K-Ras Regulates the Steady-state Expression of Matrix Metalloproteinase 2 in Fibroblasts*

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Fibroblasts constitutively express matrix metalloproteinase 2 (MMP-2), which specifically cleaves type IV collagen, a major structural component of basement membranes. The level of MMP-2 expression was not altered by serum withdrawal, suggesting that MMP-2 expression is regulated by a series of steady-state conditions that impinge on the MMP-2 promoter. Expression of a dominant-negative Ras protein significantly inhibited MMP-2 transcription, thereby suggesting a role for steady-state Ras function in the regulation of MMP-2 expression. Kirsten-Ras (K-Ras) knockout fibroblasts express undetectable basal levels of MMP-2, whereas N-Ras knockout fibroblasts expressed constitutive levels of MMP-2 similar to those observed in wild-type control fibroblasts. Using an MMP-2 promoter-luciferase reporter assay, we demonstrated that the transcription of MMP-2 in K-Ras knockout fibroblasts was partially restored by transient expression of c-K(B)-Ras but not c-K(A)-Ras. A phosphoinositide-3 (PI-3) kinase-specific inhibitor (LY294002) decreased the basal level of MMP-2 in wild-type fibroblasts. Blocking PI-3 kinase signaling by overexpression of the regulatory domain of PI-3 kinase (p85) also down-regulated the steady-state MMP-2 levels. Fibroblasts that fail to express AKT1 also expressed decreased amounts of MMP-2 compared with wild-type fibroblasts. These data suggest that steady-state MMP-2 expression is regulated by c-K(B)-Ras through a PI-3 kinase:AKT-dependent signaling pathway. Because the majority of the MMP-2 assays were performed using conditioned media from serum-starved fibroblasts, these data also highlight our previous observations that Ras proteins have functions in the absence of acute mitogenic stimulations. In addition, this is the first demonstration of a specific steady-state function attributable to K(B)-Ras.

MMP-2* belongs to an evolutionarily conserved proteinase family that requires Zn$^{2+}$ for enzymatic activity. MMP-2 is synthesized and secreted as inactive zymogen. The activation of MMP-2 requires the removal of prodomain by the membrane-type MMPs. The activity of MMP-2 is tightly regulated by its natural inhibitor TIMP2 (1–3). The expression of MMP-2 is tissue and developmentally regulated. MMP-2 is expressed in the developing murine lung and kidney, with minimum expression in bone and the central nervous system. In the adult animal, MMP-2 is minimally expressed, primarily confined to cells of mesangial origin (4). The synthesis of MMP-2 is relatively constitutive. Sequence analysis revealed that the transcription of MMP-2 is under control of a number of cis-acting regulatory elements in the 5'-flanking region, including P53, Y-box, AP2, AP-1, ETS, C/EBP, CREB, PEA3, and Sp1 (5–8).

The high specific activity of MMP-2 toward collagen and its pericellular localization imply that it is a key component in the degradation of basement membranes and cell migration (1, 9). Increased expression of MMP-2 in tumor cells correlates with invasiveness and metastatic potential (10–13). Tumor cells expressing oncogenic Ras proteins possess higher potential to metastasize, in part because of the up-regulation of MMP-2 expression (14–17). Ras proteins are a group of small G proteins. The most common forms of oncogenic Ras have mutations at either codon 12, 13, or 61 (18). These mutations result in a higher proportion of Ras proteins with GTP in their binding site. Introduction of oncogenic H-Ras into fibroblasts results in increased expression of MMP-2 (14, 17). Blocking Ras-dependent signaling decreased both expression of MMP-2 and invasion of Src-transformed cells, indicating a role for Ras in regulating MMP-2 expression (19).

