Constitutively active Ras negatively regulates Erk MAP kinase through induction of MAP kinase phosphatase 3 (MKP3) in NIH3T3 cells

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The Ras/Raf/MEK/Erk signaling pathway is important for regulation of cell growth, proliferation, differentiation, survival, and apoptosis in response to a variety of extracellular stimuli. Lack of Erk MAPK activation is observed in several cancer cells despite active activation of Ras. However, little is known about the modulation of Erk1/2 activity by active Ras. Here, we show that overexpression of active H-Ras (H-RasG12R) in NIH3T3 fibroblasts impaired FGF2-induced Erk1/2 phosphorylation, as compared to wild-type cells. Northern blot analysis revealed that prolonged expression of active Ras increased MAP kinase phosphatase 3 (MKP3) mRNA expression, a negative regulator of Erk MAPK. Inhibition of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway abrogated active Ras-induced up-regulation of MKP3 expression, leading to the rescue of Erk1/2 phosphorylation. Our results demonstrated that the Ras/Raf/MEK/Erk signaling cascade is negatively regulated by the PI3K/Akt-dependent transcriptional activation of the *MKP3* gene. [BMB Reports 2014; 47(12): 685-690]

INTRODUCTION

Ras is a small GTP-binding protein that is involved in transmitting extracellular signals within cells. The Ras signaling pathway is important for regulation of cell growth, proliferation, differentiation, survival and apoptosis in response to a variety of extracellular stimuli (1-3). Three different Ras proteins are known: H-Ras, K-Ras, and N-Ras. Upon growth factor stimulation, Ras is activated through the nucleotide exchange of GDP for GTP by guanine-nucleotide-exchange factor son of seven less (SOS). Activated Ras stimulates Raf kinases, which leads to activation of MAPK/Erk kinase (MEK). MEK is a dual-specificity protein kinase that phosphorylates Erk1/2 (p42/p44 MAP kinase) on both threonine and tyrosine in a conserved Thr-X-Tyr motif (4, 5). Activated Erk phosphorylates several cytosolic signaling molecules, including p90 ribosomal S6 kinase (RSK), in the cytoplasm and translocates into the nucleus where it regulates many downstream targets, including the transcription factors Elk-1 and c-Jun, resulting in regulation of cell growth and differentiation. The qualitative and quantitative features of Erk1/2 activity determine the physiological response to any given mitogenic or stress stimulus (6). Sustained Erk phosphorylation by nerve growth factor (NGF) induces differentiation, while transient Erk phosphorylation by epidermal growth factor (EGF) stimulates cell proliferation in PC12 cells (7). Thus, Erk1/2 phosphorylation status is strictly regulated by several positive and negative regulatory loops (8).

Paradoxically, lack of Erk MAPK activation is observed in several cancer cells despite active activation of Ras (9-12). MAPK phosphatase (MKP) belongs to the dual-specificity phosphatase (DUSP) family that functions as a negative regulator of MAPK activity by dephosphorylating both the phosphothreonine and phosphotyrosine residues in the Thr-X-Tyr motif of MAPKs. The human genome contains around 30 DUSP genes, and the various members of the MKP family have distinct specificities for Erk1/2 in either the cytoplasm (e.g., PP2A, MKP3/DUSP6/Pyst1 and MKP-X/DUSP7/Pyst2) or the nucleus (e.g., MKP1/DUSP1, PAC-1/DUSP2, B23/hVH3/DUSP5, and MKP4/DUSP9/Pyst3) (13-16). In general, MKP3 is highly specific to cytoplasmic Erk1/2 and is involved in preventing nuclear translocation of Erk1/2 (17-19). In this study, we examined the molecular mechanism by which active Ras leads to decreased Erk1/2 activity by using NIH3T3 cells conditionally expressing active H-Ras under the control of tetracycline-inducible promoter. We found that prolonged expression of active Ras resulted in up-regulation of the *MKP3* gene via the PI3K/Akt pathway.

