Filamin A-mediated Down-regulation of the Exchange Factor Ras-GRF1 Correlates with Decreased Matrix Metalloproteinase-9 Expression in Human Melanoma Cells

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The actin-binding protein filamin A (FLNa) is associated with diverse cellular processes such as cell motility and signaling through its scaffolding properties. Here we examine the effect of FLNa on the regulation of signaling pathways that control the expression of matrix metalloproteinases (MMPs). The lack of FLNa in human M2 melanoma cells was associated with constitutive and phorbol ester-induced expression and secretion of active MMP-9 in the absence of MMP-2 up-regulation. M2 cells displayed stronger MMP-9 production and activity than their M2A7 counterparts where FLNa had been stably reintroduced. Using an MMP-9 promoter construct (pMMP-9-Luc), in vitro kinase assays, and genetic and pharmacological approaches, we demonstrate that FLNa mediated transcriptional down-regulation of pMMP-9-Luc by suppressing the constitutive hyperactivity of the Ras/MAPK extracellular signal-regulated kinase (ERK) cascade. Experimental evidence indicated that this phenomenon was associated with destabilization and ubiquitylation of Ras-GRF1, a guanine nucleotide exchange factor that activates H-Ras by facilitating the release of GDP. Ectopic expression of Ras-GRF1 was accompanied by ERK activation and elevated levels of MMP-9 in M2A7 cells, whereas a catalytically inactive dominant negative Ras-GRF1, which prevented ERK activation, reduced MMP-9 expression in M2 cells. Our results indicate that expression of FLNa regulates constitutive activation of the Ras/ERK pathway partly through a Ras-GRF1 mechanism to modulate the production of MMP-9.

Matrix metalloproteinases (MMPs)5 play a crucial role in degradation of extracellular matrix (ECM) associated not only with normal growth and development but also with various pathological conditions such as tumor invasion and angiogenesis (1). Melanoma progression, as in many other cancers, is associated with invasion into surrounding tissues, which depends on MMP-mediated proteolytic degradation of the basement membrane and ECM. Among its members, the 72-kDa gelatinase A (MMP-2) and 92-kDa gelatinase B (MMP-9) are thought to be key enzymes for degrading type IV collagen, a major component of the basement membrane. Contributions of these enzymes in both physiological and pathophysiological processes such as tumor cell invasion and metastasis have been well documented (2–6). Although the recent work of Bartolome et al. (7) shows that MMP-2 is the most important contributor to melanoma cell invasion mediated by the chemokine CXCL12, others have found that MMP-9 is predominantly expressed by advanced stage melanoma cells and could have prognostic value in identifying patients at high risk of melanoma progression (8–11). The expression of MMP-9 can be induced by a variety of mitogens, including epidermal growth factor (EGF), tumor necrosis factor α and phorbol esters. There are a number of transcription factors that bind in the upstream regulatory region of the MMP-9 gene, which helps explain the multiple mechanisms of regulation of MMP-9 gene expression in tumor cell lines (12, 13). Stimulus-mediated activation of the extracellular signal-regulated kinase (ERK) pathway is required for expression of MMP-9, via the binding of AP-1 and ETS family of transcription factors to the human MMP-9 promoter (14–16). The binding site for NF-κB in the MMP-9 promoter is probably important for maximal induction of its transcription (12, 13, 17).

Filamin A (FLNa), a member of the non-muscle actin-binding protein family, is a widely expressed molecular scaffold that regulates signaling events involved in cell shape change and motility by interacting with integrins, transmembrane receptor complexes, adaptor molecules, and second messengers (18–20). Mutations in FLNa gene underlie a spectrum of human disorders, such as skeletal dysplasia and the localized cerebral cortical neuronal migration disorder known as periventricular nodular heterotopia (21, 22). In addition, the ability of tissue factor to promote vascular remodeling and tumor cell metastasis have been shown to be mediated by interaction with FLNa

NF-κB; Ras117, dominant-negative mutant of Ras; HA, hemagglutinin; WT, wild type.
(23), and remodeling of the cytoskeleton has been identified as having a role in cell migration and acquisition of invasive behavior (24, 25). Thus, the ability of FLNa to act as an integrator of cell mechanics (20) is of significant interest, and has led us to hypothesize that this scaffold protein provides a molecular pathway to support metastasis and motility through regulation of MMP expression in melanoma.

The present study was designed to determine the importance of FLNa in the regulation of MMP-2 and MMP-9 levels in the human melanoma M2 cell line. Using FLNa-deficient M2 cells and M2 cells stably expressing normal concentration of human FLNa (M2A7 cells) we found a significant reduction in MMP-9 secretion and activity upon FLNa expression, and have assessed here the intracellular mechanisms responsible. Our data indicate a dampening in the constitutive activation of the Raf-1/MEK/ERK cascade in M2A7 cells through a decrease in steady-state levels of GTP-bound Ras. A number of studies have been reported on the important role of guanine-nucleotide exchange factors (GEFs) in Ras activation (26, 27). FLNa interacts with Trio, a RhoGEF that controls RhoG/Rac1 GTPase function (28); however, it remains unclear whether FLNa can limit the GTP-bound Ras levels through down-regulation of GEFs that target Ras. In this study, we present evidence that expression of FLNa was associated with reduction of Ras-GRF1 levels through ubiquitylation-mediated proteolysis. Ectopic expression of Ras-GRF1 but not the ΔCdc25 mutant form of Ras-GRF1 was correlated with an increase in the Raf-1/ERK pathway to support metastasis and motility through regulation of cell mechanics (20) is of significant interest, and has led us to hypothesize that this scaffold protein provides a molecular pathway to support metastasis and motility through regulation of MMP expression in melanoma.

### EXPERIMENTAL PROCEDURES

**Materials**—All cell culture reagents were purchased from Invitrogen (Carlsbad, CA) and Cellgro (Herdon, VA), except fetal bovine serum (FBS), which was from Hyclone (Logan, UT). Phorbol 12-myristate 13-acetate (PMA), BMS345541, U0126, and manunycin A were purchased from CalBiochem-EMD Biosciences, Inc. (La Jolla, CA).

**Cell Culture**—Human M2 melanoma cells and FLNa-expressing M2A7 cell clone were originally described by Cunningham et al. (29). These cells were maintained in α-MEM medium supplemented with 10 mM HEPES (pH 7.4), 0.25% sodium bicarbonate, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 8% newborn calf serum, and 2% fetal bovine serum. Experiments were performed on passage 6–16 cells.

