Histone deacetylase 4 associates with extracellular signal-regulated kinases 1 and 2, and its cellular localization is regulated by oncogenic Ras

Xianbo Zhou*, Victoria M. Richon*, Audrey H. Wang†, Xiang-Jiao Yang†, Richard A. Rifkind*, and Paul A. Marks*‡

*Cell Biology Program, Sloan–Kettering Institute, Memorial Sloan–Kettering Cancer Center and Graduate School of Medical Sciences, Cornell University Medical School, New York, NY 10021; and †Molecular Oncology Group, Department of Medicine, McGill University Health Center, Montréal, QB, Canada H3A 1A1

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Histone deacetylase 4 (HDAC4) is a member of a family of enzymes that catalyze the removal of acetyl groups from core histones, resulting in a compact chromatin structure that is generally associated with repressed gene transcription. Protein phosphorylation has been implicated in the regulation of the corepressor activity of the deacetylase. Here we report that serine/threonine kinases are found in association with HDAC4 and phosphorylate HDAC4 *in vitro*, and HDAC4 is phosphorylated in cells. The extracellular signal-regulated kinases 1 and 2 (ERK1/2), also known as p44^{MAPK} and p42^{MAPK}, respectively, are two of the kinases associated with HDAC4. ERK1/2 are components of the Ras–mitogen-activated protein kinase (MAPK) signal transduction pathway. Activation of the Ras–MAPK pathway by expression of oncogenic Ras or constitutively active MAPK/ERK kinase 1 results in an increased percentage of cells (from ≈10% to ≈70%) that express HDAC4 in the nucleus in C2C12 myoblast cells. In cells transfected with oncogenic Ras, nuclear HDAC4 is associated with kinase activity. Our results provide evidence that protein kinase activity is present in a protein complex with HDAC4 and directly links the Ras–MAPK signal transduction pathway to a mechanism for chromatin remodeling (i.e., histone deacetylation).

The N-terminal tails of core histones are sites of several types of posttranslational modifications, including acetylation and phosphorylation (1). Evidence suggests that these covalent modifications play important roles in biological activities involving chromatin, e.g., DNA transcription and replication (2). The acetylation of core histone tails is a dynamic process maintained by histone acetyltransferases and histone deacetylases (HDACs; refs. 3 and 4). HDACs form complexes with transcriptional corepressors and are believed to repress transcription by removing the acetyl groups from the N-terminal tails of the core histones of chromatin (1). Hypoacetylated chromatin is often associated with a transcriptional inert state (1).

HDAC4 is a mammalian homolog of the yeast HDA1 protein that deacetylates histone *in vitro* and represses transcription when brought to a promoter through its interaction with DNA-binding transcription factors or as a Gal4-fusion protein (5–9). HDAC4 interacts with myocyte enhancer factor 2 (MEF2), represses MEF2-mediated transcription, and inhibits skeletal myogenesis (6, 9, 10). HDAC4 is present in both the cytoplasm and the nucleus (5–8, 11). Protein phosphorylation has been implicated in the regulation of HDAC4 function (8, 10–12). HDAC4 interacts with 14-3-3 (8, 11), a family of proteins that primarily interacts with phosphorylated proteins (13). It is believed that 14-3-3 proteins negatively regulate HDAC4 by preventing its nuclear localization. The HDAC4 mutant that abolishes the interaction with 14-3-3 localizes in the nucleus (8, 11). This mutant also enhances the ability of HDAC4 to repress MEF2-mediated transcription (8, 11). These findings suggest that protein phosphorylation plays a role in the regulation of HDAC4 function. The identities of the kinases that phosphorylate HDAC4 and how the intracellular localization of HDAC4 is regulated are not known.

Here we report that serine/threonine protein kinases are associated with HDAC4 and can phosphorylate HDAC4. The mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinases 1 and 2 (ERK 1/2) are two of the kinases that are complexed with HDAC4. Further, activation of the Ras-MAPK signal transduction pathway results in an increased percentage of cells expressing HDAC4 in the nucleus.