Three genes encode the four immediate members of the Ras family: H-, N-, K(A)-, and K(B)-Ras. K(A)- and K(B)-Ras proteins arise from alternative splicing of the K-Ras mRNA (20, 21). We have generally found c-Ha-Ras protein expression to be detectable only in cells of neuronal origin, whereas c-N-Ras and both K-Ras isoforms are expressed in all tested cell lines. Recent data suggest that different Ras isoforms possess distinct cellular functions. The most compelling evidence is that both the H-Ras knockout and the N-Ras knockout mice are viable, whereas the K-Ras knockout is embryonic lethal. The loss of two K-Ras proteins was not mimicked by the Ha-, N-Ras double knockout mice, which, similar to the single knockouts, were viable (22–26). Furthermore, over-expression experiments suggest that different Ras isoforms might have preferential targets (27, 28). We have shown in mouse fibroblasts that Raf-1 is the preferential binding partner for plasma membrane-associated c-N-Ras (29, 30). Using immortalized fibroblasts derived from the N-Ras knockout mice, we demonstrated that c-N-Ras provides a steady-state antiapoptotic function (31). The N-Ras and K-Ras knockout fibroblasts provide excellent tools to study the role the K- and N-Ras isoforms in regulating the expression of MMP-2.

MATERIALS AND METHODS

Reagents—Gelatin was obtained from Sigma. MMP-2 cDNA probe was obtained from American Type Culture Collection. PD098059,
SB203580, LY294002, genistein, PD153035, and the AKT-specific inhibitor were obtained from Calbiochem. Anti-HSP90 monoclonal antibody was obtained from BD Transduction Laboratories. Anti-AKT1 antibody was obtained from Cell Signaling Technology, Inc. The polyclonal antibody against K(R)-Ras, PS5, and the monoclonal antibodies against N-Ras and Ha-Ras were obtained from Santa Cruz Biotechnology, Inc.

Cell Culture—All cell lines were maintained in Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum. For the preparation of conditioned medium for zymography, cells (at ~80% confluence) were washed three times with serum-free medium. Cells were then cultured for an additional 18 h in serum-free medium before collection of the conditioned medium. For MMP assays, when using pharmacological inhibitors, cells were cultured for 24 h in the presence of the inhibitor, washed, and then placed in serum-free medium containing the same concentration of the specific inhibitor for an additional 18 h.

Preparation of Cell Lysates—All lysis buffers contained the following phosphate buffer and protease inhibitors: 30 mM β-glycerophosphate, 5 mM N-tosylphenyl phosphate, 1 mM phosphoserine, 1 mM phosphothreonine, 0.2 mM phosphothreonine, 100 mM sodium orthovanadate, 25 μg/ml each aprotinin, leupeptin, pepstatin A, and 1 mM phenylmethylsulfonyl fluoride. Cells were scraped into phosphate-buffered saline and pelleted by centrifugation (500 × g for 5 min). The cell pellet was resuspended in p21 buffer (20 mM MOPS, 5 mM MgCl2, 0.1 mM EDTA, 200 mM sucrose, pH 7.4) containing 1% CHAPS (United States Biochemical Corp.). The cells were lysed for 20 min on ice and centrifuged at 13,000 × g for 10 min to remove nuclei and cell debris. The supernatant solution was retained for further experiments. The protein concentration was determined by the method of Bradford (39).

Zymography of MMP in Conditioned Medium—Conditioned, serum-free medium was prepared as described above. The medium was centrifuged at 15,000 × g to remove cellular debris. The amount of conditioned medium was normalized to the amount of protein in the corresponding parallel cell lysates (prepared as described above). Non-reducing sample buffer was added to the appropriate amount of conditioned medium and the samples were separated by SDSPAGE using gels containing 1% gelatin in the separating gel. The gels were electrophoresed at 25 mA until the bromphenol blue dye front reached the bottom. The gels were rinsed three times for 10 min each with 50 mM sodium acetate. MMP activity was observed by the generation of a negative clear band in the blue-gelatin staining background. The gels were rinsed and stained with 0.2% Coomassie blue in 50% methanol and 10% acetic acid. The gels were electrophoresed at 25 mA until the bromphenol blue dye front reached the bottom.