**DNA Constructs and Transfections**—The pREP4 expression vector containing the human wild-type FLNa construct (GenBank™/EBI Data Bank accession number NM_001456) was a gift from Y. Ohta (Harvard Medical School, Boston, MA). The pKH3 expression vector containing the mouse HA-tagged Ras-GRF1 and pCEFLAU5 vector expressing the rat ΔCdc25 mutant of Ras-GRF1 (truncation of the Cdc25 domain) were generously provided by P. Crespo (Instituto de Investigaciones Biomedicas, Universidad de Cantabria-CSIC, Santander, Spain) (26). The pUSEamp expression vector containing the dominant negative RasN17 mutant was purchased from Upstate Biotechnology Inc. (Lake Place, NY; 21-104). The pGL3-basic vector expressing wild type human MMP-9 promoter (~670/+54 fragment, GenBank™/EBI Data Bank accession number D10051) luciferase construct and the MMP-9 promoter constructs harboring point mutation in either the AP1 or NF-kB binding site fused to luciferase were generously provided by H. Sato (Cancer Research Institute, Kanazawa University, Kanazawa, Japan) (13).

M2 and M2A7 cells were plated in duplicates, and transfected with either pcDNA3.1 or FLNa construct at a ratio of 4 μg of plasmid/60-mm dish, using Lipofectamine2000 (Invitrogen). In other experiments, RasN17 (2 μg), HA-Ras-GRF1 (1–2 μg), or AU5-ΔCdc25 (2–4 μg) were transfected into M2 and M2A7 cells (35-mm² dishes) with MMP-9 luciferase (1 μg) and pSV-β-galactosidase (0.2 μg) constructs. At 30 h post-transfection, the cells were serum-starved for different times as described, rinsed in phosphate-buffered saline, and lysed in reporter lysis buffer (Promega, Inc., Madison, WI) for luciferase and β-galactosidase assays (kits from Promega) and Western blot analysis.

**Gelatin Zymography**—Growth-arrested M2 and M2A7 cells were incubated in the serum-free MEM for 24 h at 37 °C. Gelatinase activity in conditioned medium was measured by zymography. An equal amount of proteins from the concentrated conditioned medium (25 μl) was separated on a 10% Zymogram gel containing 0.1% gelatin, and the gel was incubated in the zymogram developing buffer (Invitrogen) for 16 h at 37 °C to evaluate activities of MMP-9 and MMP-2. The gel was stained with Coomassie Blue G-250 and then destained to reveal areas of gelatinolytic activity.

**Semiquantitative RT-PCR**—Cells were serum-starved for 24 h and then cellular RNA was extracted with TRIzol, followed by RT-PCR reaction. MMP-9 forward (5′-GATGCCGTGGAAGTGGGAAAT-3′) and reverse (5′-CACCACACTGATGAGTGCTGAAAT-3′) primers, MMP-2 forward (5′-ACAAGGGTGGCAGTCCTGAA-3′) and reverse (5′-CAGAGGAGAACCGTCATCCTC-3′) primers, and GAPDH forward (5′-GAAGGTGAAGTGGAGCTGTCGAGAT-3′) and reverse (5′-GAAGATCCGTGATGCAGGAT-3′) and reverse (5′-GAAGATCCGTGATGCAGGAT-3′) were synthesized by Integrated DNA Technologies, Inc. (Corvalle, IA). For all reactions, PCR conditions comprised 25 cycles of 94 °C (45 s), 50 °C (45 s) and 72 °C (45 s). PCR products were resolved by 2% agarose gel electrophoresis, and the relative intensity of MMP-2 (303 bp), MMP-9 (329 bp) and GAPDH (220 bp) bands was determined following ethidium bromide staining and quantitated using ImageQuant software (Amersham Biosciences Life Science). Samples were standardized for equal expression of GAPDH.

**Quantitative Real Time PCR**—cDNA were prepared from treated M2 and M2A7 cells, and the quantitative PCR analysis was performed by using TaqMan® Gene Expression Assay (Part 4324018) from Applied Biosystems (Foster City, CA). Primers for MMP-9 (catalog number Hs00234579), MMP-2 (catalog number Hs00234422), and reference gene GAPDH (catalog number Hs99999905) were predesigned by Applied Biosystems according to the sequences available from NCBI with the accession numbers NM_004994.2, NM_004530.2, and NM_002046.3, respectively. Each reaction was carried out in triplicate in the ABI 7300 Real Time PCR System. To deter-
mine the relative quantitation of gene expression, the comparative CT (threshold cycle) method was used (30) and normalized to the amount of GAPDH RNA present in each sample.

Assays—To evaluate the amount of MMP-9 secretion, the conditioned medium from M2 and M2A7 cells was concentrated and assayed for MMP-9 protein by ELISA, according to the manufacturer’s protocol (Calbiochem).

Ras activation assay was performed as followed: Briefly, M2 and M2A7 cells were rinsed in PBS and then serum-starved for 4 h before cell lysis. Ras activation was then determined using a RasGTPase Chemi ELISA kit, according to the manufacturer’s protocol (Active Motif, Carlsbad, CA). B-Raf and c-Raf kinase cascade assay kits were used to measure cellular Raf activities via phosphorylation of recombinant inactive MEK1 and its inactive substrate ERK2. Activation of ERK2 leads to phosphorylation of myelin basic protein as ERK substrate. In brief, after PBS washes, cells were lysed in ice-cold Tris lysis buffer (TLB: 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 25 mM β-glycerophosphate, 2 mM EDTA, 1 mM sodium orthovanadate, 2 mM Na2HPO4, 1% Triton X-100, 10% glycerol, 0.5 mM dithiothreitol, and protease inhibitor mixture set I). The cell lysates were clarified by centrifugation (14,000 g for 20 min at 4 °C), and protein content was measured using BCA protein assay kit (Pierce). 500 µg of soluble cellular extracts were immunoprecipitated with 2 µg of polyclonal anti-B-Raf (07-453) or c-Raf (07-396) antibody (Upstate Biotechnologies, Inc.) in the presence of protein A/G PLUS agarose beads (Calbiochem) for 2 h at 4 °C. Immunoprecipitates were extensively washed and resuspended in kinase assay buffer (20 mM HEPES (pH 7.4), 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol). In vitro kinase assays were then performed in two stages, according to the manufacturer’s protocol (Upstate Biotechnologies, Inc.).

Subcellular Fractionation—Serum-starved cells were left untreated or treated either with 20 mM EGF or 100 mM PMA for 10 min. The cells were rinsed twice in ice-cold phosphate-buffered saline and scraped in lysis buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 1 mM MgCl2, 10 mM NaF, 10 mM sodium pyrophosphate, 0.5 mM sodium orthovanadate, 2 mM EDTA, 1 mM dithiothreitol, and protease inhibitor mixture set I). The cells were homogenized by passing them five times through a 23-gauge needle on ice and centrifuged at 13,000 g for 5 min to sediment nuclei and unbroken cells. The clarified homogenate was then centrifuged at 100,000 × g for 60 min at 4 °C. The supernatant was removed and saved as cytosol, and the pelleted membranes were washed twice in lysis buffer and then resuspended in TLB buffer. Protein concentration for the cytosol, and membrane fractions was determined by the BCA method.