Materials and Methods

Cell Culture, Transfection, and DNA Constructs. The 293T cells were cultured as described (14). C2C12 cells were obtained from the American Type Culture Collection. Transient transfection assays were performed by using Fugene 6 reagent (Roche Molecular Biochemicals) by following the manufacturer's instructions.

pFLAG-HDAC4 (pF-HDAC4), pFLAG-MAC (met amino cytoplasm)-HDAC4, and enhanced green fluorescent protein (EGFP)-HDAC4 (E-HDAC4) were constructed by PCR amplification of plasmid KIAA0288 (gift of T. Nagase, Kazusa DNA Research Institute, Chiba, Japan) corresponding to amino acids 118–1,084 of HDAC4 (7) in-frame with the FLAG epitope and subcloned into the pFLAG-CMV2 vector for mammalian expression and the pFLAG-MAC vector for bacterial expression (Sigma), or in-frame with EGFP and subcloned into the pEGFP-C3 vector (CLONTECH). pEGFP-HDAC4 (1–1,084) is described elsewhere (11). The constructs were confirmed by DNA sequencing. The oncogenic Ras (H-Ras^{V12}) expression construct, pCMV5-H-Ras^{V12}, was a gift of Y. G. Chen and J. Massague (Memorial Sloan–Kettering Cancer Center). The constitutively active MAPK/ERK kinase 1 (MEK1) expression construct, pUSE-MEK1 (S218D/S222D), was obtained from Upstate Biotechnology (Lake Placid, NY).

Phosphorylation Assays. Kinase assays were carried out by using FLAG peptide-released immunocomplexes in reaction buffer containing 20 mM Tris·HCl (pH 8.0), 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM ATP, 1 mCi/ml [γ-^{32}P]ATP (1 Ci = 37 GBq), 1 mM NaF, 0.1 mM Na_3VO_3, and 1 mM DTT at 30°C for 20 min. Kinase reactions were stopped by the addition of the SDS/PAGE sample buffer for reactions by using myelin basic protein (MBP) or histone H3 as a substrate, or by using phosphoric acid after applying it onto P81 phosphocellulose

Abbreviations: HDAC, histone deacetylase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; H-Ras^{V12}, oncogenic Ras; F-HDAC4, FLAG-tagged HDAC4; MEK1, MAPK/ERK kinase 1; EGFP, enhanced green fluorescent protein; E-HDAC4, EGFP-tagged HDAC4; ERK1/2, extracellular signal-regulated kinases 1 and 2.

‡To whom reprint requests should be addressed: E-mail: paula_marks@mskcc.org.

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paper for reaction by using the synthetic peptide as substrate. The synthetic peptide KKALRRQETVDAL was obtained from Upstate Biotechnology, the MBP was obtained from Sigma, and the histone H3 was obtained from Roche Molecular Biochemicals. Thin-layer, two-dimensional electrophoresis analysis of phosphoamino acids was carried out according to Boyle *et al.* (15) by using the HTLE 7000 electrophoresis apparatus (C.B.S. Scientific, Del Mar, CA). The in-gel kinase assay was carried out according to Hutchcroft *et al.* (16) by using MBP as a substrate.

For labeling cultured cells with $^{32}P_i$, 293T cells transfected with control FLAG vector and F-HDAC4 were cultured with 0.5 mCi/ml $H_3{}^{32}PO_4$ (1 Ci/ml; NEN) in phosphate-free DMEM for 3 h (17). The cells were lysed, and immunoprecipitation was performed with anti-FLAG agarose (Sigma) as described (14). Immunoprecipitated proteins were subjected to SDS/PAGE. The gel was washed three times in 10% (vol/vol) methanol/10% (vol/vol) acetic acid and dried. The phosphorylated protein was visualized by autoradiography.