RESULTS AND DISCUSSION

We previously described a NIH3T3 cell line (NIH3T3tet-on/H-RasG12R), in which the expression of active Ras (H-RasG12R) can be induced by addition of doxycycline (20). In this model system, the active Ras protein was detectable 24 h after the addition of 2 μg/ml doxycycline (Fig. 1A). Despite the continual increase in the level of active Ras, the levels of Erk1/2 phosphorylation gradually decreased after peaking at 12 h after doxycycline addition. However, Akt and Raf1, an upstream kinase of Erk1/2, phosphorylation was still increased due to expression of active Ras. To determine whether growth factor stimulation of Erk1/2 could also be affected due to sustained activation of Ras, NIH3T3tet-on/H-RasG12R cells were cultured in medium containing 0.5% serum in both the absence and presence of doxycycline, and then stimulated with FGF2 for 30 min (Fig. 1B). The level of Erk1/2 phosphorylation was increased by FGF2 in both untreated and treated cells with doxycycline for

12 h, but not in cells treated with doxycycline for 48 h, although the basal Erk1/2 phosphorylation level was similar to that in cells treated with doxycycline for 12 h. Furthermore, the phosphorylation of Erk1/2 down-stream targets, such as p90 ribosomal S6 kinase (RSK; MAPKAPK1A) and transcription factor Elk-1, was not increased by FGF2 in cells treated with doxycycline for 48 h.

We next assessed the nuclear translocation of phosphorylated Erk1/2 following doxycycline treatment. Phopho-Erk1/2 immunoreactivity was highly enriched in the nucleus following FGF2 treatment in control cultures. In contrast, Erk1/2 phosphorylation was marked in the cytosol, but sparse in the nucleus of doxycycline-treated cells (Fig. 2). These results suggest that prolonged Ras activation impairs the nuclear levels of phosphorylated Erk1/2 in NIH3T3 cells.

MKP3 is a Thr/Tyr dual-specific phosphatase of Erk1/2 that prevents nuclear translocation of Erk1/2 (18). It has been reported that MKP3 is induced by Erk1/2 upon fibroblast growth factor 2 (FGF2) stimulation in NIH3T3 cells (21), suggesting that growth factor-induced Erk1/2 activity could be controlled by negative feedback loops via Erk1/2-specific MKP3 expression. To investigate whether sustained Ras activation impairs phosphorylated Erk1/2 levels in the nucleus through induction of MKP3, NIH3T3tet-on/H-RasG12R cells were exposed to doxycycline and *MKP3* mRNA levels were examined by Northern blot analysis. A time kinetic study showed that *MKP3* mRNA was detectable within 3 h after doxycycline addition (Fig. 3A). MKP3 protein was also increased by doxycycline treatment and
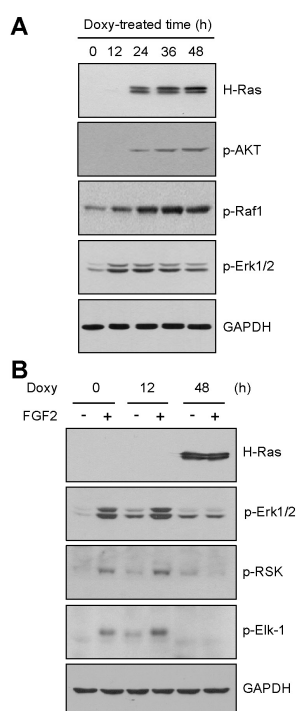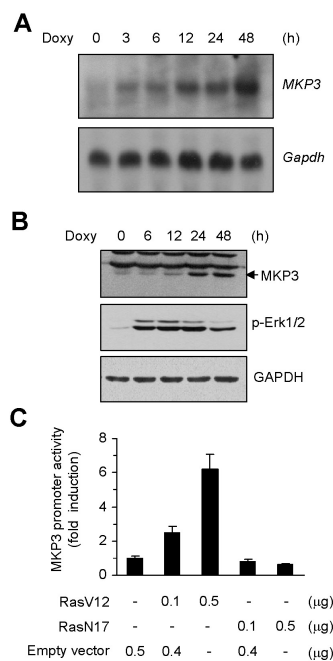