Steady-state Expression of MMP-2 in Mouse Fibroblasts—We first tested whether the production of MMP-2 maintains its tissue specificity in cultured cells. Conditioned medium from each cell line was analyzed either by gelatin zymography (Fig. 1A, top) or by immunoblotting the conditioned medium for MMP-2 (Fig. 1A, bottom). Both murine fibroblast cell lines, NIH3T3 and C3H10T1/2, secrete easily detectable amounts of MMP-2. Epithelial cells (IEC-6 and COS-7), however, did not secrete detectable levels of MMP-2 (Fig. 2A). We next tested whether the generation of MMP-2 was a constitutive, steady-state phenomenon or might be regulated through short-term signal transduction mechanisms. In Fig. 1B, the expression of MMP-2 activity in conditioned medium was monitored through a serum-deprivation time course. Cells were serum-starved for a 24-hour period. After this initial starvation period, cells were incubated with fresh serum-free medium. Aliquots of this medium were taken every 6 h and analyzed for the presence of MMP-2 by zymography. The data suggest that MMP-2 production continues at a linear rate (Fig. 1B, right), even after this prolonged period of serum withdrawal. These data suggest that the generation of MMP-2 in the extracellular medium is not regulated through a serum-dependent mechanism.

Reverse Transcription-PCR—1 μg of total RNA from K+C, K+13, K-2B, and K-6 were used for reverse transcription with Roche TagMan reverse transcription reagents (poly-dT primer). Reverse transcription was performed at 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min. PCR were performed with Roche High-Fi polymerase. The denaturation temperature was 94 °C, annealing temperature was 60 °C, and elongation temperature was 72 °C. The oligonucleotide primers were synthesized by Sigma. The sequences of primers were: gtc ggg cgc tgt gtt cgg ggt (MMP-2 sense primer), tca gac gcg cag cag gtc gtc (MMP-2 antisense primer), tcg ggg tga gag att tgg cgg (glyceraldehyde-3-phosphate dehydrogenase sense primer), and tcg ggg tga gag att tgg cgg (glyceraldehyde-3-phosphate dehydrogenase antisense primer). Immuno blotting—Lysates containing equal amounts of protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Hybond P; Amersham Biosciences). The membrane was blocked with blocker casein in phosphate-buffered saline (Pierce Chemical Co.) containing 10% newborn calf serum (Invitrogen). The washed blots were incubated with primary antibodies (1:1000) for 2 h at room temperature or overnight at 4 °C. After washing in Tris-buffered saline/0.1% Tween 20, the blots were incubated with secondary antibodies (1:1000) for 1 h at room temperature. After washing, the signals were detected using standard enhanced chemiluminescence techniques. The signals were quantitated using a Microtek scanner and NIH Image software, version 1.60 or 1.607, and normalized to untreated controls.

Luciferase Reporter Assay—1 × 105 cells per well were seeded into 12-well dishes. The MMP-2 promoter luciferase reporter and pRL-CMV were transiently introduced into fibroblasts in combination with other indicated constructs using LipofectAMINEPlus Reagent (Invitrogen).

The amount of total DNA was kept constant using the appropriate empty vectors. The luciferase activity of the MMP-2 reporter was analyzed with the dual-luciferase reporter assay system (Promega) and normalized to the activity of the Renilla reniformis luciferase. The basal level of luciferase activity was analyzed by transfection with pGL2-Basic plasmid and pRL-CMV. The relative luciferase activity is reported as the fold increase of the MMP-2 reporter luciferase activity compared with the basal level of luciferase activity.

RESULTS

K-Ras Knockout Fibroblasts Fail to Secret MMP-2—The constitutive production of MMP-2 by mouse fibroblasts allowed us to use N-Ras and K-Ras negative cell lines to examine the role of each Ras isoform in the maintenance of MMP-2 synthesis and secretion. To avoid potential artifacts arising from cell line divergence, we generated multiple immortalized cell lines from the K-Ras knockout and control MEF populations. These were represented by K-1C, K-2B, K-3, K-4, and K-6 for the K-Ras negative cell lines and K+C, K+13, K-2B, and K+14 for the control cell lines. N−/− and N+/+ indicate, for these experiments, a single N-Ras negative and control immortalized fibroblast population. We verified, by immunoblotting, the presence and absence of the different Ras isoforms in this spectrum of cell lines (Fig. 2A). As expected, the K-Ras negative cell lines fail to express K(R)-Ras and K(A)-Ras, whereas the N-Ras negative cells express both K(B) and K(A)-Ras but not N-Ras. There was no significant difference in the level of c-N-Ras expressed by the control K-Ras-expressing cells and the K-Ras negative cells (expressing 9 ng of c-N-Ras per 30 μg of cell lysate). Consistent with our previous experience using mouse fibroblasts, c-Ha-Ras was not detected. In this case, a lysate generated from C3H10T1/2 cells transformed by the minimal expression of an oncogenic G12V-Ha-Ras (11A) and PC12 cell lysate were used as positive controls for Ha-Ras expression. As has been our experience with cultured cell lines, c-Ha-Ras is
only detectable in cell lines of neuronal origin (with a 2-ng sensitivity).