Immunoprecipitation and Western Blot Analysis—After cell lysis in immune precipitation buffer (25 mM HEPES, pH 7.4, 135 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 mM NaF, 1 mM sodium orthovanadate, 0.01% sodium azide, and protease inhibitor mixture set I), the clarified lysates (500 µg) were incubated with 2 µg of rabbit anti-Ras-GRF1, anti-SOS1 or anti-c-Myc antibodies for 16 h at 4 °C followed by the addition of 20 µl of protein A/G-agarose (Upstate Biotechnologies, Inc.) for 90 min at 4 °C. The immunocomplexes were washed two times with immune precipitation buffer, two times with 50 mM HEPES, pH 7.6, 0.1% Triton X-100 supplemented with 0.5 mM NaCl, and twice with 50 mM HEPES, pH 7.6, 0.1% Triton X-100. Total cell extracts and immunoprecipitated complexes were separated by 4–12% gradient SDS-PAGE, and then subjected to immunoblotting with specific primary antibodies. The signals were quantified using a chemiluminescence detection system (Amersham Biosciences).

The antibodies used in these studies included polyclonal antibodies that were raised against phospho-MEK1/2 (9121), phospho-ERK1/2 (7071), phospho-AKT (Ser473, 9271), phospho-IκBα (9241), total STAT3 (9132), MMP-9 (2270), and c-Fos (4384), and were purchased from Cell Signaling Technology (Beverly, MA). Monoclonal anti-ubiquitin (sc-8017) and anti-phospho-Jun (sc-822), and polyclonal antibodies directed against SOS1 (sc-256), Ras-GRF1 (sc-863), clathrin (sc-9069), ERK1/2 (sc-094), c-Src (sc-18), IKKα (sc-7607), and p89TFIIH (sc-293) were from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibodies against FLNa, panRas (OP40), and phospho-Raf-1 (Ser338, 05-538) were purchased from Research Diagnostics, Inc. (Flanders, NJ), Calbiochem and Upstate Biotechnology Inc., respectively.

RESULTS

FLNa Regulation of MMP-9 Expression

MMP-9 Secretion and Activity—In vivo, MMP-2 and -9 are two major gelatinases produced by human melanomas, and their proteolytic activity is thought to be necessary in both physiological and pathophysiological processes. Gelatin zymography was carried out to resolve the two closely related activities. The level of active MMP-9 was markedly higher in M2 cells when compared with M2A7 cells, both in the absence and the presence of PMA (Fig. 1A). Under these conditions, MMP-2 activity levels were found to be very low and unresponsive to PMA stimulation (Fig. 1A). To verify whether the presence of FLNa differentially affected MMP-9 and MMP-2 at the mRNA level, cells were serum-starved, lysed, and mRNA was analyzed by RT-PCR. The results showed that the MMP-9 PCR product was present in M2 cells, but not in M2A7 cells (Fig. 1B), consistent with the gelatin zymography data. In contrast, MMP-2 mRNA level was comparable in both cell lines following normalization with GADPH mRNA. Real time PCR analysis was also carried out and demonstrated a ~30-fold increase in MMP-9 mRNA levels in PMA-stimulated M2 cells as compared with unstimulated cells (Fig. 1C, filled bars). Pretreatment with the MEK inhibitor U0126 sharply reduced the PMA response. In contrast, no significant increase in the amount of MMP-9 mRNA was observed in M2A7 cells post-PMA treatment (Fig. 1C, open bars). MMP-2 mRNA levels were unresponsive to PMA stimulation in both M2 and M2A7 cells (data not shown). In keeping with these observations, all subsequent experiments were performed studying the regulation of MMP-9.

The differential ability of M2 and M2A7 cells at promoting MMP-9 expression and activity was associated with differences in MMP-9 secretion (Fig. 1D). Using an ELISA assay to measure the amount of MMP-9 released in the medium, we showed that control M2 cells secreted MMP-9 at a rate of 0.12 ± 0.05 ng/ml/24 h, while no response was observed with M2A7 cells. A significant increase in MMP-9 secretion of ~8-fold above the...
control was found upon a 24-h exposure of M2 cells to the phorbol ester PMA (100 nM) (Fig. 1D). In contrast, MMP-9 secretion was found to be below the detection limit following stimulation of M2A7 cells with PMA. These results were corroborated by gelatinase activity (Fig. 1A, lanes 3–6). As anticipated, pharmacological inhibition of the ERK/MAPK pathway with U0126 blocked the basal and PMA-induced release of MMP-9 in M2 cells (Fig. 1D). Taken together, our data suggest that FLNa is a negative regulator of MMP-9 expression.

**FLNa Attenuates the MMP-9 Promoter**—The human MMP-9 promoter (−670/+54) has been previously found to contain binding sites for AP-1, NF-κB and SP-1 proteins (13). In a first series of experiments, wild type and mutant forms of the promoter were used to delineate the gene elements needed for constitutive and PMA-induced MMP-9 gene expression in M2 cells (Fig. 2A). More than 3.8-fold induction of the wild-type MMP-9 promoter construct was observed in response to PMA. It is interesting to note that mutation of the AP-1-binding site markedly reduced promoter activity in control and PMA-stimulated cells, whereas inactivation of the NF-κB binding site elicited a ~40% decrease in the PMA response (Fig. 2A). We then addressed whether pharmacological inhibition of the MEK/ERK or IKK/NF-κB pathway might reduce MMP-9 reporter activity. At 36 h post-transfection, the M2 cells were either untreated or treated with U0126 or the IKK inhibitor BMS345541 for 1 h prior to the addition of 100 nm PMA for 7 h (Fig. 2B). While basal MMP-9 reporter activity was reduced by ~50% in cells treated with the MEK inhibitor U0126, PMA-mediated increases in pMMP-9-Luc activity felt sharply upon inhibition of either pathway as compared with control cells (Fig. 2B). These results are consistent with earlier findings suggesting that activation of the MMP-9 promoter requires synergistic cooperation between various cis-acting elements (12, 13).

Finally, a marked reduction in MMP-9 promoter activity was observed in FLNa-expressing M2A7 cells when compared with M2 cells treated in the absence or the presence of PMA (Fig. 2C). These results recapitulate the MMP-9 activity determined by gelatin zymography (Fig. 1A). Importantly, transient expression of FLNa in M2 cells elicited an increase in FLNa protein levels (Fig. 2C, upper panel), while inhibiting both the
basal and PMA-induced MMP-9 gene promoter construct activation (Fig. 2C, lower panel). There was markedly reduced expression of MMP-9 protein in response to PMA in M2 cells transfected with FLNa (Fig. 2D, lanes 2 and 4, top). Reprobing the blots demonstrated similar expression of ERK1/2 (Fig. 2D, bottom). Taken together, these results provide evidence that the differences in MMP-9 expression between M2 and M2A7 cells are not attributable to clonal variations, but instead suggest that FLNa inhibits the expression of MMP-9.