Fig. 1. Effect of sustained active Ras expression on Erk1/2 down-regulation. (A) NIH3T3tet-on/H-RasG12R cells were cultured in the presence of doxycycline (2 μg/ml) for the indicated periods of time and Western blot analysis was performed using antibodies against H-Ras, phospho-Raf1 (Ser259), phospho-Erk1/2 (Thr202/Tyr204), phospho-Akt (Ser473), and GAPDH (internal control). (B) NIH3T3tet-on/H-RasG12R cells were cultured with 0.5% serum for 24 h and then treated with doxycycline (2 μg/ml) for an additional 12 or 48 h, followed by treatment with FGF2 (50 ng/ml) for 30 min. Western blot analysis was performed using antibodies against H-Ras, phospho-Erk1/2 (Thr202/Tyr204), phospho-RSK (T573), phospho-Elk1 (Ser383), and GAPDH (internal control).
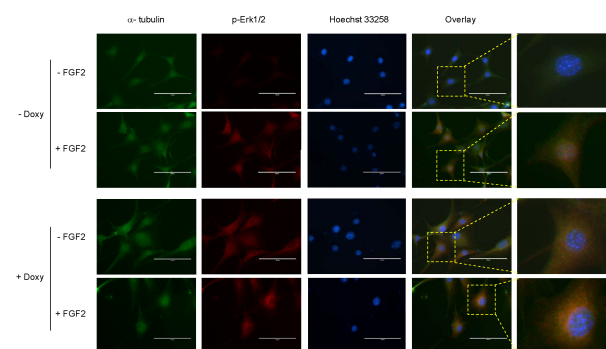
Fig. 2. Effect of sustained active Ras expression on the nuclear localization of phosphorylated Erk1/2. NIH3T3tet-on/H-RasG12R cells were cultured on glass slides and either left untreated (−Doxy) or treated (+Doxy) with doxycycline (2 μg/ml) for 24 h, followed by serum starvation with 0.5% serum for an additional 24 h in the absence or presence of doxycycline. After treatment with 50 ng/mL FGF2 for 30 min, cells were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100. The samples were incubated with primary antibodies against α-tubulin or phosphor-Erk1/2 (Thr 202/Tyr 204) for 90 min, followed by incubation with Alexa Fluor 555- (for α-tubulin) or Alexa Fluor 488-conjugated secondary antibody (for phosphor-Erk1/2) for 30 min. Nuclear DNA was stained with Hoechst 33258. Stained cells were immediately observed under an EVOSf1® fluorescence microscope (Advance Microscopy Group).
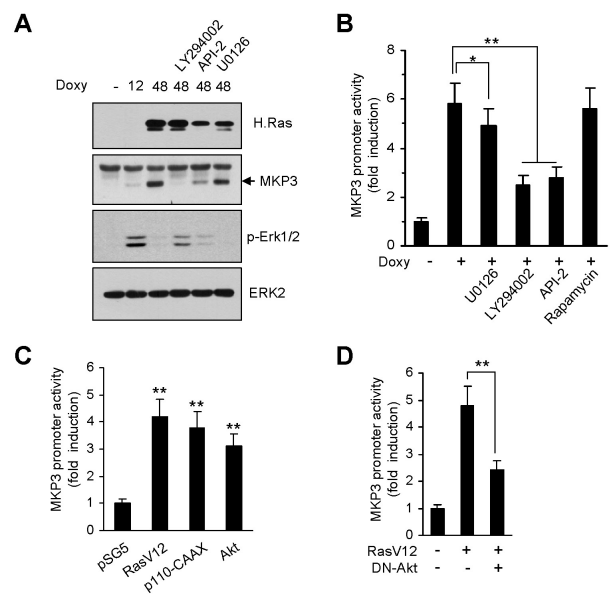
coincided with a reduction in Erk1/2 phosphorylation (Fig. 3B). To determine whether induction of *MKP3* mRNA by active Ras occurred at the transcriptional level, the 5'-regulatory region of the *MKP3* gene (nucleotides from −1597 to −10 relative to the transcription start site) was isolated and sub-cloned into a pGL3-Luc reporter vector. Transient co-transfection of active H-RasV12 and pMkp3-Luc(−1597/−10) into NIH3T3 cells led to a concentration-dependent increase in promoter reporter activity, while dominant-negative RasN17 had no effect (Fig. 3C). Thus, long-term activation of the Ras signaling pathway up-regulates MKP3 expression at the transcriptional level in NIH3T3 fibroblasts.