The five K-Ras negative fibroblast cell lines did not possess detectable MMP-2 activity. In contrast, all the wild-type, control fibroblast cell lines expressed MMP-2 activity comparable with that of other mouse fibroblast cell lines (Fig. 2B, top). Both N-Ras knockout and wild-type, N+/+, control fibroblasts expressed levels of MMP-2 activity consistent with that observed for both the NIH3T3 and C3H10T1/2 cell lines. These data were confirmed by Western analysis of the conditioned media (Fig. 2B, bottom). These data indicate that the steady-state level of MMP-2 in mouse fibroblasts requires the function(s) of a c-K-Ras gene product rather than that of c-N-Ras. The failure to detect c-Ha-Ras protein in any of these cell lines rules out its involvement in the regulation of steady-state MMP-2 expression.

K-Ras Regulates MMP-2 Levels through a Transcriptional Mechanism—We first tested whether the K-Ras negative cells were producing MMP-2 but failing to secrete the protein into the extracellular milieu. Similar to our observations with conditioned medium, whole-cell extracts generated from the K-Ras knockout fibroblasts possessed significantly less (an 85% decrease) MMP-2 protein compared with control cells (Fig. 3A). MMP-2 mRNA, as detected by reverse transcription-PCR analysis, suggested that K-Ras regulated the steady-state transcription of the MMP-2 gene (Fig. 3B). In contrast, there was a lack of MMP-2 mRNA amplification in the K-Ras negative cell lines suggesting the absence of MMP-2 mRNA. The 519-bp glyceraldehyde-3-phosphate dehydrogenase fragment was amplified in all samples as a loading control. Similar results were obtained by Northern analysis in that we did not detect significant levels of MMP-2 mRNA in the K-Ras-negative cell lines. MMP-2 mRNA was detected in both K+/+ cell lines and the positive control, HT1080, a human fibrosarcoma transformed by an activated N-Ras allele (data not shown). We confirmed this observation using a transient, luciferase reporter assay (Fig. 3C). MMP-2 transcription levels from K-Ras knockout and wild-type fibroblasts were measured by transfection of a rat MMP-2 promoter luciferase reporter construct and pRL-CMV. The luciferase activity of the MMP-2 reporter construct was analyzed with the dual-luciferase reporter assay system (Promega) and normalized to the activity of R. reniformis luciferase. The relative luciferase activity indicates the fold increase of MMP-2 reporter luciferase activity relative to the basal level luciferase activity. Only minimal reporter activity was detected in the K-Ras negative cell lines, whereas 10–30-fold higher reporter activity was detected in the K-Ras-expressing control fibroblasts. These data are consistent with the hypothesis that K-Ras function is required for the transcription of the MMP-2 gene.

