BMB
Reports

BMB Rep. 2016; 49(7): 370-375
www.bmbreports.org

Induction of MAP kinase phosphatase 3 through Erk/MAP kinase activation in three oncogenic Ras (H-, K- and N-Ras)-expressing NIH/3T3 mouse embryonic fibroblast cell lines

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Ras oncoproteins are small molecular weight GTPases known for their involvement in oncogenesis, which operate in a complex signaling network with multiple effectors. Approximately 25% of human tumors possess mutations in a member of this family. The Raf1/MEK/Erk1/2 pathway is one of the most intensively studied signaling mechanisms. Different levels of regulation account for the inactivation of MAP kinases by MAPK phosphatases in a cell type- and stimuli-dependent manner. In the present study, using three inducible Ras-expressing NIH/3T3 cell lines, we demonstrated that MKP3 upregulation requires the activation of the Erk1/2 pathway, which correlates with the shutdown of this pathway. We also demonstrated, by applying pharmacological inhibitors and effector mutants of Ras, that induction of MKP3 at the protein level is positively regulated by the oncogenic Ras/Raf/MEK/Erk1/2 signaling pathway. [BMB Reports 2016; 49(7): 370-375]

INTRODUCTION

The Ras family of small molecular weight GTPases (H-, K- and N-Ras) comprises signaling molecules that are highly conserved. Ras proteins play crucial roles in the regulation of the activity of essential signaling pathways that connect a variety of upstream signals to a wide range of downstream signaling pathways (1). This gene is one of the most critical oncogenes involved in carcinogenesis, and one of the hottest research subjects is the determination of the role of its aberrant function in carcinogenesis and the mechanism by which Ras mediates its action in normal and neoplastic cells (2). The abnormal activation of Ras corresponds with the promotion of malignant phenotypes such as transformation, proliferation, metastasis, and invasion. Additionally, the phenotypes of other Ras-related proteins are regulated by Ras-mediated signal transduction and also contribute to oncogenesis (3). Each of these three Ras proteins is a powerful transforming gene in model systems, and all forms are expressed widely in adult tissues and in tumors (4). Ras is involved in the interaction with multiple downstream effector molecules that modulate diverse cellular signaling activities, and there are strong indications of feedback and crosstalk with huge signaling networks (5, 6). The 1st Ras effector pathway to be identified was the Raf/MEK/Erk pathway (7). This pathway is a crucial shared element of mitogenic signaling involving tyrosine kinase receptors, and leads to a wide range of cellular responses including differentiation, growth, apoptosis, and inflammation (8). The intensity and duration of MAP kinase (MAPK) activation during the cellular response to external signals can be critical for cell fate. This makes MAPKs crucial players in cellular processes such as growth, proliferation, differentiation, division, and survival.

Dual specificity phosphatases (DSPs) are a subclass of the protein tyrosine phosphatase (PTP) gene superfamily, which are specific for the dephosphorylation of critical phosphothreonine and phosphotyrosine residues within MAPKs (9). DSP gene expression is induced by a host of growth factors and/or cellular stresses, thereby negatively controlling MAPK superfamily members (10). The DSP family contains approximately 30 genes, which share functional and structural properties, the most relevant being the presence of a conserved protein-tyrosine phosphatase domain, such as MAPK phosphatase 1 (MKP1), MKP2, MKP3, MKPX, and MKP4, which constitute a distinct subfamily of dual specificity MKPs (11). MKPs

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http://dx.doi.org/10.5483/BMBRep.2016.49.7.256

Received 3 December 2015, Revised 4 January 2016, Accepted 25 January 2016

Keywords: Effector mutants, Mitogen-activated protein kinase, Mitogen-activated protein kinase phosphatase 3, Oncogenic Ras, Oncogenic Ras inducible NIH/3T3 cells