**FLNa Reduces the Constitutive Activation of the Raf-1/ERK Cascade**—To investigate the signaling pathways that are influenced by FLNa expression, we focused on the Raf-MEK-ERK cascade and the PI 3-kinase/AKT pathway. Total lysates from M2 and M2A7 cells were analyzed by SDS-PAGE and immunoblotted with antibodies against phosphorylated (e.g. activated) Raf-1 (pSer338), MEK1/2, ERK1/2, and AKT. M2 cells had relatively high levels of phosphorylated forms of Raf-1, MEK1/2, and ERK1/2 in basal conditions that were increased severalfold in the presence of PMA stimulation (Fig. 3A). The stable expression of FLNa in M2A7 cells was associated with a reduction in basal phosphorylation levels of these intermediates when compared with M2 cells (Fig. 3A, lane 3 versus 2). No significant difference in AKT phosphorylation was observed between M2 and M2A7 cells (data not shown). Interestingly, one of the AP-1 subunits, the immediate-early gene c-Fos, was detected in M2 cells under basal conditions, and a decrease in c-Fos migration occurred following 15 min of PMA treatment (Fig. 3A), which is consistent with its phosphorylation. There was little c-Fos expression, if any, in M2A7 cells exposed in the absence or presence of PMA, despite the presence of ERK activation upon PMA stimulation. Because of the important role for NF-κB in regulating melanoma cell migration, we measured also the levels of phosphorylated IκBα in lysates from M2 and M2A7 cells, and found measurable IκBα phosphorylation in unstimulated M2 cells (Fig. 3A, fifth panel). Moreover, short-term treatment with PMA led to significant increase in phospho-IκBα levels in M2A7 cells, although to levels that were lower to those observed in M2 cells. These results support earlier findings about enhanced NF-κB activation in melanoma by phorbol esters (31). Western blot analysis utilizing antibodies against c-Src and IKKα confirmed equal protein loading in each lane.

Stable expression of FLNa in M2A7 cells is associated with reduction in basal Raf-1/MEK/ERK cascade activation when compared with the parental FLNa-deficient M2 cells. We ascertained whether transient expression of FLNa would also target constitutive ERK activation in M2 cells. As shown in Fig. 3B, the phosphorylation of Raf-1 and ERK1/2 was markedly reduced upon 48 h-transfection with a FLNa-expressing vector, thus corroborating the results with MMP-9 promoter (Fig. 2C).

The constitutive ERK activation in several melanomas is mediated by B-Raf mutations and/or autocrine growth factor stimulation (32). To determine whether FLNa expression influences basal Raf activity, cellular B-Raf, and Raf-1 proteins were immunoprecipitated and their phosphotransferase activities measured in a kinase cascade reaction (Fig. 3C). The results showed a significant ~40% reduction (p < 0.001) in Raf-1 activity in FLNa-expressed M2A7 cells when compared with M2 cells. In contrast, B-Raf activity was not dependent on the expression of FLNa. Thus, FLNa correlated with a selective down-regulation of Raf-1 activity.

**p21ras Is Required for Constitutive Activation of ERK1/2 and MMP-9 Expression**—The recruitment of Raf-1 to the plasma membrane by GTP-bound Ras is required for its phosphorylation and activation. To determine whether Ras is involved in the differential regulation of Raf-1 between M2 and M2A7 cells, we performed Ras-GTPase activation assays. This assay relies on the binding of active GTP-bound Ras to the Ras binding domain of Raf-1 (33). Remarkably, quantitation of three independent experiments revealed that unstimulated M2 cells exhibited ~8-fold increase in active Ras when compared with M2A7 cells (Fig. 4A). Overexpression of a dominant-negative ras mutant (Ras$^{N17}$) was utilized to determine the relative contribution of Ras-dependent pathways to constitutive ERK1/2 activation and MMP-9 expression. Efficient transfection of M2 cells resulted in robust expression of Ras$^{N17}$ after 48 h (Fig. 4B). The efficacy of Ras$^{N17}$ overexpression was confirmed by marked inhibition of basal Raf-1-MEK-ERK1/2 activation. Under these conditions, MMP-9 promoter activity was significantly inhibited by Ras$^{N17}$ overexpression (Fig. 4C). To further corroborate these data, M2 cells were treated with manumycin A, a potent and specific cell-permeable farnesyltransferase inhibitor, which blocks the membrane localization and function of Ras (34). We found that basal activation of the Raf-1/ERK cascade (Fig. 4B) and MMP-9 promoter activity (Fig. 4C) was impaired after manumycin A treatment. The ability of Ras$^{N17}$ to regulate the levels of MMP-9 was next addressed.

**FIGURE 3.** The Raf-1/ERK pathway is constitutively active in FLNa-deficient M2 cells. A, serum-starved M2 and M2A7 cells were left untreated or treated with 100 nM PMA for 15 min. Western blot analysis was performed on extracts prepared from these cells, using phospho-Raf-1, phospho-MEK1/2, phospho-ERK1/2, c-Fos, phospho-IκBα, or FLNa antibodies. c-Src and IKKα are shown as loading controls. B, cells were transfected with pcDNA (lanes 1 and 2) or with a vector encoding FLNa (M2 cells only, lane 3). Cells were incubated in serum-free medium for 7 h and then processed for Western blot analysis with the FLNa, phospho-Raf-1, or phospho-ERK1/2 antibodies. p89TFIH is shown as a loading control. C, serum-starved M2 and M2A7 cells were lysed, and B-Raf (filled bars) or Raf-1 (open bars) protein was immunoprecipitated for analysis of its kinase activity as described under “Experimental Procedures.” The results represent the means ± S.E. of three independent experiments and normalized relative to M2 cells. p ≤ 0.01.
Control pcDNA- and RasN17-transfected M2 cells were incubated in the presence of vehicle or PMA for 16 h, followed by the concentration of the conditioned medium. A ~50% reduction in the MMP-9 levels was observed in the presence of RasN17 (Fig. 4D, lanes 1 and 4). Taken together, these data indicate that FLNa is implicated in the regulation of GTP-bound Ras levels and activation of Ras-dependent signaling.