Immunoprecipitation, Western Blot Analyses, and Antibodies. The immunoprecipitation and Western blot analyses were performed as described (14). The cytoplasmic and nuclear fractionation was carried out according to Ausubel *et al.* (17). The anti-FLAG M2 agarose affinity gel, FLAG peptide, and anti-FLAG bio-M2 antibody were obtained from Sigma. The antibody against MAPKs ERK1/2 was obtained from Upstate Biotechnology. Anti-JNK1 was obtained from Santa Cruz Biotechnology. Anti-CDK7 was a gift of R. Fisher (Memorial Sloan–Kettering Cancer Center). Anti-HDAC4 raised in rabbit is described elsewhere (11). Anti-phospho-ERK1/2 was obtained from New England Biolabs. Mouse monoclonal antibody against Ras (Pan Ras) was obtained from Oncogene Research Products, and Texas-red conjugated goat anti-mouse IgG was obtained from Jackson ImmunoResearch.

Expression of F-HDAC4 in Bacteria. Expression of FLAG-tagged protein in bacteria was carried out as described (18). FLAG peptide-released F-HDAC4 was the substrate in kinase assay using purified active ERK2 (Upstate Biotechnology).

Immunofluorescence. C2C12 cells were transfected with indicated DNA in chamber slides (Falcon). At 48 h after transfection, cells were fixed in paraformaldehyde and stained with Hoechst 33342 and a mouse monoclonal antibody against Ras followed by Texas-red conjugated goat anti-mouse IgG. EGFP was visualized directly by using a Zeiss LSM 510 confocal microscope (Molecular Cytology Core Facility, Memorial Sloan–Kettering Cancer Center).

Results

Serine/Threonine Protein Kinases Are Associated with HDAC4 and Can Phosphorylate HDAC4 *in Vitro*. To determine whether protein kinases are associated with HDAC4, proteins that coimmunoprecipitated with HDAC4 were assayed for protein kinase activity. Human kidney epithelial 293T cells were transfected with control FLAG vector or F-HDAC4, and proteins associated with F-HDAC4 were immunoprecipitated by using anti-FLAG agarose. The immunoprecipitated proteins were resolved by SDS/PAGE and visualized by Commassie blue staining (Fig. 1*A*). The presence of immunoprecipitated F-HDAC4 was verified by Western blot analysis by using anti-FLAG antibody (Fig. 1*B*). The kinase activity of the immunoprecipitates was assayed by using three substrates: a synthetic peptide, KKALRRQE**T**VDAL (threonine, the only residue that can be phosphorylated, is in bold face; Fig. 1*C*); MBP (Fig. 1*D*); and histone H3 (Fig. 1*E*). The results of the kinase assays indicate that a protein kinase activity is associated with the F-HDAC4 immunocomplex and kinases present in the F-HDAC4 complex phosphorylate all three substrates and the immunoprecipitated F-HDAC4 (Fig. 1 *D* and *E*). Phosphoamino acid analysis of the phosphorylated F-HDAC4 demonstrates the presence of primarily phosphoserine and a detectable level of phosphothreonine (Fig. 1*F*). These results indicate that there is a serine/threonine protein kinase activity in the F-HDAC4 immunocomplex and the kinase(s) associated with F-HDAC4 can phosphorylate HDAC4.