Active Ras can stimulate multiple effector pathways, including Raf and PI3K (22). Numerous reports have demonstrated that MKP3 expression is dependent on Erk1/2 activity in several cell types, including NIH3T3, human pancreatic cancer,

and non-small-cell lung cancer cells (21, 23-25). The PI3K pathway also inhibits Erk1/2 activity through up-regulation of MKP3 expression in response to FGF8 stimulation during limb development (26). These studies have suggested that multiple signaling pathways are involved in MKP3 expression. To determine the downstream signaling pathway of active Ras involved in MKP3 expression, we utilized pharmacological inhibitors. Treatment with either the PI3K inhibitor (LY294002) or the Akt inhibitor (API-2) blocked doxycycline-induced accumulation of MKP3, which led to the partial recovery of the phosphorylation status of Erk1/2 (Fig. 4A), suggesting the existence of an alternative mechanism. Interestingly, treatment with the MEK inhibitor (U0126) caused a dramatic reduction

Fig. 3. Up-regulation of MKP3 expression by active Ras expression. (A) NIH3T3tet-on/H-RasG12R cells were cultured and harvested at the indicated times after the addition of doxycycline (2 μg/ml). After total RNA was isolated, *MKP3* or *Gapdh* mRNA expression was determined using Northern blotting. (B) NIH3T3tet- on/H-RasG12R cells were cultured in the absence or presence of doxycycline (2 μg/ml) for the indicated periods of time, and Western blot analysis was performed using antibodies against MKP3, phosphor-Erk1/2 (Thr202/Tyr204), and GAPDH (internal control). (C) NIH3T3 cells were transiently co-transfected with 0.2 μg of the mouse MKP3 promoter reporter plasmids [pMkp3-Luc (−1597/−10)] and an expression plasmid for RasV12 or RasN17, as indicated, along with 50 ng of the Renilla luciferase expression plasmid (pRL-null/Luc) to normalize transfection efficiency. After 24 h, the cells were treated with doxycycline (2 μg/ml) for an additional 24 h. The cells were collected and assayed for luciferase activity.

Fig. 4. Role of PI3K signaling in active Ras-induced MKP3 expression. (A) NIH3T3tet-on/H-RasG12R cells were treated with doxycycline (2 μg/ml) for 12 or 48 h in the absence or presence of LY294002 (25 μM), API-2 (10 μM), or U0126 (10 μM). The cells were collected and Western blot analysis was performed using antibodies against H-Ras, MKP3, phosphor-Erk1/2 (Thr202/Tyr204) or Erk2 (internal control). (B) NIH3T3tet-on/H-RasG12R cells were transfected with 0.2 μg of pMkp3-Luc(−1597/−10) reporter. After 24 h, the cells were treated with doxycycline (2 μg/ml) for an additional 24 h in the absence or presence of U0126 (10 μM), LY294002 (25 μM), API-2 (10 μM), rapamycin (10 μM), or GF109203X (10 μM). The cells were collected and assayed for luciferase activity. Firefly luciferase activity was normalized to Renilla luciferase activity. The data shown represent the means ± SD of three independent experiments performed in triplicate (**P < 0.01). (C and D) NIH3T3 cells were co-transfected with 0.2 μg of pMkp3-Luc(−1597/−10) reporter and 0.2 μg of either empty vector (pSG5) or expression plasmid (pSG5/RasV12, pSG5/p110-CAAX, pSG5/Akt, or pSG5/DN-Akt), as indicated. After 24 h, the cells were collected and assayed for luciferase activity. Firefly luciferase activity was normalized to the Renilla luciferase activity. The data shown represent the means ± SD of three independent experiments performed in triplicate (**P < 0.01).