ISSN: 1976-670X (electronic edition)
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differ in their physiological functions, expression patterns, and subcellular localizations, and play tissue- and/or developmentally-specific roles related to the regulation of MAPK pathways (12). For instance, MKP3, MKP-X, and MKP4 are predominantly cytosolic, whereas inducible MKP1 is found only in the nucleus. The expression of several DSPs depends in part on MAPK activation, and many of these are transcriptionally upregulated in response to growth factors, both in developing embryos and in cell culture experiments (11, 13). Among these DSPs, MKP3 is specific to Erk1/2 and Erk5, and may play a role in determining the cytosolic localization of Erk1/2 during early development (10, 14, 15). Upon induction, phosphorylated Erk (pErk) and MKP3 are expressed in the same compartments, suggesting a possible role of MKP3 in sustained levels of pErk. These results implicate a conserved negative regulatory feedback loop, mediated by MKP3, on Ras/MAPK signaling, and suggest a pivotal role of MKP3 in processes in which the level of Ras/MAPK signaling is essential for normal development (16). The extent to which MKP3 is directly regulated by Ras/MAPK signaling remains known. Despite a poor level of understanding, the regulatory mechanisms of MKP3 expression appear to be cell type-dependent and induced by certain growth factors such as FGF and NGF. For instance, MKP3 expression is dependent on Erk activity in certain cell types, such as human pancreatic cancer (17), MCF-7 breast cancer (18), and non-small-cell lung cancer (19). However, it has been proposed that the induction of MKP3 by Erk is mediated by the PI3K pathway in chick embryos and cell culture experiments (20). However, another report has suggested that the PI3K pathway is not involved in the induction of MKP3 expression in response to FGF (13). These observations indicate that other regulatory inputs besides Ras/MAPK signaling contribute to the generation of the expression pattern of MKP3, and that the discrepancy may be due to the different biological contexts. The research group led by Jose de Celis, conducting studies in *Xenopus*, could not discriminate whether MKP3 is induced by the MAPK and/or PI3K pathway (16). In the present study, we demonstrated a molecular mechanism by which the induction of MKP3 at the protein level is positively regulated by active Erk, using the inducible NIH/3T3 cell lines for three types of oncogenic Ras under the tight control of doxycycline. Pharmacological inhibitors of either Erk/MAPK or PI3 kinase and effector mutants of H-Ras, which each engage only one effector pathway, were used to invoke these pathways as essential mediators of MKP3 induction.

RESULTS AND DISCUSSION

Expression of oncogenic Ras proteins results in the chronic stimulation of the Raf/MEK/Erk protein kinase cascade. We previously described three types of NIH/3T3/Ras/G12V Tet-On expression cell lines for oncogenic H-, K- and N-Ras, the induction of which is under the tight control of doxycycline (21). These cells gradually expressed the three active oncogenic Ras proteins from 12 h post-induction in response to 2 μg/ml doxycycline (Fig. 1). In addition, pErk and pAkt, which represent the activation status of Erk and Akt, were increased in the three kinds of oncogenic Ras-expressing NIH/3T3 cells ((A) NIH/3T3/H-Ras/G12V, (B) NIH/3T3/K-Ras/G12V, and (C) NIH/3T3/N-Ras/G12V). In this unique inducible expression system in response to the antibiotic doxycycline, three oncogenic Ras-expressing cell lines showed the typical morphological phenotype for oncogenic transformation, exhibiting a spindle-like shape with small round cell bodies following induction (data not shown). These morphological changes are in good agreement with previous reports (21, 22). It has been shown that oncogenic Ras protein expression is sufficient for transformation of cells based on anchorage-independent growth in soft agar (data not shown). Thus, these three oncogenic Ras-expressing cell lines are useful for discriminating cellular functions in the elucidation of its networks. In order to investigate the regulation of MAPKs in NIH/3T3 cells upon the induction of oncogenic Ras expression

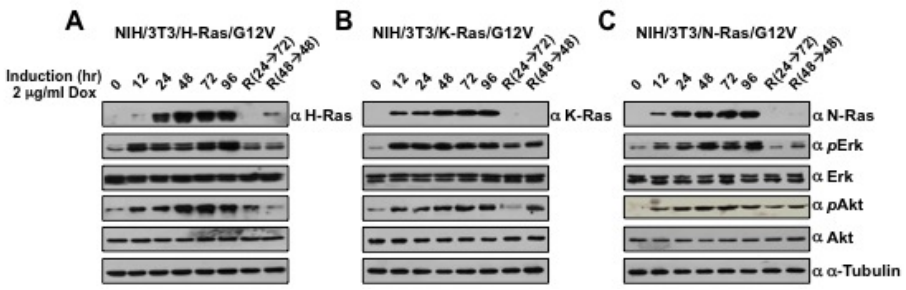

Fig. 1. Expression levels of three different Ras-inducible NIH/3T3 cell lines. The expression levels of three different Ras-inducible NIH/3T3 cell lines (NIH/3T3/H-Ras/G12V (A), NIH/3T3/K-Ras/G12V (B), and NIH/3T3/N-Ras/G12V (C)) treated with or without 2 μg/ml doxycycline for the indicated periods of time were monitored by immunoblotting analyses using specific antibodies. For reverted cells, NIH/3T3/Ras/G12V cells were treated with doxycycline (2 μg/ml) for the indicated periods of time, and following replacement with fresh medium, allowed to grow for the indicated periods of time without doxycycline. The quantity of the applied protein was normalized by Western blotting analysis with an anti-α-tubulin antibody. The influence of oncogenic Ras transformation on MAPK and Akt/PKB pathways was monitored.