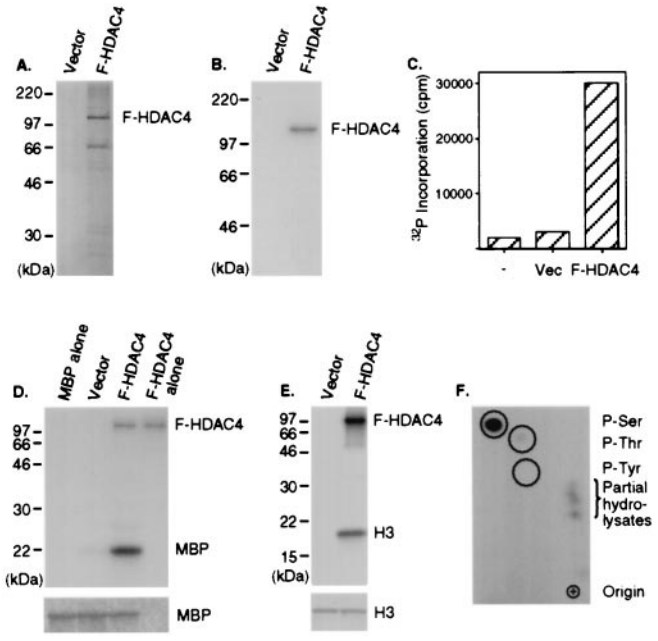

Fig. 1. Serine/threonine protein kinases associate with F-HDAC4 complexes and phosphorylate F-HDAC4. (*A* and *B*) Anti-FLAG immunoprecipitates were resolved by SDS/PAGE. Commassie blue staining (*A*) and Western blot analysis (*B*) with anti-FLAG antibody after blotting proteins onto a nitrocellulose filter. Vec, vector. (*C–E*) The presence of protein kinase activity was assayed by using the following substrates: synthetic peptide KKALRRQETVDAL (*C*), MBP (*D*), and histone H3 in the presence of [γ-^{32}P]ATP (*E*) . *D* and *E* show results from autoradiography (*Upper*) and Commassie blue stain (*Lower*). (*F*) Two-dimensional-electrophoresis analysis of acid-hydrolyzed phosphorylated F-HDAC4 from the gel shown in *E*. The positions of the origin, cold phosphoamino acid standards, and partially hydrolyzed peptide are shown. P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine.

F-HDAC4 Is Phosphorylated in Cells. To determine whether F-HDAC4 is phosphorylated in cells, 293T cells were transiently transfected with control FLAG vector or F-HDAC4 and cultured in the presence of $^{32}P_i$. F-HDAC4 was recovered from these cells by immunoprecipitation. F-HDAC4 is labeled by $^{32}P_i$ and phosphorylated in cells (Fig. 2).

ERK1/2 Are Associated with HDAC4, and ERK2 Phosphorylates HDAC4 *in Vitro*. To identify the kinases present in the HDAC4 complexes, in-gel protein kinase assays were performed by using MBP as a substrate. Protein kinase activity was associated with a protein that migrated with a molecular mass of approximately 43 kDa in both the F-HDAC4 immunoprecipitate from transfected human 293T cells (Fig. 3*A*) and the endogenous HDAC4 immunoprecipitate from mouse C2C12 myoblast cells (Fig. 3*B*) but was not detected in the immunoprecipitate from the FLAG vector control or normal rabbit serum. Several protein kinases were present in the whole-cell lysates (Fig. 3 *A* and *B*).

Protein kinases with an apparent molecular mass of approximately 43 kDa include ERK1/2, cyclin-dependent kinase 7

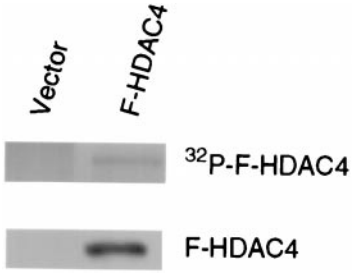

Fig. 2. HDAC4 is phosphorylated in cells. Anti-FLAG immunoprecipitated proteins from lysates of control FLAG vector, and F-HDAC4-transfected 293T cells that were cultured with $H_3{}^{32}PO_4$ were resolved on SDS/PAGE. (*Upper*) Autoradiography of dried gel. (*Lower*) Western blot analysis with anti-FLAG antibody.

(CDK7), JNK1, cAMP-dependent protein kinase, MEK1, and MEK3. F-HDAC4 immunoprecipitates were assayed for the presence of these protein kinases by using antibodies specific to each of these kinases (Fig. 3*C* and data not shown). These assays identified ERK1/2 as kinases associated with HDAC4 (Fig. 3*C*). JNK1 and CDK7 were not found to be associated with HDAC4 (Fig. 3*C*), nor were cAMP-dependent protein kinase, MEK1, and MEK3 (data not shown).