Effects of FLNa on Ras-GRF1, an Upstream Regulator of Ras—Ras-GRF1 and SOS1 are two members of the guanine-nucleotide exchange factor (GEF) family that have an important role in signal transduction by increasing active Ras-GTP levels (27). Next we addressed whether Ras-GRF1 and SOS1 were differentially expressed in M2 and M2A7 cells. The full-length form of Ras-GRF1 (140 kDa) was remarkably absent; however, two truncated forms of this protein (~100 and 64 kDa) were detected utilizing a commercially available antibody developed against the protein C-terminal region (Fig. 5, A and B, second panels). The quantitation of several immunobots demonstrated similar levels of expression of SOS1 in the two cell lines, but showed a 50% reduction in the levels of the 100-kDa form of Ras-GRF1 in FLNa-expressing M2A7 cells relative to M2 cells (Fig. 5A). Note that the absence of the 64-kDa species correlated with sharp reduction in the levels of phosphorylated Raf-1 in M2A7 cells (Fig. 5A). In the presence of competing peptide, the signal associated with both Ras-GRF1 protein forms was eliminated (data not shown). These results are consistent with the existence of truncated versions of Ras-GRF1 lacking the N-terminal moiety (35, 36).

The ability of RasGEFs to stimulate GDP/GTP exchange on Ras and to activate ERK cascade requires their recruitment to cellular membranes. Therefore, it was important to determine whether FLNa might impair GEFs movement and thus reduce Ras activation. Following cell lysis in detergent-free buffer, cellular extracts were subjected to 100,000 × g centrifugation to resolve the crude membrane fraction from the cytosol. These experimental conditions allowed the detection of Rac mainly in the membrane fraction, whereas STAT3 was detected preferentially in the cytosolic fraction of unstimulated cells (Fig. 5B). While SOS1 and the 64-kDa Ras-GRF1 were largely found in the cytosolic fraction, the ~100-kDa Ras-GRF1 was distributed in the two compartments. In fact, there was reduced targeting of Ras-GRF1 to cellular membranes prepared from unstimulated M2A7 cells relative to M2 cells (Fig. 5B, lane 4 versus 2). These experiments were repeated at least two to three times with comparable results.

Unlike Ras-GRF1, the upstream connection of SOS1 involves receptor tyrosine kinases via the adaptor protein GRB2 (37, 38). To further define the effect of FLNa on membrane association of SOS1 and Ras-GRF1, M2, and M2A7 cells were first stimulated with EGF (Fig. 5C), and the membrane fraction was isolated. Western blot analysis was performed and the results demonstrated similar levels of membrane-associated SOS1 in unstimulated cells (Fig. 5C, top panel, lanes 2 and 3). Exposure to EGF elicited slower migration of SOS1 on SDS-PAGE gel (Fig. 5C, top panel, lanes 1 and 4 versus 2 and 3), which is consistent with phosphorylation (9, 19). There was a marked reduction in the ~100-kDa Ras-GRF1 level in M2A7 cell membranes as compared with that seen in membranes from M2 cells (Fig. 5C, second panel). At 10 min post-EGF treatment, Ras-GRF1 levels were not affected even though there was significant increase in phosphorylated (active) forms of Raf-1 and ERK1/2 in the membrane fractions of both cell lines (Fig. 5C, third and fourth panels). Of significance, a clear increase in Raf-1 and ERK1/2 phosphorylation levels was consistently seen in membranes from unstimulated M2 cells relative to M2A7 cells (Fig. 5C, lanes 2 versus 3). Similar expression of clathrin was observed in each lane (Fig. 5C, bottom panel). These experiments were repeated following cell treatment with PMA for 15 min. The results indicated that there was no detectable increase in membrane association of the ~100-kDa Ras-GRF1 following PMA stimulation as compared with that seen in the untreated cells (data not shown). Taken together, our results support the notion that the reductions in constitutive Ras/ERK cascade activation and MMP-9 promoter activity in M2A7 cells correlate with a decrease in the amount of membrane-associated Ras-GRF1 protein as compared with FLNa-deficient M2 cells.

A number of proteins, including tumor suppressors and transcriptional activators are processed and degraded via an
ubiquitin-mediated pathway (39, 40). We addressed whether Ras-GRF1 might be selectively targeted for ubiquitylation and subsequent proteolysis in M2A7 cells. To evaluate the constitutive ubiquitylation of Ras-GRF1, immunoprecipitation of endogenous Ras-GRF1 was performed, followed by Western blot analysis with the anti-ubiquitin antibody (Fig. 6A). The results showed significant levels of ubiquitylated Ras-GRF1 in M2A7 cells as compared with that seen in the parental M2 cells, even though more immunoprecipitated Ras-GRF1 was observed from the latter cell line (Fig. 6A). In a second experiment, SOS1 immunoprecipitates were also analyzed for the levels of ubiquitylated and total SOS1 (Fig. 6B). There were similar levels of SOS1 ubiquitylation in both cell lines under the conditions where Ras-GRF1 was selectively ubiquitylated in FLNa-expressing M2A7 cells (Fig. 6B, lanes 2 and 5 versus lanes 3 and 4). Ubiquitylated proteins were not detected when these extracts were immunoprecipitated with rabbit IgG (Fig. 6B, lane 1).

To address whether ectopically expressed Ras-GRF1 was also a target for ubiquitylation, M2A7 cells were transiently transfected with HA-tagged Ras-GRF1, and the extracts were immunoprecipitated using either anti-Ras-GRF1, which recognizes the C-terminal region, or anti-HA followed by Western blot analysis (Fig. 6C). There was marked ubiquitylation signal in the anti-Ras-GRF1 and anti-HA immunoprecipitates, which is consistent with efficient post-translational modification of HA-Ras-GRF1 in M2A7 cells.

Role of Ras-GRF1 in Constitutive Activation of ERK Signaling and MMP-9 Expression—The sequestration of upstream activators by ΔCdc25 mutant of Ras-GRF1 prevents endogenous Ras-GRF1 to efficiently activate its effector H-Ras (41). To address whether overexpression of ΔCdc25 altered constitutive MMP-9 promoter activation, the M2 cells were transiently transfected with ΔCdc25 together with the MMP-9 luciferase reporter and β-galactosidase expression vector. At 48-h post-transfection, the cells were serum-starved for 6 h prior to assaying luciferase activity (Fig. 7A). There was a dose-dependent reduction in MMP-9 gene reporter activity in ΔCdc25-transfected cells when compared with M2 cells transfected with control vector. Western blot analysis showed a clear loss in phosphorylation of ERK1/2 in the extracts prepared from the ΔCdc25-transfected cells (data not shown). Moreover, there was a 30–40% reduction in the levels of MMP-9 protein and activity in the conditioned medium prepared from ΔCdc25-transfected M2 cells treated with PMA for 16 h relative to pcDNA-transfected cells (Fig. 7B, lanes 1 and 4).