and hFGF-basic treatment, we performed a comprehensive analysis of the expression of MKP3. The results demonstrate a significant increase in MKP3 protein levels depending on the oncogenic H-Ras expression (Fig. 2A and 2D) and also the expression of two other oncogenic Ras proteins (Fig. 2B & 2E for K-Ras and 2C & 2F for N-Ras) in NIH/3T3 cells. Additionally, using activation-specific anti-phospho-antibodies and pharmacological inhibitors, we monitored the activation status of the Erk1/2 and PI3K/Akt pathways under inhibition conditions with the expression of three types of oncogenic Ras protein (Fig. 2D-F). Erk/MAPK activity was effectively repressed with U0126, as was PI3K activity with LY294002, and the DMSO control had no effect on the expression of MKP3. As shown in Fig. 2, blockade of Erk1/2 activity by incubation of the oncogenic Ras-expressing NIH/3T3 cells with the Erk1/2 specific inhibitor, U0126, significantly inhibited the induction of MKP3, demonstrating that phosphorylated Erk1/2 drives the induction of the MKP3 protein. Conversely, the PI3K/Akt-specific inhibitor, LY294002, showed no effect and maintained the elevated MKP3 protein levels (Fig. 2D-F). A good positive correlation was observed between the degree of activation of Erk1/2 under distinct conditions and the extent of MKP3 protein upregulation. This is in accordance with MKP3 being upregulated as a consequence of Erk1/2 activation, as has been reported in the developing chick somite and human breast and colon cancer cells (23-25). Evidence shows that injection of 1 ng constitutively-activated Ras mRNA promotes ectopic MKP3 expression in *Xenopus* development (16). To determine whether growth factor stimulation of Erk1/2 and Akt could be affected by sustained activation due to oncogenic H-Ras, NIH/3T3/H-Ras/G12V cells were cultured in medium containing 0.5% bovine serum in the absence or presence of the inducer for the indicated times (12, 24, or 48 h), followed by incubation in both the absence and presence of hFGF-basic (50 ng/ml) for 30 min (Fig. 2A). Previously, it has been found that MKP3/Pyst1 expression is mediated by Erk activation, and that negative feedback predominates in limiting the extent of FGF-induced Erk activity (26). Signaling cascades activated through hFGF-basic binding to FGFR include the Ras/Raf/MAPK, PLCγ/PKC, and PI3K/Akt pathways (27). Treatment with FGF, during NIH/3T3/H-Ras/G12V cell incubation in the absence of doxycycline, significantly increased the phosphorylated Erk1/2 level. In contrast, the cells incubated with hFGF-basic in the presence of doxycycline for 12 and 24 h did not show an increase in phosphorylated Erk1/2 compared with the level of induction seen without hFGF-basic, despite the same induction level of MKP3 (Fig. 2A). However, in the case of the 48 h induction of oncogenic H-Ras, the level of activated Erk1/2 in the presence of hFGF-basic, as measured by its phosphoactive content, showed a significant increase over that seen in the absence of FGF, and even over that seen in the extracts with and without induction of oncogenic H-Ras.

We next addressed whether sustained activation of the Erk1/2 pathway was necessary for the accumulation of MKP3