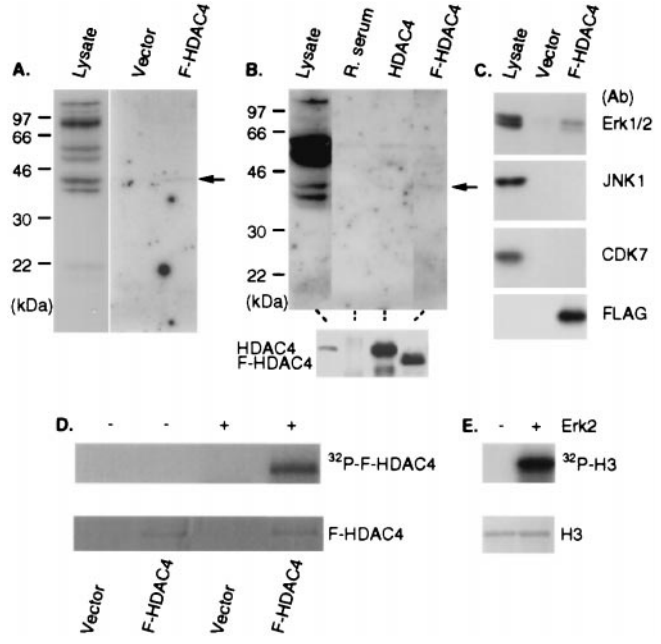

Fig. 3. ERK1/2 are associated with HDAC4 and phosphorylated F-HDAC4 *in vitro*. (*A*) In-gel protein kinase assays with the 293T whole-cell lysate or anti-FLAG immunoprecipitates from 293T cells transfected with vector or with F-HDAC4 as the enzyme source. MBP was the substrate. A 10-min exposure time was used for the lysate, and a 24-h exposure time was used for the immunoprecipitates. (*B*) In-gel protein kinase assays with the C2C12 whole-cell lysate, normal rabbit serum (R. serum), or anti-HDAC4 immunoprecipitates from C2C12 cells as the enzyme source and MBP as the substrate. An F-HDAC4 immunoprecipitate from 293T cells transfected with F-HDAC4 was included as a control. (*Upper*) Autoradiography. (*Lower*) Western blotting with anti-HDAC4 antibody. Arrowheads in *A* and *B* indicate the locations of kinases that were present in the immunoprecipitates. (*C*) Western blot analyses of the 293T whole-cell lysate and anti-FLAG immunoprecipitates from 293T cells that were transfected with vector or with F-HDAC4 by using antibodies to ERK1/2, JNK1, CDK7, and FLAG. (*D* and *E*) ERK2 phosphorylated the immunoprecipitated F-HDAC4 expressed in bacteria (*D*) and histone H3 (*E*). (*Upper*) Autoradiography. (*Lower*) Commassie blue stain.