To corroborate the specific role of Ras-GRF1 in the regulation of MMP-9 expression, FLNa-expressing M2A7 cells were

FIGURE 5. Targeting of the exchange factor Ras-GRF1 to cellular membranes. A, following lysis of M2 and M2A7 cells, extracts were immunoblotted with antibodies raised against SOS1, Ras-GRF1, phospho-Raf1, and clathrin, which served as loading control. Similar results were obtained in at least three independent experiments. The signal associated with these proteins in M2A7 cells are expressed relative to M2 cells. B, cells were lysed and fractionated as described under “Experimental Procedures.” Extracts from the cytosol (C) and membrane (M) fractions were separated by SDS-PAGE and then Western blot analyses with the SOS1, Ras-GRF1, STAT3, or panRas antibodies were performed. Ras-GRF1 and SOS1 signals from M2A7 cell fractions were obtained after longer exposure of the blots to the ECL film. C, serum-starved M2 and M2A7 cells were either untreated (lanes 2 and 3) or treated with 20 ng/ml EGF for 10 min (lanes 1 and 4). The membrane fraction (100,000 × g) was prepared from each sample, and Western blot analysis was performed to analyze the levels of SOS1, Ras-GRF1, phospho-Raf1, and phospho-ERK1/2. The membrane was reprobed with anti-clathrin to show equal loading.

FIGURE 6. Stability and ubiquitination of Ras-GRF1 protein. A, serum-starved M2 and M2A7 cells were lysed and following immunoprecipitation (IP) of the extracts with a Ras-GRF1 antibody, SDS-PAGE, and Western blot analysis were then performed using the ubiquitin (Ub, top) or Ras-GRF1 (bottom) antibody. B, immunoprecipitation of the extracts was carried out either with rabbit IgG (lane 1), or a SOS1 (lanes 2 and 5) or Ras-GRF1 (lanes 3 and 4) antibody. Western blot analysis was performed using Ub and then SOS1 or Ras-GRF1 antibodies. C, M2A7 cells were transfected with a vector encoding HA-tagged Ras-GRF1. At 40 h post-transfection, the cells were serum-starved for 6 h and then harvested. Extracts prepared from these cells were immunoprecipitated with rabbit IgG (lane 1), Ras-GRF1 (lane 2), or HA (lane 3) antibody. Western blot analysis was performed with the indicated antibodies. The respective positions of C-terminal 66-kDa fragment and full-length HA-Ras-GRF1 species (arrow) are indicated. Grf-1-Ubn, ubiquitylated Ras-GRF1.
FLNa Regulation of MMP-9 Expression

Data presented here show that in M2 melanoma lacking FLNa, there is a significant increase in basal level Raf-1/ERK activation, which correlated with the expression and secretion of active MMP-9 under basal conditions. Increased expression of MMP-9 through up-regulation of AP-1 is thought to be necessary for the acquisition of invasive phenotype in many tumors, including melanoma (42). c-Jun and c-Fos are components of the AP-1 transcription factor complex, which are placed downstream of ERK signaling pathway (43). It is interesting to note that NF-κB and activation of AP-1 complex by phosphorylation are both induced in M2 cells and functional binding sites for these factors in the MMP-9 promoter are crucial for induction of transcription. Our findings indicate that FLNa functionally restricts transcriptional regulation of the MMP-9 promoter in the absence of stimuli in M2A7 cells and following ectopic expression of FLNa in M2 cells. The fact that PMA did cause a significant 5–6-fold increase in MMP-9 reporter activity over basal levels in these cells compared with 4-fold increase in parental M2 cells suggests that FLNa functions mainly in attenuating signaling events elicited under basal conditions. Consistent with these data, expression of FLNa resulted in the reduction in basal levels of downstream kinases MEK and ERK through the decrease in constitutively elevated Raf-1 activity, but not that of B-Raf. Because FLNa restricts the hyperactivity of the Raf-1/ERK pathway, we focused upon the role of FLNa on signaling events located upstream in the signal transduction cascade. M2 cells exhibit elevated levels of GTP-bound form of p21ras under basal

transiently transfected with HA-tagged Ras-GRF1 or ΔCdc25 along with the MMP-9 luciferase reporter and β-galactosidase expression vector (Fig. 7C). MMP-9 reporter activity was increased ~2-fold in cells transfected with HA-Ras-GRF1 (2 μg) as compared with cells transfected with empty vector. However, the constitutive increase in MMP-9 reporter activity was sharply reduced upon cotransfection with the ΔCdc25 mutant of Ras-GRF1 (Fig. 7C). Finally, we addressed whether ectopic expression of HA-Ras-GRF1 or ΔCdc25 altered the constitutive activation of Raf-1/ERK pathway in M2A7 cells. The extracts from this experiment were analyzed for the levels of phospho-Raf-1, phospho-ERK, phospho-Jun and total c-Fos (Fig. 7D). There was enhanced phosphorylation of Raf-1 and ERK1/2 following overexpression of Ras-GRF1 in M2A7 cells, which was abolished by coexpression of the RasN17 dominant negative mutant. Total c-Fos and phospho-Jun levels were also increased in Ras-GRF1-transfected cells, which were consistent with ERK1/2-mediated activation of the AP-1 transcription complex (Fig. 7D, panels 3 and 4). However, phosphorylation of these signaling intermediates was not detected upon transfection of M2A7 cells with ΔCdc25 (Fig. 7D, lane 2). A marked increase in MMP-9 levels was consistently seen in extracts prepared from Ras-GRF1-transfected cells as compared with that seen in the control pcDNA-treated M2A7 cells (Fig. 7D, panel 7, lanes 1 and 3). Coexpression of Ras-GRF1 with RasN17 resulted in ~80% reduction in MMP-9 levels, shown here to exist as a dimeric form (lanes 3 and 4). Western blot analysis demonstrated similar levels of FLNa (Fig. 7D, bottom). These results indicated that the association between Ras/ERK cascade activation and MMP-9 expression correlates with changes in Ras-GRF1 levels and/or its intrinsic GEF activity.

