf Up-Regulate MMP-3/MMP-10, but not MMP-2/MMP-9, Propeptide Expression

To assess whether the up-regulation in MMP-3 and MMP-10 transcription corresponded to up-regulation in protein expression, we examined conditioned media from ROSE cells stably expressing activated Ras or Raf. We then analyzed protease activity using gel zymography assays in the presence of casein as a protease substrate. Media collected from equal numbers of cells revealed single, 53-kDa bands corresponding to the propeptide, latent forms of rat MMP-3 and MMP-10 (Fig. 8). We were not able to differentiate between MMP-3 and MMP-10 as a result of the identical size and substrate specificity of these two related protease family members (71% amino acid identity). Nevertheless, consistent with our Northern blot analyses, Ras and Raf activation increased MMP-3/MMP-10 protein levels, whereas activation of the PI3K and Rlf signaling pathways did not.

Interestingly, although MMP-9 has been described to be up-regulated in a variety of Ras-transformed cells, it was not identified in the subtractive suppression hybridization analyses. To examine protease activity of MMP-2 and MMP-9, we further subjected conditioned media to gel zymography using gelatin as a protease substrate to determine the levels of MMP-2 and MMP-9 in cells expressing oncogenic Ras or its various activated downstream effectors. Surprisingly, neither MMP-2 nor MMP-9 propeptide levels were increased by H-Ras(12V) or Raf-CAAX expression, demonstrating that specific MMP up-regulation by Ras is cell context dependent (Fig. 8).

MMP-3/MMP-10 Up-Regulation Is Not Required for Ras Transformation in Vitro

In addition to promotion of tumor cell invasion, metastasis, and angiogenesis, there is evidence that MMP-3 can contribute to tumor initiation and uncontrolled growth (31). To address
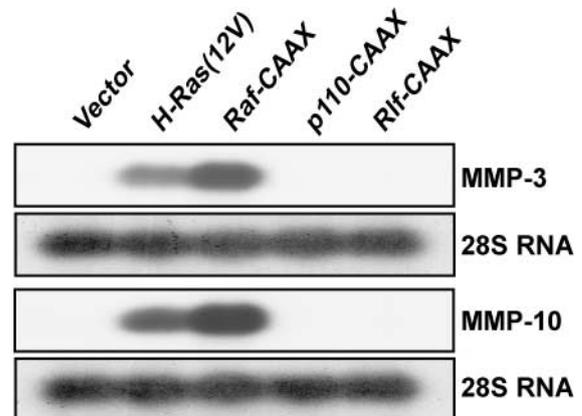
FIGURE 6. Activated Raf is sufficient to mediate Ras up-regulation of MMP-3 and MMP-10 gene expression. MMP-3 and MMP-10 transcript levels in ROSE 199 cells stably infected with the empty pBabe-puro vector or vectors expressing H-Ras(12V), Raf-CAAX, p110-CAAX, or HA-Rlf-CAAX were assayed by Northern blotting with rat MMP-3- and MMP-10-specific probes. Total RNA was collected from cells plated to equal density and maintained in growth media for 48 h. Blots were stripped and reprobed with 28S RNA-specific probes to determine total RNA levels.
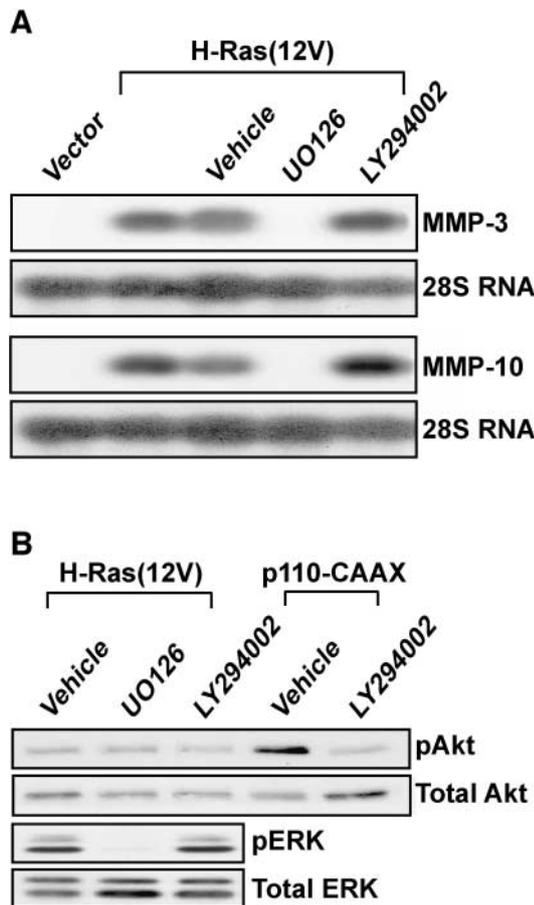
A