Fig. 1. Fibroblasts constitutively secrete MMP-2. A, subconfluent cells were exhaustively washed with serum-free medium and cultured in serum-free medium overnight. Conditioned medium from each cell line was analyzed either by gelatin zymography (top) or by immunoblotting for MMP-2 (bottom). The amount of conditioned medium was normalized to the protein content of the corresponding cell lysates. Top, the white unstained band is proportional to MMP activity. NIH3T3 and C3H10T1/2, murine fibroblasts; IEC-6, intestinal epithelial cells; VSMC, vascular smooth muscle cells; COS-7, monkey epithelial cells. These data are representative of two separate experiments. B, cells were switched to serum-free medium. 24 h later, cells were again switched to fresh serum-free media. At the indicated times, aliquots of conditioned medium (20 μl) were removed and analyzed for the presence of MMP-2 by zymography. The relative amounts of MMP-2 were quantitated (using NIH image) and are shown in the adjacent graphs. C, cells that had been serum-starved for 24 h (0) were challenged with 10% serum for the indicated times, either 5 min or 24 h. Once stimulated with serum for the indicated time intervals, the medium was removed, and the cells were washed in phosphate-buffered saline and incubated an additional 24 h in serum-free medium. Conditioned, serum-free medium (20 μl) was analyzed for MMP-2 and MMP-9 activity by zymography as described under “Materials and Methods.”
Ras Signaling Is Required for Transcription of MMP-2—To explore the possible role of Ras signaling in the regulation of steady-state MMP-2 expression, a dominant-negative Ha-Ras-N17 vector was co-transfected with the previously described Rat MMP-2 promoter luciferase reporter construct. Ras-N-17 blocks Ras activity by tying up Ras exchange factors into a non-productive cycle (32). The luciferase activity of the MMP-2 reporter construct was analyzed as described in Fig. 3C. Expression of the dominant-negative Ha-Ras-N17 inhibited MMP-2 promoter activity by \( \frac{80}{100} \) in the K-Ras-expressing control fibroblasts (Fig. 4A). We next used expression plasmids encoding the separate K-Ras isoforms in an attempt to modulate MMP-2 reporter activity (Fig. 4B). Only c-K(B)-Ras restored MMP-2 reporter activity in the transient transfection assay described in Fig. 4A (\( p < 0.05 \)). In this instance, we used phorbol 12-myristate 13-acetate addition as a positive control. In separate experiments, we found that phorbol ester addition increased the basal expression of MMP-2 protein by 2-fold (data not shown). Therefore, the level of transcriptional activation by K(B)-Ras, though only \( 50\% \) above the vector control, is needed for the regulation of MMP-2 expression.
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consistent with the level of transcriptional activation by the positive control. Combining both K(A)- and K(B)-Ras encoding plasmids did not give rise to a synergistic activation of MMP-2 luciferase activity. These results suggest that constitutive expression of MMP-2 is controlled through the function(s) of only c-K(B)-Ras and not c-K(A) or c-N-Ras.

A series of pharmacological inhibitors were used to test the potential contribution of one or more Ras-dependent signaling pathways that might regulate the steady-state transcription of the MMP-2 gene. Secretion of MMP-2 into the medium of K-Ras-expressing control fibroblasts was analyzed by immunoblotting for MMP-2 after a 48-h treatment with one of the following signaling inhibitors: PD098059 (mitogen-activated protein kinase kinase-specific inhibitor, 37 μM), SB203580 (p38-specific inhibitor, 10 μM), LY294002 (PI-3 kinase-specific inhibitor, 20 μM), genistein (tyrosine kinase inhibitor, 100 μM) or PD153035 (epidermal growth factor receptor tyrosine kinase inhibitor, 10 μM). LY294002 treatment dramatically inhibited the secretion of MMP-2 in wild-type fibroblasts (Fig. 5A). There was also a less pronounced, but reproducible, effect using the general tyrosine kinase inhibitor genestein. Others have also reported the inhibition of MMP-2 expression by genestein (34).

The PI-3 kinase-dependent, constitutive expression of MMP-2 was mimicked upon examination of the protein levels in whole-cell lysates (Fig. 5B). The specific PI-3 kinase inhibitor LY294002 was effective in blocking the steady-state accumulation of MMP-2 in whole-cell lysates, again suggesting that the expression of MMP-2 is regulated through a K-Ras-dependent pathway that flows through PI-3 kinase. We used overexpression of the regulatory p85 subunit of PI-3 kinase as a dominant negative to block basal signaling through receptor-mediated tyrosine kinases. Overexpression of the p85 regulatory subunit inhibited MMP-2 promoter activity in both K-Ras-expressing control cell lines (Fig. 5C). The K+C control cell line was more susceptible to this inhibition, which is consistent with this cell line’s increased sensitivity of MMP-2 promoter activity to the tyrosine kinase inhibitor, genestein (Fig. 5B). These data all support the hypothesis that constitutive MMP-2 expression is regulated through a steady-state signaling event passing through c-K(B)-Ras and PI-3 kinase.