DISCUSSION

The data presented here show that in M2 melanoma lacking FLNa, there is a significant increase in basal level Raf-1/ERK activation, which correlated with the expression and secretion of active MMP-9 under basal conditions. Increased expression of MMP-9 through up-regulation of AP-1 is thought to be necessary for the acquisition of invasive phenotype in many tumors, including melanoma (42). c-Jun and c-Fos are components of the AP-1 transcription factor complex, which are placed downstream of ERK signaling pathway (43). It is interesting to note that NF-κB and activation of AP-1 complex by phosphorylation are both induced in M2 cells and functional binding sites for these factors in the MMP-9 promoter are crucial for induction of transcription. Our findings indicate that FLNa functionally restricts transcriptional regulation of the MMP-9 promoter in the absence of stimuli in M2A7 cells and following ectopic expression of FLNa in M2 cells. The fact that PMA did cause a significant 5–6-fold increase in MMP-9 reporter activity over basal levels in these cells compared with ~4-fold increase in parental M2 cells suggests that FLNa functions mainly in attenuating signaling events elicited under basal conditions. Consistent with these data, expression of FLNa resulted in the reduction in basal levels of downstream kinases MEK and ERK through the decrease in constitutively elevated Raf-1 activity, but not that of B-Raf. Because FLNa restricts the hyperactivity of the Raf-1/ERK pathway, we focused upon the role of FLNa on signaling events located upstream in the signal transduction cascade. M2 cells exhibit elevated levels of GTP-bound form of p21ras under basal
conditions, and its activity is significantly lower in FLNa-expressing M2A7 cells. The mechanism by which Ras activity is decreased in response to FLNa has not been totally elucidated. We showed that FLNa negatively correlates with the levels of Ras-GRF1, a signal transduction molecule located upstream of H-Ras. The fact that Ras-GRF1 catalyzes the GDP/GTP exchange needed for Ras activation (27) suggests that this GEF could modulate the activation of key components of the ERK signaling cascade in M2 cells in the absence of stimuli. However, there are alternative means of regulating Ras-Raf activity. For example, the GTPase-activating protein SynGAP has been reported to enhance Ras GTPase activity, which results in its inactivation (44). On the other hand, RIN1 acts as a negative regulator of Ras by competing with Raf-1 to bind to Ras (45). Interestingly, RasGAP interacts with muscle-specific filamin C to regulate Cdk7 and S6 ribosomal levels and myocyte growth (46). The scaffolding property of the C-terminal region of filamin (isoform A, B, or C) has been proposed to localize distinct downstream effectors and facilitate their functional communication. This would allow the recruitment and/or activation of negative regulators of Ras, thereby restricting p21\(^{ras}\) from inappropriately inducing Raf-1/ERK downstream signals in the absence of stimuli. However, to assess the impact of these and other negative regulators of Ras upon ERK activity and MMP-9 gene expression, the silencing of these genes will be required.

In this study, PMA was found to be ineffective at eliciting an increase in the endogenous levels of MMP-9 (mRNA and protein) in FLNa-expressing M2A7 cells, despite the fact that MEK activity (as measured by phosphorylated ERK levels) was induced to levels similar to those observed in PMA-treated M2 cells. Of significance, IKK activation (as measured by the extent of IκBα phosphorylation) and expression of c-Fos were significantly lower in M2A7 cells post-PMA treatment (Fig. 3A). It is unclear whether FLNa deregulates an effector pathway downstream of ERK activation. Moreover, FLNa could exert a substantial inhibition of ERK-independent pathway(s) that controls inducible MMP-9 expression. Ongoing efforts are to identify the mechanism of ERK sensitivity to FLNa, which may involve spatio-temporal redistribution of active ERK and other yet to be defined signaling intermediates.

To independently examine the requirement of p21\(^{ras}\) in MMP-9 transcriptional activation, two approaches were used. The results showed that pharmacological inhibition of farnesyltransferase with manumycin A or expression of RasN17 lessens the basal Raf-1/ERK cascade activation while mediating the transcriptional down-regulation of the MMP-9 reporter in M2 cells in the absence of stimuli. Because this down-regulation was only ~50%, our data suggest that Ras-independent signaling events are likely to play a role in the control of MMP-9 expression. For example, a number of studies report that other members of the MAPK signaling pathways such as p38 and the c-Jun N-terminal kinase (JNK) are involved in MMP-9 expression and activation in response to various stimuli (47–50). In addition, p38 and JNK are also upstream modulators of AP-1 transcriptional activity through phosphorylation (43). The combinatorial diversity of Jun and Fos proteins, that makes up the functional dimeric form of AP-1, and their association with other interacting factors (e.g. NF-κB, SMADs, CBP/p300) contributes to both basal and stimulus-activated gene expression (51). It is unclear which of the AP-1 family members are the most specifically affected by FLNa in human melanoma. Nevertheless, because of the hyperactivity of p21\(^{ras}\) in the parental M2 cells and the selective reduction of Ras-GRF1 levels in FLNa-expressing M2A7 cells, we chose to focus on the FLNa-dependent regulation of Ras-GRF1 expression and stability and on the role of Ras-GRF1 in the transcriptional regulation of the MMP-9 promoter.

The N-terminal-half of full-length 140-kDa Ras-GRF1 contains a pleckstrin homology domain, a coiled-coil region, an IQ motif, and a Dbl-homologous (DH) domain (52). In addition to these structural domains, Ras-GRF1 also contains a PEST-like region that is cleaved by the Ca\(^{2+}\)-dependent proteinase calpain to generate C-terminal fragments that appear to be more effective at GDP/GTP exchange of H-Ras than the full-length protein (53). The fact that calpain 3 was found as one of the genes most highly expressed in melanoma tissues (54) suggests that activation of intracellular proteinases may act on Ras-GRF1 to up-regulate the ERK pathway. Pharmacological inhibition of calpain has been reported to delay ERK activation and reduce cell growth and survival in melanoma cell lines (55, 56), while reducing leukemic cell invasiveness via decreased mRNA expression and secretion of MMP-9 (57). Furthermore, inhibition of calpain and cathepsin B provides a neuroprotective effect after focal cerebral ischemia in rats by inhibiting MMP-9 up-regulation (58). However, the precise molecular mechanisms of calpain in these processes are poorly understood. In our study, we show that parental M2 melanoma cells express two truncated forms of Ras-GRF1, which likely contain the C-terminal catalytic domain based on our Western blot analysis. The presence of a mixture of protease inhibitors during cell lysis, and the identification of two well-defined protein bands that were specifically competed by the immunogenic peptide antigen support the notion that multiple forms of Ras-GRF1 exist in melanoma and other cell types (35, 36). The presence of truncated forms of Ras-GRF1 may be biologically significant to malignant melanoma, as ectopic expression of N-terminally truncated versions of Ras-GRF1 results in transformation of NIH 3T3 cells (59). Thus, it is reasonable to speculate that the increase in catalytic function of these truncated forms of Ras-GRF1 contributes to the constitutive activation of the Ras/ERK signaling cascade and ultimately to greater MMP-9 production, at least in M2 cells.