B

FIGURE 7. ERK activation is necessary for Ras up-regulation of MMP-3 and MMP-10 gene expression. **A.** MMP-3 and MMP-10 transcript levels in ROSE 199 cells stably expressing the indicated proteins were assayed by Northern blot analyses with rat MMP-3- and MMP-10-specific cDNA probes. Total RNA was collected from cells plated to equal density and maintained in growth medium for 24 h followed by treatment with growth medium supplemented with DMSO (*vehicle*), 15 μM UO126, or 10 μM LY294002 for an additional 24 h. The filters were stripped and reprobed with a 28S RNA-specific probe to determine total RNA levels. **B.** ERK and Akt activation were assayed by immunoblot analyses with phosphospecific ERK and Akt antibodies of cell lysates collected from duplicate plates. Blots were stripped and reprobed with ERK and Akt antibodies to determine total ERK and Akt protein expression.

this possibility, we used small interfering RNA (siRNA) to suppress MMP-3 or MMP-10 expression in Ras-transformed cells and assessed the consequences on Ras-mediated morphological or growth transformation.

For these analyses, we used the pSUPER.retro retrovirus vector (36) to stably express short hairpin RNA to silence MMP-3 or MMP-10 gene expression in Ras-transformed cells (Fig. 9A). We found that infection of H-Ras(12V)-expressing cells with the control empty vector reduced MMP-3 expression by 40%, an effect that we have seen commonly in doubly infected cells. While the 311 MMP-3-specific siRNA construct (designated by an arrow) reduced MMP-3 gene expression by ~70% relative to H-Ras(12V) + vector-expressing cells, the 328 and 331 constructs only partially suppressed MMP-3 transcription (52% and 55%, respectively). Cells infected with

the 321 missense siRNA control construct showed a slight (20%) reduction in MMP-3 transcript levels. The 1021 siRNA construct (designated by an arrow) decreased MMP-10 transcript levels to a greater degree (~90%) compared with vector-only and 1036 missense control siRNA-infected cells. Although the MMP-3- and MMP-10-specific siRNA constructs did not appear to significantly alter gene expression of MMP-10 and MMP-3, respectively, the 1021 siRNA oligonucleotide did partially suppress MMP-3 transcription (36%), suggesting some cross-reactivity of the 1021 siRNA construct with MMP-3 transcripts.

To obtain a cell population in which MMP-3 gene expression was more significantly suppressed, we isolated clonal populations from the 311 siRNA mass population of cells. We found only one population, clone 311-1 (designated by an arrow), in which MMP-3 gene expression was lower (15% of vector control) than in the 311 mass population of cells (30% of vector control; Fig. 9B). Although clone 311-3 also showed significantly reduced levels of MMP-3 transcript, H-Ras(12V) expression was markedly low, suggesting that lower MMP-3 gene expression in this clonal population was due to decreased expression of activated Ras. The low percentage of clonal populations that demonstrated siRNA-mediated MMP-3 gene suppression was surprising, given the significant (70%) reduction of MMP-3 gene expression in the pooled population of cells (311) infected with MMP-3-specific siRNA. This observation may suggest that cells in which MMP-3 expression was not suppressed have a growth advantage during the clone selection and expansion process.