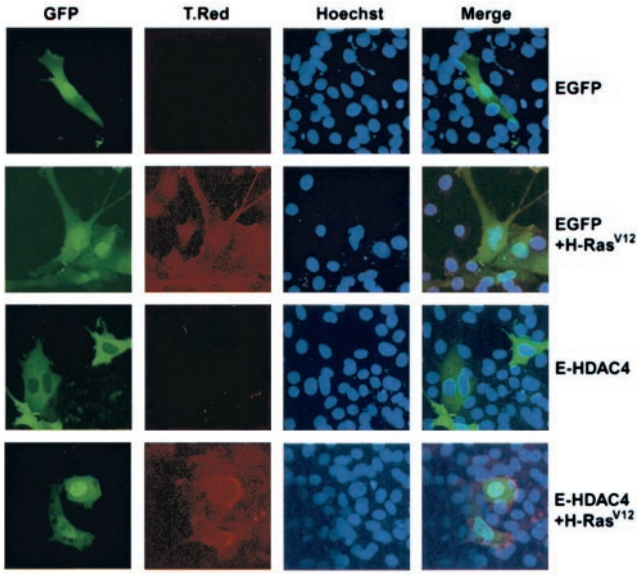

Fig. 4. H-Ras^{V12} increased the percentage of cells that express HDAC4 in the nucleus. C2C12 cells were transfected with expression vectors containing EGFP, EGFP plus H-Ras^{V12}, E-HDAC4, or E-HDAC4 plus H-Ras^{V12}, as indicated. The localization of EGFP and E-HDAC4 was examined by using a confocal microscope (Zeiss) LSM 510 at ×63. GFP, green fluorescent protein; T. Red, Texas Red detection of H-Ras^{V12}; Hoechst, Hoechst 33342 detection of DNA (Blue); Merge, overlay of the three other panels.

To determine whether ERK1/2 may be the kinases responsible for phosphorylating HDAC4 *in vitro*, kinase assays were performed with purified ERK2 by using anti-FLAG-immunoprecipitated F-HDAC4 expressed in bacteria as a substrate. ERK2 phosphorylated F-HDAC4 (Fig. 3*D*). ERK2 also phosphorylates histone H3 (Fig. 3*E*). These results demonstrated that ERK2 has a similar substrate-specificity as kinases associated with F-HDAC4.

Activation of ERK1/2 by Oncogenic Ras or Constitutively Active MEK1 Increases the Percentage of Cells That Express HDAC4 in the Nucleus. ERK1/2 are components of the Ras-MAPK signal transduction pathway that mediate the transduction of extracellular signals to the nucleus (19–21). HDAC4 is present in both the cytoplasm and the nucleus (5–7, 11) suggesting that HDAC4 may be regulated in part by modulating its intracellular localization.

To determine whether activation of the MAPK signal transduction pathway altered the intracellular localization of HDAC4, E-HDAC4 or EGFP vector was transfected with or without H-Ras^{V12} into murine myoblast C2C12 cells. Oncogenic Ras activates ERK1/2 through the Ras-MAPK signal transduction pathway (22). The localization of EGFP and E-HDAC4 was examined by confocal fluorescent microscopy. EGFP is expressed in both the cytoplasm and the nucleus. H-Ras^{V12} does not affect the localization of EGFP in the cells (Fig. 4). In ≈90% of cells expressing E-HDAC4, E-HDAC4 localized in the cytoplasm (Fig. 4), and in the remaining 10% of the cells, it was detected in both the cytoplasm and the nucleus. F-HDAC4 has a similar expression pattern when assayed by indirect fluorescent microscopy by using FITC conjugated anti-FLAG antibody (data not shown). Coexpression of H-Ras^{V12} and E-HDAC4 results in an increase from ≈10% to ≈70% of the cells expressing nuclear E-HDAC4 (Fig. 4). Similar results were obtained when using the EGFP-HDAC4 (1–1,084) construct (data not shown). Cotransfection of constitutively activated MEK1, the kinase that activates ERK1/2 (19–21), similarly causes an increased percentage of cells expressing E-HDAC4 in the nucleus (data not shown).

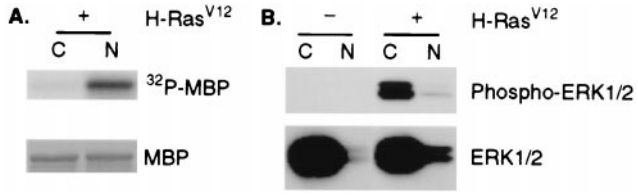

Fig. 5. Higher kinase activity is associated with nuclear F-HDAC4 rather than with cytoplasmic F-HDAC4 in oncogenic Ras-cotransfected cells. (*A*) F-HDAC4 immunoprecipitates containing an equal amount of F-HDAC4 protein from the cytoplasmic (C) and nuclear (N) fractions of F-HDAC4- and H-Ras^{V12}-cotransfected 293T cells were assayed for protein kinase activity by using MBP as a substrate. (*Upper*) Autoradiography. (*Lower*) Commassie Blue stain. (*B*) Western blotting analyses of equal amount of protein of the cytoplasmic and nuclear extracts from transfected 293T cells without (−) and with (+) H-Ras^{V12}. Proteins (5 μg) were resolved by SDS/PAGE. Western blot analyses were performed by using antibodies against phospho-ERK1/2 (*Upper*) and ERK1/2 (*Lower*).