Oncogenic B-Raf expression can vary significantly in different melanoma lines, thus resulting in marked difference in the degree and/or duration of ERK pathway activation (60). As a consequence, it is unlikely that a simple relationship can be defined between Ras-GRF1 expression and changes in the synthesis and secretion of MMP-9 in different melanoma lines. Interestingly, MMP-9 gelatinase activity depends not only on the amount of MMP-9 protein, but also the amount of co-localized tissue inhibitors of metalloproteinases (TIMPs) (61). Thus, constitutive and/or inducible activation of the RasGRF-1/ERK pathway may also affect expression of TIMP family members resulting in net increase in MMP-9 proteolytic activity. Such a query can only be resolved by further experiment.
Cell fractionation experiments revealed that p100-Ras-GRF1 is bound to a membrane fraction, indicating that ~1/3 of the N-terminal part of full-length Ras-GRF1 is not involved nor required for membrane localization. In contrast, p65-Ras-GRF1 does not attach to a 100,000 × g pellet after subcellular fractionation and immunoblotting, which let us to conclude that p65-RasGRF1 lacks the region required for its anchoring to membranes. The fact that the DH-PH2 module is present in p100-Ras-GRF1 but not in p65-Ras-GRF1 suggests that DH-PH2 probably acts as membrane targeting signal. Deletion of the DH domain has been reported to weaken localization of overexpressed Ras-GRF1 to the endoplasmic reticulum, thus resulting in impaired H-Ras activation (41). Moreover, Ras-GRF1 interaction with microtubules has been recently proposed to occur via the DH-PH2 module (62). The Cdc25p Ras exchange factor from Saccharomyces cerevisiae also contains a membrane-anchoring domain; however, it is located within the C-terminal region (63), and shares a significant homology only with human SOS2.6 Investigation exploring the interaction of p100-Ras-GRF1 with putative membrane-associated scaffolds is under way in our laboratory and might have therapeutic potential for controlling the growth and invasiveness of melanoma and other malignant tumors.

Another finding in our study is the decrease in the expression of the two C-terminal fragments of Ras-GRF1 in FLNa-expressing M2A7 cells. There are likely multiple mechanisms that can down-regulate Ras-GRF1 protein levels. In many instances, phosphorylation precedes polyubiquitylation and proteasomal degradation of proteins, such as with IκBα, an inhibitor of NF-κB nuclear translocation (64). Phosphorylation of Ras-GRF1 has been shown to occur on multiple sites, which could down-regulate its activity and/or stability (65–68). This concept is consistent with our data showing that FLNa may indirectly influence Ras-GRF1 levels, in part, via constitutive activation of protein kinases that phosphorylate Ras-GRF1, resulting in increased ubiquitylation and proteasomal degradation. Moreover, the effects of FLNa were relatively specific in that it did not alter the ubiquitylation and/or stability of SOS-1, a GEF reported to be involved in Ras activation in response to growth factor stimulation (69). Thus, in addition to Ras-GRF1, only a subset of GEFs that have been reported to activate Ras may potentially be targets of FLNa. Whether the up-regulation of basal level Ras/ERK activation and MMP-9 promoter activity stems from N-terminal truncation of HA-Ras-GRF1 remains to be elucidated. Expression of a dominant negative form of Ras blocks the hyperactivation of the Raf-1/ERK pathway induced by HA-Ras-GRF1, in agreement with our data with endogenous Ras-GRF1. These results suggest that multiple mechanisms, including post-translational modification such as ubiquitylation, are likely involved in the FLNa ability to both destabilize Ras-GRF1 protein and down-regulate Ras/ERK pathway necessary for expression of MMP-9 in melanoma.

Ras activates a number of downstream cellular activities, including invasion and metastasis. Whereas HA-Ras-GRF1 could elicit basal Raf-1/ERK cascade activation and MMP-9 promoter activity in FLNa-expressing M2A7 cells, ΔCdc25 mutant of Ras-GRF1 could not. Because ΔCdc25 mutant contains all the functional domains with the exception of the catalytic domain, these data suggest that the GEF-dependent induction of Ras-GTP levels by Ras-GRF1 is likely to play an important role in the regulation of signal transduction pathways leading to MMP-9 expression. However, it is possible that GEF activity-independent interactions of Ras-GRF1 with downstream effectors elicit biological responses that are yet to be discovered.

To conclude, we have identified Ras-GRF1 as a positive modulator of MMP-9 expression. In human melanoma, the loss of FLNa triggers Ras-GRF1-mediated hyperactivation of the Ras/Raf-1/MEK/ERK cascade to elicit constitutive and PMA-mediated increase in MMP-9. The involvement of filamin in carcinogenesis has been well documented. In particular, silencing of the FLNa gene was recently identified in human gastric cancers (70), and binding of FLNa to prostate-specific membrane antigen reduces its metalloproteinase activity within the prostate cancer cells (71). Moreover, the anticancer activity of 1α,25-dihydroxyvitamin D3 is associated with up-regulation of FLNa in human SW480-ADH colon cancer cells (72). It is therefore tempting to speculate that filamin deficiency could be involved in certain cancer phenotypes, at least in part, through abrogation of normal actin polymerization and impaired formation of protein scaffolds involved in proliferation and differentiation. We propose that FLNa mitigates the sustained activation of ERK cascade by promoting selective ubiquitylation and degradation of Ras-GRF1 and sharply reducing the deregulated signaling events that lead to MMP-9 expression.

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REFERENCES
1. Sternlicht, M. D., and Werb, Z. (2001) Annu. Rev. Cell Dev. Biol. 17, 463–516
2. Himelstein, B. P., Canete-Soler, R., Bernhard, E. J., Dilks, D. W., and Muschel, R. J. (1994) Invasion Metastasis 14, 246–258
3. Itoh, T., Tanioka, M., Matsuda, H., Nishimoto, H., Yoshioka, T., Suzuki, R., and Uehira, M. (1999) Clin. Exp. Metastasis 17, 177–181
4. Johansson, N., and Kahari, V. M. (2000) Histol. Histopathol. 15, 225–237
5. Liabakk, N. B., Talbot, I., Smith, R. A., Wilkinson, K., and Balkwill, F. (1996) Cancer Res. 56, 190–196
6. Parsons, S. L., Watson, S. A., Collins, H. M., Griffin, N. R., Clarke, P. A., and Steele, R. J. (1998) Br. J. Cancer 78, 1495–1502
7. Bartolome, R. A., Molina-Ortiz, I., Samaniego, R., Sanchez-Mateos, P., Bustelo, X. R., and Teixido, J. (2006) Cancer Res. 66, 248–258
8. Hofmann, U. B., Westphal, J. R., Van Muijen, G. N., and Ruiter, D. J. (2000) J. Investig. Dermatol. 115, 337–344
9. MacDougall, J. R., Bani, M. R., Lin, Y., Muschel, R. J., and Kerbel, R. S. (1999) Br. J. Cancer 80, 504–512
10. MacDougall, J. R., Bani, M. R., Lin, Y., Rak, J., and Kerbel, R. S. (1995) Cancer Res. 55, 4174–4181
11. Nikkola, J., Vihinen, P., Vuoristo, M. S., Kellokumpu-Lehtinen, P., Kahari, V. M., and Pyrhonen, S. (2005) Clin. Cancer Res. 11, 5158–5166
12. Ma, Z., Shah, R. C., Chang, M. J., and Benveniste, E. N. (2004) Mol. Cell. Biol. 24, 5496–5509
13. Sato, H., and Seiki, M. (1993) Oncogene 8, 395–405
14. Gum, R., Wang, H., Lengyl, E., Juarez, J., and Boyd, D. (1997) Oncogene 14825

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