We next determined the contribution of MMP-3 and MMP-10 up-regulation to Ras-mediated morphological and growth transformation. Because MMP-3 and MMP-10 are expressed as prozymogens in Ras-transformed cells but need to be activated to mediate their biological effects, we treated H-Ras(12V)-expressing cells infected with empty vector or various siRNA constructs with plasmin and examined cell
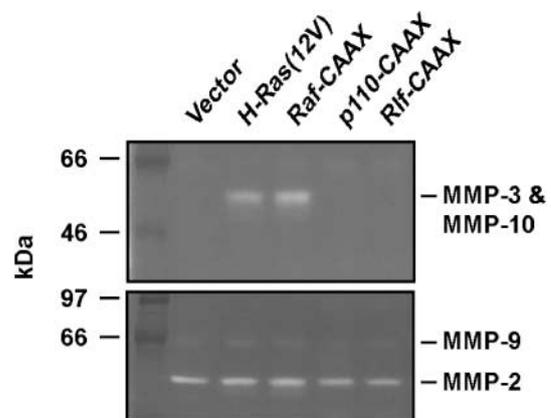
FIGURE 8. Activated Ras and Raf up-regulate MMP-3/MMP-10, but not MMP-2 or MMP-9, propeptide expression. MMP-3/MMP-10 and MMP-2 or MMP-9 prozymogen expression were assayed by gel zymography with casein- or gelatin-containing SDS-PAGE gels, respectively. ROSE 199 cells stably expressing the indicated proteins were plated to equal cell density in growth medium for 24 h and then serum starved for 48 h. Conditioned media were collected and clarified for gel zymography.

FIGURE 9. Down-regulation of MMP-3 and MMP-10 transcription in Ras-expressing cells using siRNA antisense constructs. **A.** MMP-3 and MMP-10 transcript levels in ROSE 199 cells stably expressing H-Ras(12V) and infected with the indicated siRNA antisense constructs were assayed by Northern blot analyses with rat MMP-3- a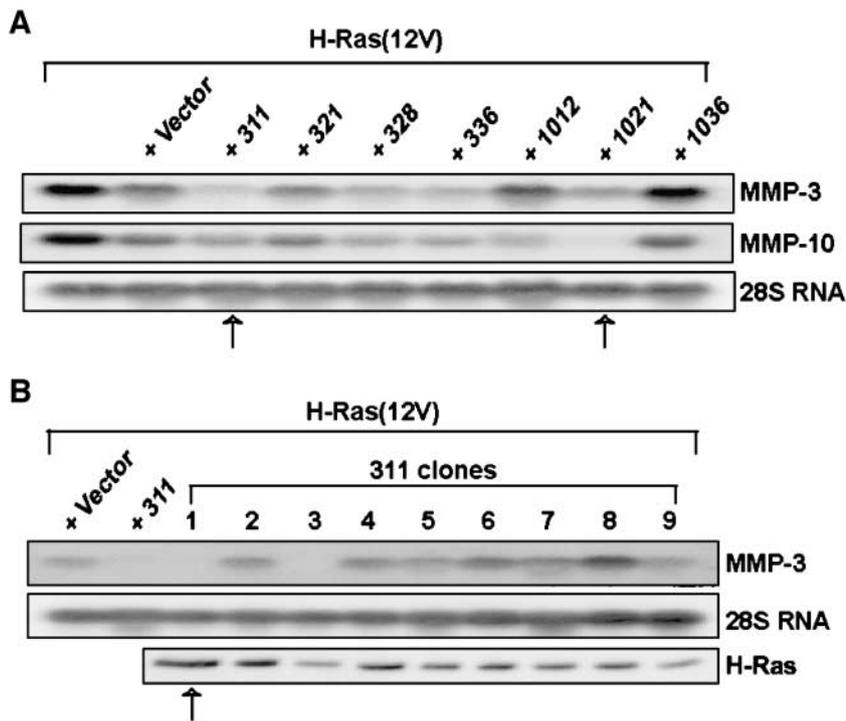nd MMP-10-specific cDNA probes. Total RNA was collected from cells plated to equal density and maintained in growth medium for 48 h. Blots were stripped and reprobed with 28S RNA-specific probes to determine total RNA levels. **B.** Northern blot analyses for MMP-3 and 28S transcript levels were performed as described above using total RNA collected from clones isolated using 96-well dilution cloning. Duplicate plates were lysed, normalized for total protein, and used for Western blot analysis. Ras expression was determined using Ras antibodies.

morphology and anchorage-independent growth. In the presence of plasmin, we observed no reversion of morphological transformation in Ras-transformed cells expressing siRNA constructs (Fig. 10). H-Ras(12V)-expressing cells in which MMP-3 or MMP-10 gene expression was suppressed had similar cell morphology to those with elevated levels of MMP-3 and MMP-10 transcript. Similarly, colony formation in soft agar assays was not altered by suppression of MMP-3 or MMP-10 gene expression (data not shown). These results suggest that up-regulation of MMP-3 or MMP-10 alone is not necessary for Ras-mediated morphological transformation or anchorage-independent growth of ROSE cells.