in the level of phosphorylated Erk1/2, but only slightly prevented MKP3 accumulation. Moreover, doxycycline-induced *MKP3* promoter activity was strongly inhibited by LY294002 or API-2, whereas only slightly, but significantly, inhibited in the presence of U0126 (Fig. 4B). In contrast, the mTOR inhibitor rapamycin had no significant effect on doxycycline-induced *MKP3* promoter activity. We next assessed the effect of forced expression of PI3K. Transient transfection of active RasV12, p110-CAAX (constitutively active p110 subunit of PI3K), or Akt led to a strong increase in reporter activity of the pMkp3-Luc(−1597/−10) construct (Fig. 4C). Furthermore, RasV12-induced *MKP3* promoter activity was significantly abrogated by expression of dominant negative (DN)-Akt (Fig. 4D). These data suggest that the PI3K pathway plays a critical role in MKP3 expression induced by active Ras activation.

The present study proposes a novel mechanism by which prolonged activation of active Ras can modulate Erk1/2 activity through induction of the *MKP3* gene in NIH3T3 cells. Activated PI3K converts phosphatidylinositol 4,5-bisphosphate (PIP$_2$) into phosphatidylinositol 3,4,5 phosphate (PIP$_3$), which promotes membrane localization of PI-dependent kinase 1 (PDK1), leading to the activation of Akt, which in turn, can cause the activation or inhibition of specific target proteins involved in transcription, translation, cell growth, and survival. Ras positively cross-talks through direct interaction with the p110 catalytic subunit of PI3K to promote PI3K pathway activation (27). In addition, constitutive Ras activation can promote the down-regulation of PTEN, a lipid phosphatase which counterbalances the effects of PI3K by removing the phosphate from PIP$_3$ (28). On the contrary, Akt can inhibit the Raf through phosphorylation on Ser-259 (29, 30). These findings suggest that the Ras and PI3K pathways can be mutually regulated by several cross-talk mechanisms in different cellular contexts. Taking these observations into consideration, our data suggest that the PI3K/Akt pathway transmits the active Ras signal to MKP3 expression, which in turn leads to the down-regulation of the Erk1/2 activity in NIH3T3 cells. On the contrary, it has been reported that negative feedback regulation of FGF signaling (FGF2/4/8) is not mediated by the PI3K pathway, but by an Erk1/2-dependent Ets transcription factor, in MKP3 expression (21). This discrepancy might be due to the different cellular contexts. High levels of constitutively active Ras may affect multiple signaling pathways that are not activated by normal Ras (31). It is speculated that transient activation of Ras by growth factor signaling may induce MKP3 expression through an Erk1/2-dependent Ets-family transcription factor, but sustained activation of Ras may preferentially use the PI3K-dependent pathway to induce MKP3 expression.

In summary, we showed that the PI3K-Akt signaling pathway negatively regulates Erk1/2 through induction of MKP3 expression in NIH3T3 cells expressing constitutively active Ras. However, it remains possible that other MKPs of the DUSP family could affect active Ras-induced Erk1/2 activity. An understanding of the precise molecular mechanism of the negative feedback loop induced by active Ras would be beneficial to the design of future therapeutic development against active Ras-mediated tumorigenesis.

MATERIALS AND METHODS

Cells and reagents

Tetracycline-inducible NIH3T3tet-on/H-RasG12R cells are described elsewhere (20). Kinase inhibitors (LY294002, API2 and U0126) were purchased from Calbiochem (San Diego, CA, USA). The firefly and Renilla Dual-Glo™ Luciferase assay systems and the pRL-null plasmid, which encodes Renilla luciferase, were purchased from Promega (Madison, WI, USA). The expression plasmids for active H-Ras (pSG5/RasV12), dominant negative (DN)-Akt (pSG5/DN-AKT) and dominant-active (DA)-PI3K (pSG5/p110-CAAX) were donated by Julian Downward (Cancer Research UK London Research Institute, London, UK).