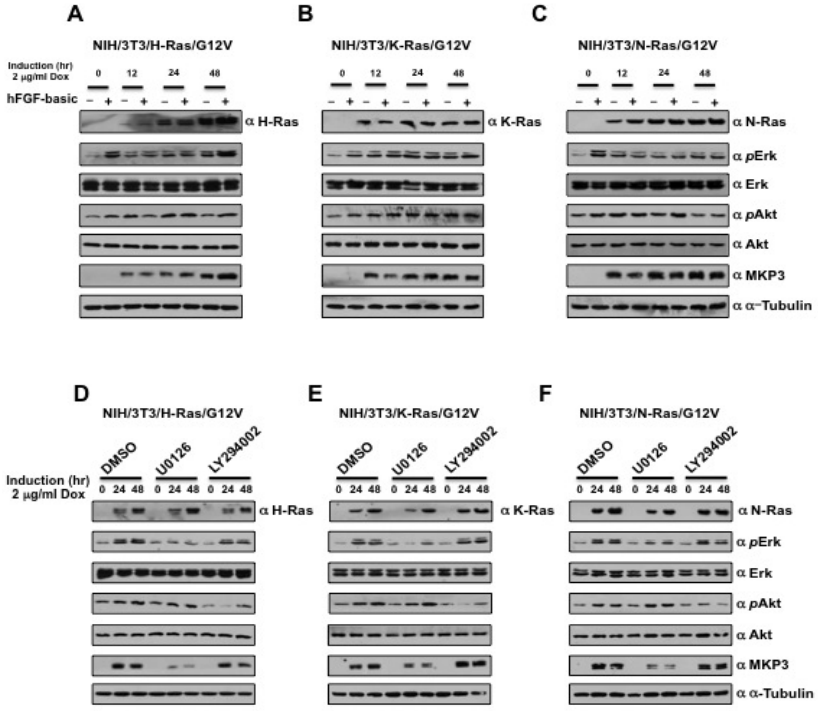

Fig. 2. Effect of oncogenic Ras expression on MKP3 induction and the role of pErk signaling in oncogenic Ras-induced MKP3 expression. NIH/3T3/H-Ras/G12V (A), NIH/3T3/K-Ras/G12V (B), and NIH/3T3/N-Ras/G12V (C) cells were cultured with 0.5% bovine serum for 24 h and then incubated with 2 μg/ml doxycycline for additional time (12, 24, or 48 h), followed by culture in either the absence or the presence of hFGF-basic (50 ng/ml) for 30 min. NIH/3T3/H-Ras/G12V (D), NIH/3T3/K-Ras/G12V (E), and NIH/3T3/N-Ras/G12V (F) cells were treated with 2 μg/ml doxycycline for 0, 24, or 48 h in the presence of DMSO, U0126 (25 μM), or LY294002 (25 μM).

proteins in the specifically-established cell lines. Ample evidence supports that notion that Ras can cascade multiple signaling networks and utilize a variety of diverse proteins. The specific H-Ras mutants in the effector loop give Ras the ability to discriminate between different effectors, facilitating specific interaction and activation. Certain delicate mutations in the effector-interacting region of Ras (residues 32-40) may lead to partial loss of function in which the interaction with certain effectors is retained, but with others is abolished, leading to the promotion of selective Ras signaling events. The Ras/G12V/T35S mutant preferentially interacts with, and triggers the activation of, Raf1 over PI3K, and Ras/G12V/Y40C preferentially interacts with, and triggers the activation of, PI3K over Rad1 (5, 28, 29). In addition, Ras/G12V/E37G specifically binds the Ral-GDS effector molecule. This concrete set of effector loop mutants, each of which specifically engage one effector network, allows one to demonstrate that a variety of signaling systems are required for efficient transformation, and that oncogenic Ras performs multiple roles in cells. Increases in Ras effector mutants were shown in response to the inducer, depending on the amount and duration of doxycycline present in the medium (Fig. 3). Cells were examined for the effect of Ras protein expression on the activation of direct effectors. In the case of our inducible expression system, in response to 2 μg/ml doxycycline, these cells gradually expressed effector mutants of Ras. pErk1/2 and pAkt, which represent the specific activation status of Erk and PI3K/Akt of the effector molecules for either the Raf1/MEK/Erk/MAPK or PI3K cascades, were increased in each of the inducible NIH/3T3 cell lines for the specific effector mutants of H-Ras (in NIH/3T3/H-Ras/G12V/T35S and NIH/3T3//H-Ras/G12V/Y40C, respectively). The effector Ral-GDS mutant from NIH/3T3/H-Ras/G12V/E37G did not show any effect on the pErk or pAkt levels. The phosphorylated Erk1/2, as judged by the expression of the effector Raf1 mutant from NIH/3T3/H-Ras/G12V/T35S, drove the induction of the MKP3 protein. The influence of the effector mutants on the induction of MKP3 is in agreement with previous experiments that blocked Erk1/2 activity by incubating the inducible oncogenic Ras-expressing NIH/3T3 cells with the Erk1/2 specific inhibitor, U0126. These results suggest that the Raf1/MEK/Erk/MAPK pathway, rather than the PI3K signaling pathway, plays a pivotal role in oncogenic Ras activation-induced MKP3 expression.

