Regulation of histone deacetylase-2 by protein kinase CK2

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Running Title: Phosphorylation of HDAC2

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Histone deacetylase-2 (HDAC2) is a member of a large family of enzymes that alter gene expression by catalyzing the removal of acetyl groups from core histones. Originally isolated as a transcriptional corepressor, HDAC2 possesses extensive amino acid sequence homology to HDAC1 (the founding member and most extensively studied HDAC enzyme). Because of this high degree of sequence similarity between HDAC1 and HDAC2, coupled with the fact that the two always co-exist in the same complexes, it is difficult to assess whether different properties exist between these two proteins. Here, we report that similar to HDAC1, HDAC2 is a phosphoprotein. In addition, like HDAC1, the phospho-acceptor sites in HDAC2 are located in the C-terminal portion of the protein. However, unlike HDAC1, which can be phosphorylated by protein kinase CK2, PKA, and PKG, HDAC2 is phosphorylated uniquely by protein kinase CK2 in vitro. Studies using unfractionated cell extracts with CK2 inhibitors suggest that protein kinase CK2 is the major source of HDAC2 kinase. Finally, and perhaps most interesting, HDAC2 phosphorylation promotes enzymatic activity, selectively regulates complex formation, but has no effect on transcriptional repression. Together, our data indicate that like many HDACs, HDAC2 is regulated by posttranslational modification, particularly phosphorylation. Furthermore, we demonstrate for the first time that there are similarities and differences in the regulation of HDAC1 and HDAC2 by phosphorylation.
In eukaryotes, DNA is tightly bound to histones, forming repeating units of DNA-protein particles called nucleosomes. Each nucleosome contains a nucleosomal core particle, consisting of 146 base pairs of supercoiled DNA wrapped twice around a complex of eight histone molecules. The histone core complex consists of two molecules each of histones H2A, H2B, H3, and H4. Linker DNA of variable length connects the core particles to one another.

The four core histones can undergo different posttranslational modifications: acetylation, phosphorylation, methylation, ADP-ribosylation, and ubiquitination (1). Many thousands of different combinations of histone modification are possible, providing an abundance of regulatory potential. In fact, it was proposed that the combinatorial nature of histone modification may form a "histone code" that is read by other proteins to bring about distinct downstream events (2, 3). The existence of this code would considerably extend the information potential of the genetic code.

All core histones undergo postsynthetic acetylation of one or more lysine residues in the N-terminal third of the molecule, and numerous studies suggest that acetylation of histones correlates with gene expression. Although supporting experimental evidence is still lacking, it is reasonable to assume that acetylation of core histones can weaken their interaction with DNA. Recent evidence is also accumulating in support of a model in which acetylation/deacetylation, in addition to affecting the intrinsic folding properties of nucleosomal arrays, generates specific docking surfaces for proteins that, in turn, regulate chromatin folding and/or transcription. For instance, unacetylated tails may perhaps provide interaction sites for transcriptional repressors, while the acetylated tails may provide interaction sites for activating complexes (4, 5). In addition to its effect on transcription and chromatin assembly, acetylation/deacetylation of histones may have important roles in many cellular processes including DNA replication and repair, recombination, and chromosome segregation. Comprehensive knowledge of the mechanisms regulating histone acetylation and deacetylation is a definite prerequisite for elucidation of the potential histone code and eventually understanding the complex mechanisms of gene regulation in eukaryotic cells.

The first histone acetyltransferase (HAT) enzyme was identified in *Tetrahymena* and found to possess a high degree of amino acid sequence similarity to the yeast transcriptional adapter
GCN5 (6). Subsequently, over twenty proteins, including some very well characterized transcription activators and co-activators, were found to contain histone acetylating activities (reviewed in refs. 7-10). Each of these HATs may have a particular histone substrate specificity, and different HATs are specific with regard to which histone amino acids they will acetylate. Moreover, some "histone" acetyltransferases have a wide range of protein substrates in addition to histones.