Western blot analysis

Cells were lysed in buffer that contained 20 mM HEPES (pH 7.2), 1% Triton X-100, 10% glycerol, 400 mM NaCl, 10 μg/ml leupeptin, and 1 mM PMSF. Western blot analysis was performed according to standard procedures, as described previously (32), using an antibody against either H-Ras (1 : 500; Oncogene), phospho-Raf1 (Ser259; 1 : 1,000; Cell Signaling), phospho-Erk1/2 (Thr202/Tyr204; 1 : 1000; Cell Signaling), phospho-RSK (Thr573; 1 : 1,000; Cell Signaling), phospho-Akt (Ser473; 1 : 1,000; Cell Signaling), Erk2 (1 : 5,000; Santa Cruz Biotechnology), phospho-Elk1 (Ser383; 1 : 1,000; Cell Signaling), MKP3 (1 : 5,000; Cell Signaling), or GAPDH (1 : 2,000; Santa Cruz Biotechnology).

Northern blot analysis

Total RNA samples (10 μg) were separated by electrophoresis on a formaldehyde/agarose gel and transferred to a Hybond N$^+$ nylon membrane (Amersham Pharmacia Biotech). Northern blotting was performed with [γ-^{32}P]dCTP-labeled MKP3 cDNA probes using a High Prime DNA Labeling Kit (Roche), followed by hybridization with a glyceraldehyde-3-phosphate dehydrogenase (Gapdh) cDNA probe, as described previously (32).

Immunofluorescence staining

Cells seeded on glass slides were cultured in the absence or presence of doxycycline. After 24 h, the cells were serum starved with 0.5% serum for an additional 24 h. The cells were left untreated or treated with 10 ng/ml FGF2 for 30 min, and then fixed in 4% paraformaldehyde for 10 min, and permeabilized in 0.1% Triton X-100 and 2% bovine serum albumin (BSA) for 10 min. The samples were incubated with a primary antibody against α-tubulin or phosphor-Erk1/2 (Thr 202/Tyr 204) for 90 min, followed by incubation with a goat Alexa Fluor 555-conjugated anti-rabbit IgG (for α-tubulin) or Alexa Fluor 488-conjugated anti mouse IgG (for phos-

phor-Erk1/2) antibody for 30 min. Nuclear DNA was stained with Hoechst 33258. Stained cells were immediately observed under an EVOSf1® fluorescence microscope (Advance Microscopy Group).

Construction of the MKP3 promoter-reporter construct

A fragment of the mouse *MKP3* gene spanning nucleotides −1597 to −10 (transcription start site numbered as +1) was amplified from mouse genomic DNA (Promega) by PCR using the primers 5'-agctcctttccctgggacc-3' (forward; −1597/−10) and 5'-agagaatgtatccattgagacgc-3' (reverse; −34/−10). The amplified PCR products were ligated into a T&A vector (RBC Bioscience), digested with *Hind*III, and then subcloned into the luciferase reporter plasmid pGL3-basic (Promega), yielding pMkp3-Luc (−1597/−10).

Transient transfection and luciferase promoter reporter assay

For the promoter reporter assay, cells were seeded into 12-well plates and transfected with 0.5 μg of the *MKP3* promoter constructs using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Where indicated, mammalian expression vectors were also included. Transfection efficiency was monitored by co-transfection with 50 ng of pRL-null plasmid encoding Renilla luciferase as described previously (32). At 24 h post-transfection, the levels of firefly and Renilla luciferase activities were measured using a Dual-Glo™ Luciferase assay system (Promega), and luminescence was measured with a dual luminometer (Centro LB960; Berthold Tech). The firefly luciferase activity was normalized to the Renilla activity, and the relative amount of promoter activity in the control cells was set to "1".

ACKNOWLEDGEMENTS

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