The lipid products of PI-3 kinase activity result in the activation of a downstream serine kinase, AKT. Using a similar strategy, we examined the levels of MMP-2 secretion in cells that were wild-type, heterozygous, or homozygous knockouts for AKT1. In complete agreement with our previous observations and the documented relationship between PI-3 kinase lipid products and AKT activation, there was a significant decrease in constitutive MMP-2 expression in the AKT1-negative MEF populations (Fig. 6A, top). It is interesting that the decreased MMP-2 expression was similar between the AKT1+/− and the AKT1+/− MEFs, suggesting a possible threshold effect for AKT expression. We have observed a similar phenomenon in N-Ras heterozygous MEFs in their heightened response to apoptotic agents; N-Ras+/− cells responded in a manner more similar to the N−/− cells than the N+/− cells. Western analysis of AKT1 verified that the AKT1+/− cells expressed AKT1 at about 50% of the level observed in the AKT1+/+ MEFs. To verify the threshold nature of the MMP-2 expression of AKT levels, we quantitated the amount of AKT in the different MEF populations (Fig. 6A, bottom). To confirm the role of AKT signaling in mediating MMP-2 expression, we used an AKT-specific inhibitor (20 μM) to block AKT-dependent gene regulation.2

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expression in control K-Ras-expressing fibroblasts. Similar reductions in the steady-state levels of MMP-2 in whole-cell lysates were observed in the presence of either the PI-3 kinase inhibitor LY294002 or the specific AKT inhibitor (20 μM). The level of MMP-2 expression in the whole-cell lysates was determined by SDS-PAGE and immunoblot analysis. The corresponding levels of 90-kDa heat-shock protein (HSP90) expression were used as loading controls. These data are representative of at least three separate determinations.

expression in control K-Ras-expressing fibroblasts. Similar reductions in the steady-state levels of MMP-2 in whole-cell lysates were observed in the presence of either the PI-3 kinase inhibitor LY294002 or the specific AKT inhibitor (Fig. 6B). These data support the hypothesis that constitutive MMP-2 expression is regulated through the steady-state functions of c-K(B)-Ras, PI-3 kinase and AKT.

fibroblasts were treated with the indicated signaling inhibitors: Me2SO (control), PD098059 (mitogen-activated protein kinase kinase-specific inhibitor, 37 μM), SB203580 (p38-specific inhibitor, 10 μM), LY294002 (PI-3 kinase-specific inhibitor, 20 μM), genistein (tyrosine kinase inhibitor, 100 μM), or PD153035 (epidermal growth factor receptor tyrosine kinase inhibitor, 10 μM) in complete Dulbecco’s modified Eagle’s medium containing 10% serum for 24 h. The medium was then replaced with serum-free medium containing the indicated inhibitors. The conditioned medium was harvested and normalized according to the protein amounts of the corresponding whole-cell lysates. MMP-2 was analyzed by immunoblotting as described previously. These data are representative of at least three separate determinations. B, cells were untreated or treated with LY294002 (20 μM) as described in (A). Equal amounts of whole-cell lysates were analyzed by SDS-PAGE and immunoblot analysis for MMP-2 expression. Levels of the 90-kDa heat-shock protein (HSP90) were used to demonstrate equal protein loading. These data are representative of three separate determinations. C, the regulatory subunit of PI-3 kinase, p85, was transiently overexpressed in the K-Ras-expressing control cell lines along with the MMP-2 luciferase reporter construct and components of the dual luciferase reporter system. The transient overexpression of the p85 subunit was detected by immunoblotting (IB) with p85-specific antibody. The level of MMP-2 reporter activity was assessed as described previously. The data are averages of triplicate determinations. The error bars represent the standard deviation of the mean.
DISCUSSION