Equally swift and significant advances have been made in the last six years toward identification of histone deacetylase (HDAC) enzymes. The first HDAC, HDAC1, was purified and cloned by Schreiber and colleagues using a trapoxin affinity matrix (11). Sequence analysis revealed that HDAC1 is related to the yeast protein RPD3. At the same time, a second human histone deacetylase protein, HDAC2, also with high homology to yeast RPD3, was identified in our laboratory based on a yeast two-hybrid screen with the YY1 transcription factor as bait (12). Human HDAC1 is highly homologous to the human HDAC2 protein with 75% identity in DNA sequence and 85% identity in protein sequence. HDAC1 and HDAC2 exist together in at least three distinct multi-protein complexes called the Sin3, the NuRD/NRD/Mi2, and the CoREST complexes (13-21). In addition, many transcription factors interact directly with HDAC1/2 and thus may target HDAC1/2 to specific promoters (17, 22). Detailed analyses of the HDAC1/2 complexes revealed an unprecedented connection between deacetylation, DNA methylation, and chromatin remodeling.

Since the initial discovery of HDAC1 and HDAC2, many additional HDACs have been identified in various species including human, mouse, chicken, Xenopus, Drosophila, C. elegans, yeast, and maize. In humans and in mice, HDACs are divided into three categories (22-25): the class I RPD3-like proteins (HDAC1, HDAC2, HDAC3, and HDAC8); the class II HDA1-like proteins (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10); and the class III SIR2-like proteins (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, SIRT7). The class III proteins do not show any sequence resemblance to class I/II HDACs and are unique in that their activity requires the cofactor NAD.
Many observations suggest that the function of the class II HDACs is tightly regulated by phosphorylation. For example, the 14-3-3 proteins negatively regulate the actions of HDAC4 by excluding it from the nucleus (26, 27), and calcium/calmodulin-dependent protein kinase (CaMK) signaling induces nuclear export of HDAC4 and HDAC5 by phosphorylating these proteins (28). In contrast, activation of the Ras-MAPK pathway by expression of Ras or constitutively active MAPK/ERK kinase 1 increases the nuclear:cytoplasmic ratio of HDAC4 (29). Recently, it was reported that like many class II HDACs, HDAC1 (the prototype HDAC), is also a phosphoprotein (30, 31).

Because HDAC2 typically co-exists in the same protein complexes as HDAC1 and plays a crucial role in gene regulation, a complete picture of how phosphorylation may regulate HDACs requires that HDAC2 kinases be identified and the functional consequences of HDAC2 phosphorylation clearly determined. Here, we report that HDAC2 is phosphorylated at the C-terminal portion of the protein exclusively at serine residues. Mutational analysis revealed that Ser$^{394}$ and potentially Ser$^{411}$, Ser$^{422}$, and Ser$^{424}$ of HDAC2 are phosphorylated in vivo. Further, we found that the protein kinase, CK2, is responsible for phosphorylation of HDAC2. Our data also indicate that phosphorylation of HDAC2 is more critical for complex formation with mSin3 and Mi2 than with HDAC1. Finally, our results suggest that phosphorylation of HDAC2 is essential for enzymatic activity but not for transcriptional repression.
EXPERIMENTAL PROCEDURES

Cell culture-- 2X10^5 HeLa cells were cultured in 60-mm tissue culture dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin.

Plasmids -- pBJ5-HD1-F (11), pME18S-HDAC2 (32), pCS2-MT-mSin3 (16), pGal4-HDAC2 (32), pGEX-HDAC3 (32), pGEX4T-3-HDAC8 (33), pGST-HDAC1 (32), and pGST-H2B (34) have been described. pGEX4T-1-HDAC2, pCEP4FLAG-HDAC8, pGal4E1B-luc, and plasmids that express different FLAG-tagged or Gal4-fused HDAC2 mutants were constructed by standard PCR and recombinant DNA methods. All constructs were verified by automated DNA sequencing analysis.