Discussion

Aberrant Ras activation mediates cellular transformation, tumorigenesis, and invasion via multiple downstream effectors. Although several downstream signaling pathways are well characterized, the contribution of distinct pathway activation to specific aspects of Ras-induced malignant transformation is complicated by cell context differences (12). In this study, we examined the involvement of Raf, PI3K, and Ral GEF, the three best characterized Ras effectors, in mediating Ras transformation and up-regulation of MMP-3 and MMP-10 gene expression in ROSE cells. We determined that Raf activation alone was sufficient to cause morphological and growth transformation of
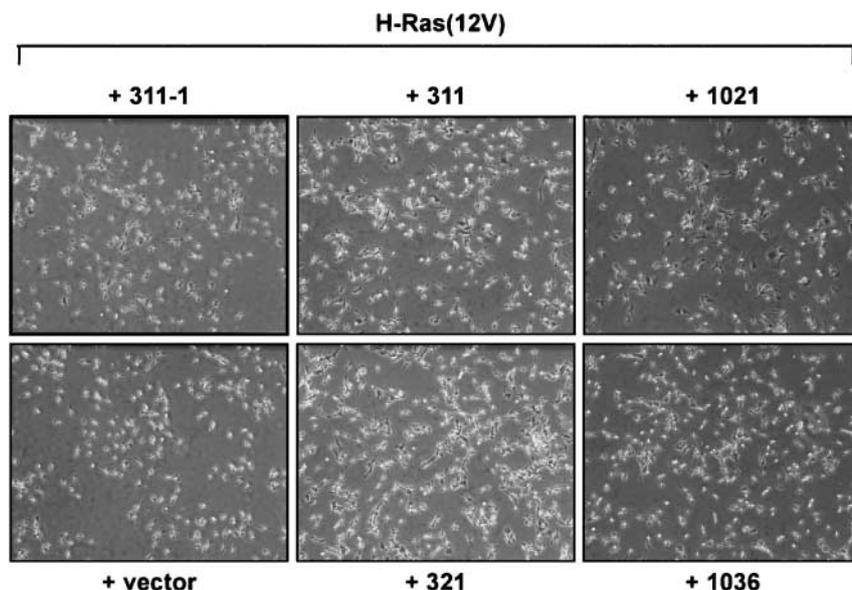
FIGURE 10. Inhibition of MMP-3 or MMP-10 expression alone does not revert morphological transformation in Ras-transformed ROSE cells. ROSE 199 cells stably infected with H-Ras(12V) and the indicated siRNA antisense constructs were seeded at equal density and maintained in growth medium supplemented with 10 μg/ml plasmin for 24 h. Photographs were taken to demonstrate cell morphology. Cells infected with the MMP-3- and MMP-10-specific siRNA constructs 311 and 1021, respectively, are compared with cells infected with empty vector only or siRNA missense controls 321 and 1036.

ROSE cells. Thus, in contrast to other epithelial cell types (*e.g.*, RIE-1, MCF-10A, and HEK; 14, 17, 18), Raf activation alone is sufficient and necessary to promote Ras transformation. We also found that, in contrast to other cell types (14, 21, 32, 37), oncogenic Ras did not cause up-regulation of the PI3K/Akt pathway in ROSE cells and that basal PI3K activity was required for anchorage-independent, but not morphological, transformation. Finally, whereas Ras transformation of rodent fibroblasts is associated with up-regulation of MMP-9, we found that Ras transformation of ROSE cells was associated with up-regulation of MMP-3 and MMP-10, but not MMP-9, expression. Taken together, these results emphasize the striking cell context dependence of Ras signaling and transformation.

We first used activated variants of Raf, PI3K, and Ral GEF to determine if activation of any one effector pathway alone was sufficient to promote Ras transformation of ROSE cells. We showed that constitutive activation of Raf-1 alone partially reconstituted the morphological and growth transformed phenotype caused by activated Ras, whereas PI3K or Ral GEF activation alone did not result in ROSE cell transformation. Activated MEK alone also caused a partial transformed phenotype. We also found that pharmacological inhibition of MEK completely reversed H-Ras(12V)-induced morphological transformation and anchorage-independent growth, indicating that the Raf/MEK/ERK pathway is necessary and sufficient for Ras-mediated ROSE cell transformation. Taken together, our observations indicate that Raf/MEK/ERK pathway activation plays a significant role in Ras-mediated transformation of ROSE cells. These observations contrast with previous studies in RIE-1 rat intestinal (17, 38), MCF-10A human mammary (18), and HEK human embryonic kidney (14) epithelial cells in which Raf activation alone was not sufficient to cause any aspect of Ras transformation. These results emphasize the importance of cell context in understanding the signaling pathways involved in Ras-mediated neoplastic transformation. Interestingly, recent observations determined that B-Raf, as well as Ras, mutations are found in cancers derived from ovarian epithelial cells (19, 20). The fact that Ras and Raf mutations were not found in the same cancers suggests the possibility that activation of Ras and Raf serve equivalent roles in ovarian cancer development. Our finding that Raf alone can promote ROSE transformation provides evidence to support this possibility.