The data presented in this manuscript support the newly established consensus that the different immediate Ras isoforms do not have completely overlapping functions (20, 21). Several groups have shown that the Ras isoforms possess different potencies in their respective abilities to activate Raf-1 and PI-3 kinase (27, 28). We have also shown, in mouse fibroblasts, that plasma membrane associated c-N-Ras is the preferential binding partner for Raf-1 (29, 30). Here, using immortalized N-Ras and K-Ras knockout fibroblasts, we have shown that, in the absence of short-term mitogenic stimulation, the expression of MMP-2 is regulated through a c-K(B)-Ras dependent process. Every K-Ras-negative cell line tested was devoid of MMP-2 secretion, whereas the N-Ras-negative cells expressed levels of MMP-2 consistent with that observed in control fibroblasts. The decreased expression of MMP-2 in K-Ras-negative cells was mimicked by incubation of control cells with either a PI-3 kinase or AKT inhibitor, by expression of a dominant-negative PI-3 kinase, or by the failure to express AKT-1, suggesting the involvement of these signaling intermediates in the regulation of MMP-2 levels. Indeed, cells failing to express AKT1 were also deficient in the expression of MMP-2. The data presented in this manuscript are the first to document a specific signaling pathway endpoint regulated solely by K(B)-Ras. It is likely that other Ras isoforms regulate PI-3 kinase and AKT. In previous work, we have shown that the N-Ras-negative cell lines also have depressed steady-state levels of activated AKT (31, 35), although this does not apparently affect their ability to regulate MMP-2 expression. The mechanism that renders the PI-3 kinase/AKT-dependent regulation of MMP-2 expression specific for K(B)-Ras is unclear. It is possible that the subcellular localization of the specific K(B)-Ras, PI-3 kinase and AKT molecules that function to control the MMP-2 promoter significantly contribute toward specificity between c-K(B)-Ras, AKT and MMP-2 expression. Based on previous results and those reported in this communication, regulation of a specific downstream target is not necessarily sufficient to obtain an identical phenotype. Both c-N-Ras and c-K(B)-Ras can regulate steady-state AKT activity, but only c-K(B)-Ras does so in a manner that results in the corresponding regulation of MMP-2 expression. These subtle differences highlight the importance of performing experiments at endogenous levels of protein expression to avoid upsetting the delicate balance between these unique steady-state signaling pathways.

Signal transduction has traditionally been examined by short-term stimulation of cells with agents that induce mitogenesis, differentiation, or apoptosis (36–38). We have found, however, that maintenance of basal cell function also requires “steady-state” signal transduction. In this specific instance, the steady-state production of a single matrix metalloproteinase, MMP-2, is regulated through the steady-state functions of c-K(B)-Ras, PI-3 kinase, and AKT. Our previous work using N-Ras-negative cells suggested that short-term signaling pathways readily adapt to the absence of essential signal transduction components (31, 35). In contrast, we have found that cellular steady-state functions do not adapt as rapidly. Using immortalized N-Ras- and K-Ras-negative cell lines, we have identified a novel steady-state function for c-K(B)-Ras. The K-Ras-negative cell lines expressed detectable levels of only c-N-Ras. Consistent with our previous observations, we have not detected detectable levels of c-Ha-Ras in mouse fibroblasts, even as a compensatory mechanism for the loss of either the N-Ras or K-Ras genes. The K-Ras-negative cells failed to synthesize detectable levels of MMP-2, one of the matrix metalloproteinases involved in cell detachment, migration, and metastasis of invasive tumors. Failure to express the c-N-Ras gene product did not alter the levels of MMP-2 generation, suggesting a direct relationship between one of the K-Ras gene products and the steady-state production of MMP-2. Transient assays confirmed that only one of the K-Ras gene products, c-K(B)-Ras, is responsible for controlling the transcriptional activity of the MMP-2 gene. This observation is supported by the inhibition of basal MMP-2 production by transient expression of a dominant-negative Ras construct, N17-Ha-Ras, which functions through the sequestering of Ras exchange factors.

The identification of steady-state Ras dependent functions opens novel possibilities regarding the role of oncogenic Ras isoform in specific cancers. The common hypothesis of the involvement of Ras in the development of tumors has always stemmed from their role in short-term signal transduction. Given the data presented in this manuscript, we could now predict that tumors containing an activating K(B)-Ras rather than N-Ras should expressed elevated levels of MMP-2. This should result in a higher metastatic potential and probably a poorer overall prognosis. Therefore, although much of the transforming potential of each Ras isoform might arise from “continuous short-term signaling,” there is probably a secondary component that arises from changes in Ras isoform-dependent “steady-state” signal transduction that has been overlooked.

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