Immunochemical reagents and techniques -- Polyclonal anti-HDAC2, anti-HDAC1, and anti-mSin3 have been described (16, 35). Anti-FLAG M2 and anti-MYC antibodies were obtained from Sigma Biochemical. Anti-Mi2 antibody was obtained from Santa Cruz Biotechnology. For immunoprecipitations, cells were rinsed with ice-cold Tris-buffered saline, and lysed in 1 ml of either a modified RIPA buffer containing protease and phosphatase inhibitors (1% NP-40, 0.5% DOC, 0.1% SDS, 0.15 M NaCl, 0.05 M Tris [pH 7.5], 2 mM EDTA, 0.05 M NaF, 0.2 mM Na_3VO_4, 0.5 mM PMSF) or in a low stringency buffer (PBS containing 0.1% NP-40). After incubation for 20 min at 4°C, the lysates were clarified by centrifugation at 4°C for 10 min in a microcentrifuge at maximum speed (14,000xg), and primary antibodies were added to the resultant supernatants. After incubation for 1 h at 4°C, immunocomplexes were collected, washed four times with the buffer used for lysis, and applied to 8% SDS- polyacrylamide gels.

In vivo phosphate labeling -- HeLa cells were washed once with phosphate-free DMEM and incubated in phosphate-free DMEM containing 10% dialyzed fetal calf serum and 2 mCi of [32P]orthophosphate for 4 h. For experiments that involved the labeling of FLAG-HDAC2 proteins in vivo, expression plasmids were transfected into cells using a standard calcium phosphate coprecipitation method. Cells were harvested 48 h after transfection.
Phosphoamino acid analysis and phosphopeptide mapping -- Phosphoamino acid analysis was performed as described (36). Briefly, $^{32}$P-labeled HDAC2 was transferred onto a polyvinylidene difluoride (PVDF) membrane. The transferred product was visualized by autoradiography, excised from the membrane, and hydrolyzed in 200 µl of 6N HCl at 110°C for 60 min. After centrifugation for 1 min at 14,000Xg, the hydrolysate was dried under vacuum and resuspended in 5 µl of buffer I (0.58 M formic acid, 1.36 M glacial acetic acid [pH 1.9]) plus a mixture of phosphoamino acid standards (1 µl of a mixture of phosphoserine, phosphothreonine, and phosphotyrosine at 1 mg/ml each). Approximately 3,000 cpm of the hydrolysate was spotted on a 20-cm$^2$, 100-µm-thick glass-backed cellulose thin-layer chromatography plate (EM Science). The first dimension was resolved by electrophoresis at 1,500 V for 25 min in buffer I at 16°C. Electrophoresis of the second dimension was carried out at 1,300 V for 20 min in buffer II (0.87 M glacial acetic acid, 0.5% pyridine, 0.5 mM EDTA [pH 3.5]). After being dried, plates were sprayed with 0.25% (wt/vol) ninhydrin in acetone and developed at 65°C for 10 min to visualize the phosphoamino acid standards. Autoradiography was performed to visualize labeled HDAC2 fragments.

Kinase assays -- Two µg of GST-fusion proteins were incubated with either PKA (New England Biolab), PKC (Boehringer Mannheim), PKG (Calbiochem), or CK2 (New England Biolab) in the presence of 5 µCi of [$\gamma$-$^{32}$P]ATP, 100 µM ATP, and manufacturer supplied kinase buffers in a total volume of 20 µl for 30 min at 30°C. The reactions were terminated by the addition of 2X SDS loading buffer and boiled for 5 min. Proteins were separated on 8% SDS-polyacrylamide gels, and phosphorylated proteins were visualized by autoradiography.