In contrast to Ras transformation of rodent fibroblasts and other cell types, we found that Ras transformation of ROSE cells did not involve persistent up-regulation of PI3K. These observations are similar to those that we have made with RIE-1 rat intestinal cells (33), where Ras transformation was also not associated with persistent PI3K activation. However, our analyses involving pharmacological inhibition of PI3K yielded contrasting findings. Although pharmacological inhibition of PI3K in Ras-transformed ROSE cells did not revert morphological transformation or anchorage-dependent growth, we observed a significant inhibition of growth in soft agar. This observation suggests that basal levels of PI3K and Akt activation contribute to some aspects of Ras-mediated transformation. Previous studies with Ras-transformed Madin-Darby canine kidney epithelial cells determined that Ras promoted anchorage-independent growth by PI3K/Akt-mediated inhibi-

tion of anoikis (39). Because oncogenic Ras does not activate PI3K in ROSE cells, perhaps basal PI3K activity is critical to block this form of matrix deprivation-induced apoptosis in ROSE cells.

Of the MMP family of proteases, MMP-9 (gelatinase-A) up-regulation has been most commonly associated with Ras transformation (40–44). However, we found that MMP-3 and MMP-10, but not MMP-9, up-regulation, was seen in Ras-transformed ROSE cells. These results also contrast with analyses in human ovarian carcinoma cells, where the introduction of activated Ras into OV3121-1 human ovarian carcinoma cells caused high MMP-2 and MMP-9 gelatinolytic activity, but no increase in MMP-3 expression was seen (45). MMP-9 and the related MMP-2 (gelatinase-B) degrade gelatin, laminin, and nidogen (28–30). The MMP-3/10 stromelysins degrade basement membrane components type IV collagen, nidogen, and fibronectin. Thus, depending on cellular and genetic context, oncogenic Ras will cause activation of distinct MMPs and consequently promote distinct features of invasion and metastasis.

We demonstrated that Raf, but not PI3K or Ral GEF, activation alone was sufficient for up-regulation of MMP-3 and MMP-10 gene and prozymogen expression. Further, UO126, but not LY294002, treatment of H-Ras(12V)-expressing ROSE cells inhibited MMP-3 and MMP-10 gene expression, indicating that MEK, but not PI3K, activation is necessary for MMP up-regulation. Thus, Raf activation alone is sufficient and necessary for oncogenic Ras up-regulation of MMP-3 and MMP-10 gene and protein expression.

We found that siRNA down-regulation of MMP-3 or MMP-10 expression did not alter the transformed morphology or growth in soft agar of Ras-transformed cells. These results suggest that MMP-3/10 up-regulation is not necessary for these aspects of Ras transformation *in vitro*. However, because MMP function may be more relevant for tumor cell invasion and metastasis (46), it is still possible that the up-regulation of MMP-3/10 may contribute to the tumorigenic and metastatic growth of Ras-transformed ROSE cells. Using *in vitro* models of invasion, we sought to determine whether H-Ras(12V)-induced MMP-3 and MMP-10 expression resulted in increased ROSE cells invasion. Surprisingly, in two *in vitro* invasion assays (Boyden chambers coated with Matrigel and G8 myoblast monolayer invasion) as well as in one *in vitro* assay that correlates with metastatic potential (cell organization on Matrigel), we did not detect increased invasion of cells expressing activated Ras as compared with cells infected with empty vector (data not shown). Because MMP-3 and MMP-10 are expressed and secreted in their prozymogen forms, we treated H-Ras(12V)-expressing cells with plasmin, a known activator of MMP-3. Although we observed cleavage and activation of MMP-3 and MMP-10 via plasmin, activation of MMP-3 and MMP-10 also did not enhance the invasive properties of Ras-transformed ROSE cells (data not shown). The difficulty in developing *in vitro* models of ROSE cell invasion may result from the inability of established assays to recapitulate necessary *in vivo* conditions, including the tumor microenvironment in which multiple cell types interact. Alternatively, although aberrant Ras activation is sufficient to mediate several aspects of malignant transformation of ROSE cells, activation of Ras alone may not induce ROSE cell invasion.