Finally, we examined our biological contexts to investigate the subcellular localization in the absence (Fig. 4A) or presence (Fig. 4B) of hFGF-basic in NIH/3T3/H-Ras/G12V cells. Karlsson *et al*., (30) have shown, using an MKP3-GFP fusion protein, that MKP3 shuttles between the nucleus and cytosol, but that under steady-state conditions the export process predominates, resulting in a largely cytosolic localization of

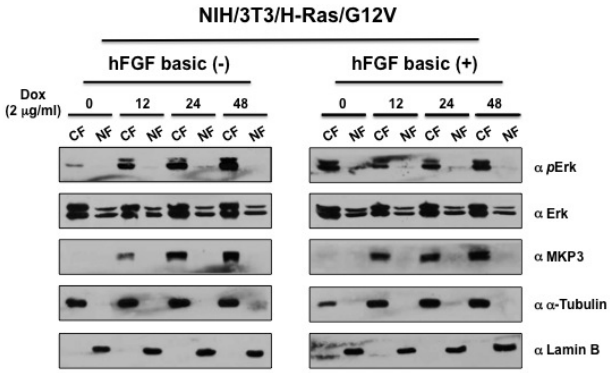

Fig. 4. Effect of oncogenic H-Ras on the subcellular localization of pErk and MKP3. The expression levels of pErk, Erk, and MKP3 in 30 μg prefractionated cytosolic fraction (CF) and nuclear fraction (NF) of NIH/3T3/H-Ras/ G12V cells cultured with 0.5% bovine serum for 24 h and then incubated with 2 μg/ml doxycycline for additional time (12, 24, or 48 h), followed by culture in the absence or presence of hFGF-basic (50 ng/ml) for 30 min, were monitored by immunoblotting analysis. α-tubulin and lamin B were used as controls to confirm the presence of cytosolic and nuclear fractions.

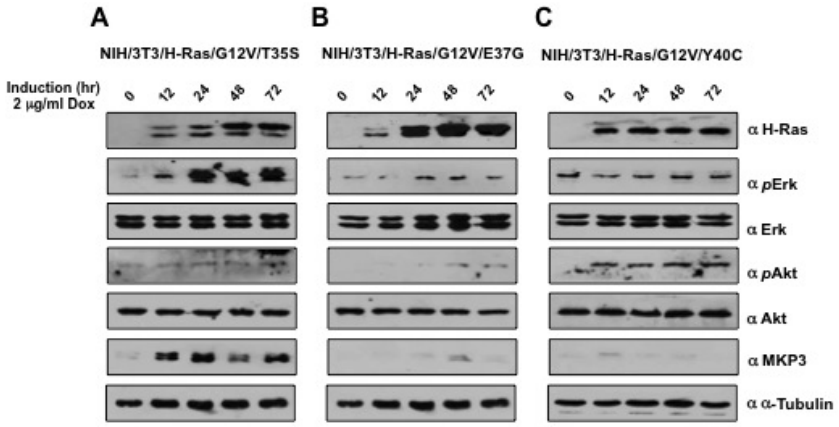

Fig. 3. Role of Erk signaling in oncogenic Ras-induced MKP3 expression evaluated using specific H-Ras effector loop mutants, each of which engages only one effector pathway. The set of Ras effector mutants have been described previously (32). Following treatment of the cells with or without 2 μg/ml doxycycline for the indicated periods of time, the cells were collected, and the influence of oncogenic Ras transformation on the MAPK and Akt/PKB pathways was monitored.

MKP3. It is known that cellular events triggered by Erk can be connected to various signaling networks that affect the duration and/or magnitude of Erk activation, as well as its subcellular localization. It has been proposed that its localization controls distinct cellular responses (31). Although MKPs may differentially regulate nuclear or cytosolic pools of activated MAPK, little is known about the mechanisms that govern its physiological significance or subcellular localization. As shown in Fig. 4A and 4B, phosphorylated Erk1/2 and induced MKP3 were detected only in the cytosol based on immunoblotting analysis, although total Erk1/2 proteins were detectable in both the nuclear and cytosolic extracts. This specific localization of activated Erk1/2 is in agreement with previous reports that MKP3 may play a role in determining the cytosolic localization of Erk1/2. Moreover, we showed the same localization of MKP3 in NIH/3T3/K-Ras/G12V and NIH/3T3/N-Ras/G12V cells (Fig. S1)