For analytical phosphorylation reactions, total cell extracts prepared from HeLa cells and 1 µg of purified FLAG-HDAC2 were incubated in a total volume of 20 µl at 30°C for 30 min with recombinant CK2 and 5 µCi of [$\gamma$-$^{32}$P]ATP (or [$\gamma$-$^{32}$P]GTP) in CK2 buffer (25 mM Tris/HCl [pH 8.5], 10 mM MgCl$_2$, 1 mM DTT). The kinase reactions were stopped by the addition of 2X sample loading buffer, boiled, centrifuged, and analyzed in SDS-polyacrylamide gels followed by
autoradiography. CK2 inhibitors apigenin (chrysin) and 6-dichloro-1-D-ribofuranosylbenzimidazole (DRB) were obtained from Sigma Biochemical.

Western blot analysis -- Proteins were transferred from SDS-polyacrylamide gels onto PVDF membranes. After blocking with 4% nonfat dried milk, the membranes were treated with diluted primary antibodies followed by 1:7500 diluted alkaline phosphatase-conjugated rabbit anti-mouse IgG (Promega). Subsequently, the blots were developed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Promega).

Luciferase assays -- 2X10^5 HeLa cells were seeded in 60-mm culture dishes 18 h before transfection. Plasmids directing the synthesis of various effector proteins and luciferase reporters were introduced into cells using a standard calcium phosphate coprecipitation method. Each transfection contained 5 µg each of effector and reporter DNAs, and all transfections were normalized to equal amounts of DNA with parental expression vectors. Forty-eight hours after transfection, cells were collected and luciferase activity was determined with the Dual Luciferase Reporter Assay System (Promega).

Histone deacetylation assay -- [3H]acetate-incorporated histones were isolated from butyrate-treated HeLa cells by acid extraction as described (37). Briefly, 5,000 cpm of purified core histones were incubated with immunoprecipitates in 150 µl of ice-cold HD buffer (20 mM Tris, [pH 8.0], 150 mM NaCl, and 10% glycerol) at room temperature overnight with mild shaking. The reaction was terminated by the addition of an equal volume of stop solution (0.16 M acetic acid, 1.0 M HCl) and mixed well by vortexing. The released [3H]acetate was extracted with ethyl acetate and combined with scintillation mixture for analysis.

Sequence analysis -- Searches for protein kinase recognition motifs in HDAC1/2 were done using the PhosphoBase 2.0, a database of phosphorylation sites at www.cbs.dtu.dk/databases/phosphobase.
RESULTS

**HDAC2 is phosphorylated at serine residues in vivo** -- To determine if HDAC2 is subject to phosphorylation modification, we prepared an extract from metabolically $^{32}$P-labeled HeLa cells, immunoprecipitated the endogenous HDAC2 protein, and resolved the product on an SDS-polyacrylamide gel. As a negative control, a mock immunoprecipitation was carried out side-by-side with the same labeled extract. As shown in Fig. 1A, HDAC2 is radioactively labeled by inorganic phosphate indicating that the protein is constitutively phosphorylated in vivo.

Using partial acid hydrolysis in HCl followed by two-dimensional thin layer electrophoresis of the labeled phosphoamino acid, the presence of phosphoserine but not phosphothreonine or phosphotyrosine were unambiguously identified in HDAC2 (Fig. 1B).

**Phosphorylation sites in HDAC2 include Ser$_{394}$, and potentially Ser$_{411}$, Ser$_{422}$, and Ser$_{424}$** -- To identify the exact phosphorylation sites on HDAC2, we transfected HeLa cells with FLAG-tagged full-length wildtype HDAC2 or various C-terminal deletion mutants, repeated the in vivo labeling, and immunoprecipitated the FLAG-fusion proteins with an anti-FLAG antibody for analysis on SDS-polyacrylamide gel. Fragments corresponding to residues 1-420 and 1-400 of HDAC2 were phosphorylated to similar levels compared with the full-length HDAC2 (1-488) (Fig. 2A, compare lane 1 to lanes 2 and 3; Fig. 2B). A fragment containing residues 1-380 of HDAC2 (lane 4), however, clearly was not phosphorylated in vivo indicating that at least one phosphorylation site exists at 380-400 of HDAC2.