In summary, we have demonstrated that activation of the Raf/MEK/ERK signaling pathway plays a more significant role in Ras-induced ROSE cell transformation than some other epithelial cell types. Furthermore, although H-Ras(12V) does not activate PI3K in ROSE cells, basal PI3K activity is necessary for certain aspects of Ras-mediated neoplastic transformation. Our results emphasize that multiple Ras effectors contribute to distinct aspects of transformation and that cell context plays a role in downstream effector pathway contribution. We are currently investigating the contribution of MMP-3 and MMP-10 up-regulation to Ras-mediated ovarian carcinoma cell invasion *in vivo*. Given the recent findings that MMPs also play a role in cell proliferation and apoptosis, future studies will also determine whether MMP up-regulation contributes to aspects of Ras-induced tumor progression other than invasion (29, 47).

Materials and Methods

Molecular Constructs

The pBabe-puro mammalian expression vector regulates the expression of introduced cDNA sequences from a cytomegalovirus promoter and encodes puromycin resistance. pDCR-H-Ras(12V) (provided by M. White) was used as template to generate a 590-bp fragment by PCR-mediated approaches using a 5′ primer containing a *Bam*HI site and a 3′ primer containing an *Eco*RI site. The product was digested with *Bam*HI and *Eco*RI and ligated into the *Bam*HI and *Eco*RI sites of pBabe-puro. Activation of human Raf-1, the p110 catalytic domain of PI3K, and the Ral guanine nucleotide dissociation stimulator Rlf was achieved by the addition of the COOH-terminal plasma membrane targeting sequences of Ras onto their COOH termini and designated Raf-CAAX (48, 49), p110-CAAX (22), and HA-Rlf-CAAX (50), respectively. pZIP-Raf-CAAX and HA-Rlf-CAAX as well as pS5G-p110-CAAX (provided by J. Downward) were subcloned into pBabe-puro and pBabe-hygrovia *Bam*HI and *Eco*RI digestions, respectively (33).

Materials and Inhibitors

Antibodies used for Western blot analyses were specific for H-Ras (Ab-1; Oncogene Science, Tarrytown, NY), hemagglutinin (HA) epitope (MMS-101R; BAbCO, Richmond, CA), activated ERK1 and ERK2 (E10; Santa Cruz Biotechnology, Santa Cruz, CA), activated Akt (phospho-Akt Ser473; Cell Signaling Technology, Beverly, MA), ERK1 C-16 (Santa Cruz Biotechnology), RalA (BD Transduction Laboratory, Lexington, KY), and β-actin (Sigma Chemical Co., St. Louis, MO). Antibodies used for immunoprecipitation were specific to Raf-1 C-12 (Santa Cruz Biotechnology) and the p110α catalytic subunit of PI3K (Upstate Biotechnology, Inc., Lake Placid, NY).

Chemical inhibitors used in this study were dissolved in DMSO and their effects were measured relative to DMSO (vehicle)-treated controls. The MEK1/2 inhibitor UO126 (provided by J. Trzaskos; DuPont, Wilmington, DE) and the PI3K inhibitor LY294002 (AG Scientific, San Diego, CA) were used at 15 and 10 μM final concentrations, respectively. H-Ras(12V)-expressing cell cultures were initially seeded in medium containing agar and a final concentration of 15 μM UO126 or 10 μM LY294002 was then replenished with medium containing UO126 or LY294002 every 72 h to maintain final concentrations of inhibitors.

Cell Culture and Retroviral Infection

ROSE 199 (51) and 293T cells were cultured in DMEM supplemented with 10% heat-inactivated FCS (Hyclone Laboratories, Logan, UT). Mass populations of cells stably infected with pBabe-puro containing various cDNA were selected by the supplementation of growth medium with 1 μg/ml of puromycin. Cell lines were used within 7–10 days after the completion of drug selection. Production of infectious, replication-incompetent retrovirus was achieved by cotransfection of 3 μg each pBabe-puro expression constructs with pVPack-GP and pVPack-Eco (Stratagene, La Jolla, CA) into 293T cells by calcium phosphate precipitation (52).