Principally, although the Ras signaling pathways are fundamental to the existence of normal, as well as tumor cells, high levels of oncogenic Ras proteins may affect multiple signaling systems that are not influenced by normal Ras proteins. A simple explanation is that the differential regulation of oncogenic Ras proteins determines their relative importance in oncogenesis rather than endogenous Ras. Thus, although novel ways of identifying proteins that depend on Ras for malignant transformation are being evaluated, tumors bearing Ras mutations remain among the most difficult to treat. We have demonstrated that the expression of PTEN is suppressed by oncogenic Ras at both the protein and MRNA level, which leads to the selection of cells with increased survival signaling. This suppressive activity of oncogenic Ras is also mediated by Raf1/Erk/MEK signaling events (32). The present findings propose a novel mechanism by which the activation of three types of oncogenic Ras proteins affect the induction of MKP proteins through activated Erk1/2 activity, rather than PI3K activity, in the cytosol of NIH/3T3 cells. This supports the notion that MKP3 is negatively involved in an Erk1/2-dependent feedback loop that inhibits Erk1/2 under conditions where the expression of three types of oncogenic Ras is the predominant stimulus of the Raf/MEK/Erk signaling pathway.

In summary, we demonstrated that MKP3 induction requires the expression of oncogenic Ras proteins in an Erk-dependent manner. Recent experience has underscored how a pathway that appeared simple and linear is extremely complex and poorly understood at the level of detail required to shut it down effectively. A much deeper analysis of the molecular mechanisms underlying Ras regulation and effector engagement is required before we can expect to interfere with these mechanisms effectively.

MATERIALS AND METHODS

Chemical reagents and antibodies

The primary antibodies used were as follows: anti-H-Ras (sc-29, Santa Cruz Biotech *Inc.*, Santa Cruz, CA, USA), anti-K-Ras (Sc-30, Santa Cruz), anti-N-Ras (sc-31, Santa Cruz), anti-Erk1 (sc-94, Santa Cruz), anti-pErk1/2 (Thr^{202}/Tyr^{204}, Cat. No. 4370, Cell Signaling Technology, Danvers, MA, USA), anti- (sc-1619, Santa Cruz), anti-pAkt (Ser^{473}, Cat. No. 4060, Cell signaling), anti-MKP3 (sc-377070, Santa Cruz), anti-α-tubulin (sc-398103, Santa Cruz), and anti-lamin B (sc-374015, Santa Cruz). Secondary antibodies were from KPL Inc. (Gaithersburg, MD, USA). Human FGF-basic (hFGF-basic, Cat. No. 8910) and U0126 (Cat. No. 9903) were purchased from Cell Signaling Technology.

Cell culture and treatment

The cell culture procedures for the three types of oncogenic Ras-expressing cells have been previously described (21, 22). For the treatment of hFGF-basic (50 ng/ml), the cells were cultured with 0.5% serum for 24 h and then treated with doxycycline (2 μg/ml) for an additional 0, 12, 24, or 48 h, followed by incubation in the absence or presence of hFGF-basic for 30 min. For the treatment of inhibitors, the cells were treated with doxycycline (2 μg/ml) for 0, 24, or 48 h in the absence or presence of DMSO, U0126 (25 μM), or LY294002 (25 μM).

Preparation of cell lysates, subcellular fractionation of the nuclear fraction, and immunoblotting analysis

The cells were washed with ice-cold PBS and lysed using lysis buffer A (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100 containing protease inhibitor cocktails) for the collection of Ras proteins or conventional RIPA buffer containing protease inhibitors for the collection of other proteins. Preparation of cytosolic and nuclear fractions from the cultured NIH/3T3/Ras cells with or without doxycycline was performed using a nuclear extraction kit from Cayman Chemical Co. (Ann Arbor, MI, USA) according to the manufacturer's instructions (33). The procedures for immunoblotting analysis were essentially performed as previously described (22, 34, 35).

ACKNOWLEDGEMENTS

This work was supported by the DGIST R&D Program of the Ministry of Science, ICT, and Technology of Korea (15-HRLA-02), and by grants from the NRF of Korea (No. 2013R1A1A2009145 (J.H.K.), No. NRF-2010-0024199 (Y.Y.B.))

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