Consistent with these results, dominant negative Ras^{N17} has no effect on the intracellular localization of E-HDAC4 (data not shown). Thus, activation of the Ras-MAPK pathway modulates the intracellular localization of HDAC4.

Higher Kinase Activity Is Associated with Nuclear F-HDAC4 than with Cytoplasmic F-HDAC4 in Oncogenic Ras-Cotransfected Cells. To determine whether kinase activity is differentially associated with cytoplasmic or nuclear F-HDAC4, kinase assays were performed on anti-FLAG immunoprecipitates from the cytoplasmic and nuclear fractions of 293T cells cotransfected with F-HDAC4 and H-Ras^{V12}. The amount of F-HDAC4 in the immunoprecipitates was quantified by Western blot analysis (data not shown), and equal amounts of F-HDAC4 were used in the kinase assays. The kinase activity is higher in the F-HDAC4 immunoprecipitate from the nuclear fraction compared with that from the cytoplasmic fraction (Fig. 5*A*). Similar results were obtained by using an ERK-specific substrate, Elk1, as a substrate for the kinase assays (data not shown). Activation of ERK1/2 by oncogenic Ras was verified by Western blot analysis by using an antibody against activated ERK1/2 (Fig. 5*B*).

Discussion

In these studies, we show that a serine/threonine kinase activity is present in a complex with HDAC4 and can phosphorylate HDAC4 *in vitro*. HDAC4 is phosphorylated in intact cells. ERK1/2 are identified as two of the kinases that are associated with HDAC4.

Regulation of the intracellular localization of proteins has been recognized as a means to regulate the activity of a number of proteins involved in cell cycle control and signal transduction (23). We show that intracellular localization of HDAC4 is regulated, at least in part, by the Ras-MAPK signal transduction pathway. Activation of the Ras-MAPK signal transduction pathway in C2C12 cells results in an increased percentage of cells expressing E-HDAC4 in the nucleus. There is both a potential nuclear localization signal and a nuclear export signal sequence in the deduced amino acid sequence of HDAC4. It is currently unclear whether the activation of the Ras-MAPK signal transduction pathway modulates HDAC4 intracellular localization by regulating the nuclear import or export. The role of the interaction between HDAC4 and ERK1/2 in the regulation of HDAC4 localization is also unclear. We show that higher kinase activity is associated with nuclear F-HDAC4 than with cytoplasmic F-HDAC4 in oncogenic Ras-cotransfected cells. A fraction of activated ERK1/2 translocates to the nucleus (24, 25). It is possible that HDAC4 and activated ERK1/2 enter the nucleus as a complex.

Differentiation of myoblast cells such as C2C12 is inhibited by oncogenic Ras and mitogens (26, 27). H-Ras^{V12} inhibits the expression of MyoD and myogenin, two transcription factors required for myogenesis (28). MEF2 activity is required for the expression of MyoD and myogenin during muscle differentiation (29). HDAC4 interacts with MEF2 and inhibits both MEF2-mediated transcription and muscle differentiation (6, 9, 10). Further, mutations in HDAC4 that increase HDAC4 nuclear localization enhance the ability of HDAC4 to inhibit MEF2-mediated transcription (8, 11). HDAC inhibitors have been reported to reverse Ras-mediated transformation (30–32). Our results are consistent with oncogenic Ras inhibiting muscle differentiation by increasing nuclear expression of HDAC4. Taken together, the present findings support a model in which the chromatin-modifying enzyme HDAC is a target of the Ras-MAPK signal transduction pathway.

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