SDS-PAGE and Western Blot Analyses

Cells stably expressing pBabe-puro expression constructs were seeded at a density of 3×10^5 per 10-cm dish 24 h prior to starvation. Cells were washed once with $1\times$ PBS and maintained for 48 h in starvation medium consisting of DMEM supplemented with 0.5% heat-inactivated FCS. Cell lysates were generated by lysis in buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 50 mM NaF, and 1% NP40. Lysates were clarified by centrifugation at $14,000 \times g$ for 9 min at $4°C$ prior to use. Total cell lysate protein concentrations were determined using the BCA Protein Assay Kit (Pierce Chemical Co., Rockford, IL) and 20–30 μg of total lysate were loaded. Proteins were separated by SDS-PAGE, transferred to Immobilon-P (Millipore, Bedford, MA) membranes, blocked, and incubated in primary antibodies as instructed by manufacturer. Secondary antibodies (Immunopure, Pierce Chemical) were horseradish peroxidase conjugated for detection by enhanced chemiluminescence (Amersham Pharmacia Biotech, Uppsala, Sweden).

Soft Agar Growth Assays

To assess growth of ROSE cells in soft agar, cells stably infected with pBabe-puro empty vector or cDNA encoding H-Ras(12V), Raf-CAAX, p110-CAAX, or HA-Rlf-CAAX were seeded in duplicate at 5×10^3 per 60-mm dish in growth medium containing 0.4% agar over a base layer of 0.6% agar (52). For inhibitor-treated cells, UO126 or LY294002 were added during cell seeding at final concentrations of 15 and 10 μM, respectively, with DMSO treatment as a vehicle control. Cells were fed with 200–250 μl of growth media every 72–96 h. Cells treated with inhibitors were fed every 72 h with 250 μl of growth media supplemented with DMSO, UO126, or LY294002 to a final soft agar concentration of 7.5 and 5 μM, respectively. Colonies larger than 1–1.5 mm were counted after 16–18 days to quantitate growth in soft agar.

RalA Activation Assay

Bacterially expressed glutathione *S*-transferase (GST)-RalBD was used for pull-down analyses to detect formation of Ral-GTP by procedures similar to those described previously

(53). Briefly, cells were lysed in detergent buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 0.5% NP40, 1 mM Pefabloc, 1 mM NaVO$_4$, 1 μg/ml leupeptin, and 2 μg/ml aprotonin], clarified, and quantitated as described for Western blot analysis. Activated, GTP-bound Ral protein from total cell lysates (75 μg) was collected on glutathione-Sepharose beads using GST-RalBD fusion protein (3–6 μg). The presence of RalA-GTP bound to RalBD was determined by Western blot analysis. Total cell lysates (10–20 μg) were subjected to Western blot analysis using β-actin-specific antibody to confirm equivalent total protein loading.

Northern Blot Analyses

Cells were seeded at a density of 3 × 10^5 per 10-cm dish 48–72 h prior to RNA collection. Cells treated with pharmacological inhibitors were seeded 24 h prior to 24 h treatment with DMSO (vehicle control), 15 μM UO126, or 10 μM LY294002 in growth media. Total RNA from cells was isolated by the guanidine thiocyanate, acid-phenol method (54). For Northern blot analyses, 25 μg of total RNA were size fractionated over 1.4% formaldehyde gels, transferred to Hybond-N nylon membrane (Amersham Biosciences, Piscataway, NJ), and hybridized to ^{32}P-labeled cDNA probes. Hybridizations were with 10^7 cpm in PerfectHyb Plus Hybridization Buffer (Sigma) at 65°C for 6 h and washed in 2× SSC with 0.1% SDS at 65°C followed by 0.5× SSC with 0.1% SDS at 65°C. Control for equivalent RNA loading was determined by hybridization to human 28S rRNA cDNA probe (BD Biosciences Clontech, Palo Alto, CA) with 10^6 cpm in PerfectHyb at 37°C for 2–3 h. Washes were performed with described solutions at 37°C. Autoradiographs were quantitated using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Gel Zymography Assay