Sequence analysis reveals that the only potential phosphorylation site located between 380-400 of HDAC2 is Ser$_{394}$ (Fig. 2C). To confirm that Ser$_{394}$ is indeed a phosphorylation site, we tested the ability of the mutant 1-398 (S394A) to be phosphorylated. Our results showed that 1-398 (S394A) cannot be phosphorylated in vivo (Fig 2A, lanes 5 and 7; Fig. 2B), unequivocally demonstrating that Ser$_{394}$ of HDAC2 is a phosphoacceptor site.

To determine if additional phosphoserines are present in HDAC2, we repeated the transfection, labeling, and immunoprecipitation with a full-length HDAC2 expression construct mutated in Ser$_{394}$. As shown in Fig. 2A (lanes 8 and 12) and Fig. 2B, 1-488 (S394A) was
phosphorylated in vivo, arguing for the existence of additional phosphorylation site(s). Besides Ser\textsuperscript{394}, four additional conserved serine residues (Ser\textsuperscript{407}, Ser\textsuperscript{411}, Ser\textsuperscript{422}, Ser\textsuperscript{424}) are present between 380 to 488 of HDAC2 (Fig. 2C). Further analysis showed that 1-414 (S394/411A) was not labeled and 1-488 (S394/411/422/424A) was labeled very modestly in the presence of γ\textsuperscript{32}P-ATP indicating that Ser\textsuperscript{407} is not a phosphorylation site in HDAC2, and any combination of Ser\textsuperscript{394} with Ser\textsuperscript{411}, Ser\textsuperscript{422}, or Ser\textsuperscript{424} could be phosphorylated (Fig. 2A, lanes 9 and 13; Fig. 2B).

**HDAC2 is phosphorylated by protein kinase CK2** -- A careful inspection of the amino acid sequence of HDAC2 revealed that while Ser\textsuperscript{394}, Ser\textsuperscript{422}, and Ser\textsuperscript{424} all lie within protein kinase CK2 recognition motifs, Ser\textsuperscript{411} could potentially be phosphorylated by PKA and PKC (Table 1). To determine if CK2, PKA, or PKC were indeed the kinases for HDAC2, we first tested the ability of purified CK2 to phosphorylate HDAC2 in vitro. As presented in Fig. 3A, GST-HDAC2 was readily phosphorylated by CK2 in vitro (lane 3). For comparisons, we examined the other class I HDACs, and, interestingly, GST-HDAC1 and GST-HDAC3 but not GST-HDAC8 were phosphorylated in similar fashions (lanes 2, 4, 5).

Intriguingly, we found that PKA does not phosphorylate GST-HDAC2 or GST-HDAC3 in vitro, although it efficiently phosphorylated GST-HDAC1 and GST-HDAC8 (Fig. 2B). Furthermore, PKC did not phosphorylate any class I HDACs under the same condition (data not shown). In addition to consensus sequences for CK2, PKA, and PKC, residues 380-488 of HDAC2 contains recognition motifs for PKG in Ser\textsuperscript{407} and Thr\textsuperscript{454}. As predicted, GST-HDAC2 was not phosphorylated by PKG in vitro (Fig. 3C, lane 3) confirming that Ser\textsuperscript{407} is not a phosphorylation site in vivo (Fig. 2A) and that HDAC2 is phosphorylated exclusively at serine residues (Fig. 1B). Like GST-HDAC2, GST-HDAC3 and GST-HDAC8 were not substrates for PKG (Fig. 3C, lanes 4 and 5). However, unlike GST-HDAC2, GST-HDAC1 is phosphorylated by PKG (lane 2). Further experiments using FLAG-HDAC2 and FLAG-1-488 (S394/422/424A) expressed and purified from bacteria conclusively confirmed that HDAC2 is a substrate for CK2 in vitro (Fig. 3D).
To confirm that CK2 is the major protein kinase and not just one of many kinases that phosphorylate HDAC2, we used a total HeLa cell lysate to phosphorylate purified HDAC2 in the presence or absence of specific CK2 inhibitors, apigenin and DRB. Results presented in Fig. 4A and 4B clearly showed that phosphorylation of HDAC2 was markedly reduced in the presence of CK2 inhibitors arguing that CK2 is the major and perhaps sole kinase responsible for phosphorylation of HDAC2. One of the unique features of CK2 among eukaryotic protein kinases is that CK2 can use both ATP and GTP as phosphoryl donors. Consistent with the observation that CK2 is the major kinase for HDAC2, we found that phosphorylation of HDAC2 by a total cell lysate was equally efficient using either ATP or GTP as phosphoryl donors (Fig. 4B).