Cells were seeded and starved as described. Conditioned media were collected and clarified by centrifugation at 1500 × *g* for 5 min. Equal volumes of media were incubated with nonreducing SDS sample buffer at 37°C for 20 min. Equal volumes of samples were loaded and proteins were separated by SDS-PAGE in 10% and 12% gels polymerized in the presence of gelatin and casein, respectively (Bio-Rad, Hercules, CA). Gels were washed for 1 h in 2.5% Triton X-100 and incubated at 37°C for 12–48 h in renaturing buffer consisting of 50 mM Tris (pH 7.5), 150 mM NaCl, and 10 mM CaCl$_2$. Gels were stained in 1% Coomassie blue and destained to visualize bands of gelatin or casein substrate digestion.

siRNA Inhibition of MMP-3 and MMP-10 Gene Expression

siRNA constructs were designed using Oligoengine, Seattle, WA (Molecular Biology Insights, Inc; Qiagen, Inc.). Complementary 64-bp oligonucleotides homologous to rat *mmp*-3 or *mmp*-10 genes were annealed and cloned into the *Bgl*II and *Hin*dIII sites of pSUPER.retro, which provided puromycin resistance (Oligoengine). The MMP-3-specific siRNA oligonucleotide sequences are as follows: 311 antisense (5′ oligo: 5′-GAT CCC CTG GTC TTG GCT CAT GCC TAT TCA AGA GAT AGG CAT GAG CCA AGA CCA TTT TTG GAA A-3′ and 3′ oligo: 5′-AGC TTT TCC AAA AAT GGT CTT GGC TCA TGC CTA TCT CTT GAA TAG GCA TGA GCC AAG ACC AGG G-3′), 321 missense (5′ oligo: 5′-TAG CCC CAA TCT CTC AGG ACC TCT GAT TCA AGA GAT CAG GGG TCC TGA GAG ATT TTT TTG GAA A-3′ and 3′ oligo: 5′-AGC TTT TCC AAA AAA ATC TCT CAG GAC CCC TGA TCT CTT GAA TCA GAG GTC CTG AGA GAT TGG G-3′), 328 antisense (5′ oligo: 5′-GAT CCC CAA TCT CTC AGG ACC CCT GAT TCA AGA GAT CAG GGG TCC TGA CAG ATT TTT TTG GAA A-3′ and 3′ oligo: 5′-AGC TTT TCC AAA AAA ATC TCT CAG GAC CCC TGA TCT CTT GAA TCA GGG GTC CTG AGA GAT TGG G-3′), and 336 antisense (5′ oligo: 5′-GAT CCC CCT TTC CAG GCA TTG GCA CAT TCA AGA GATGAG CCA ATG CCT GGA AAG TTT TTG GAA A-3′ and 3′ oligo: 5′-AGC TTT TCC AAA AAC TTT CCA GGC ATT GGC ACA TCT CTT GAA TGT GCC AAT GCC TGG AAA GGG G-3′). The MMP-10-specific siRNA oligonucleotide sequences are as follows: 1012 antisense (5′ oligo: 5′-GAT CCC CCC ATG GAT CTT GCT CAG CAT TCA AGA GAT GCT GAG CAA GAT CCA TGG TTT TTG GAA A-3′ and 3′ oligo: 5′-AGC TTT TCC AAA AAC CAT GGA TCT TGC TCA GCA TCT CTT GAA TGC TGA GCA AGA TCC ATG GGG G-3′), 1021 antisense (5′ oligo: 5′-GAT CCC CAC CTG AGA CCC CAG TCA AT TCA AGA GAT TTG ACT GGG GTC TCA GGT TTT TTG GAA A-3′ and 3′ oligo: 5′-AGC TTT TCC AAA AAC CAT GGA TCT TGC TCA GCA TCT CTT GAA TGC TGA GCA AGA TCC ATG GGG G-3′), and 1036 missense (5′ oligo: 5′-GAT CCC CGG CCG CCC GAG ACT GAT AAT TCA AGA GAT TAT CAG TCT CGG GAA GCC TTT TTG GAA A-3′ and 3′ oligo: 5′-AGC TTT TCC AAA AAG GCT TCC CGA GAC TGA TAA TCT CTT GAA TTA TCA GTC TCG GGC GGC CGG G-3′) ROSE cells stably expressing H-Ras(12V) under hygromycin selection were retrovirally infected with pSUPER.retro containing the indicated siRNA oligos. Cells were doubly selected with hygromycin and puromycin. Total RNA was collected for Northern blot analyses using MMP-3-, MMP-10-, and 28S-specific cDNA probes as described above.

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