Phosphorylation of HDAC2 may affect its enzymatic but not transcriptional activity -- HDAC2 was originally identified as a transcriptional corepressor (12). Early studies showed that a Gal4-HDAC2 fusion repressed transcription when targeted to promoters containing Gal4 binding sites (12). Our findings that HDAC2 is modified by phosphorylation raise the important question of whether phosphorylation modulates the transcriptional activity of HDAC2 in the cell. To address this issue, we constructed plasmids that expressed Gal4-1-488 (S394A) and Gal4-1-488 (S394/411/422/424A), and transfected them into cells together with a reporter containing Gal4-binding sites. Surprisingly, both of these Gal4 fusion mutant proteins repress transcription similar to wildtype Gal4-HDAC2 suggesting that phosphorylation of HDAC2 is not important for transcriptional repression (Fig. 5A). Consistent with this observation, a Gal4-1-380 fusion protein that can no longer be phosphorylated in vivo retained full repression activity (Fig. 5A).

To determine if phosphorylation influences HDAC2 enzymatic activity, we expressed various FLAG-HDAC2 mutants, immunoprecipitated the proteins with anti-FLAG antibody, and tested their abilities to deacetylate core histones. As shown in Fig. 5B, histone deacetylase activity of HDAC2 is strictly correlated with its ability to be phosphorylated in vivo. 1-488 (S394A), which is phosphorylated slightly less than wildtype HDAC2 (1-488), had slightly less enzymatic activity. 1-488 (S394/411/422/424A), which is severely impaired in phosphorylation in vivo, had approximately 30% deacetylase activity compared to wildtype HDAC2. An HDAC2 mutant that
was totally not phosphorylated \textit{in vivo}, 1-380, did not possess any enzymatic activity. Taken together, our data suggest that while phosphorylation of HDAC2 has no role in transcriptional repression, it is absolutely critical for enzymatic activity.

\textit{Phosphorylation of HDAC2 may affect its interaction with cellular factors} -- HDAC2, together with HDAC1, exists in two major complexes called the Sin3 complex and the NuRD/NRD/Mi2 complex. To determine if phosphorylation of HDAC2 changes the protein's ability to interact with other proteins, we compared the amount of coimmunoprecipitated HDAC1, mSin3, and Mi2 from nuclear extracts prepared from cells that expressed wildtype or mutant HDAC2. In multiple experiments, we repeatedly found that compared to wildtype HDAC2, a significant (although somewhat smaller) fraction of endogenous HDAC1 coprecipitated with 1-488 (S394A) and 1-488 (S394/411/422/424A), as detected via Western blot analysis (Fig. 6A, compare lane 2 to lanes 3 and 4 in mid-top panel). However, neither mSin3 nor Mi2 coprecipitated with 1-488 (S394A) or 1-488 (S394/411/422/424A) suggesting that mSin3 and Mi2 preferentially bind phosphorylated HDAC2 (compare lane 2 to lanes 3 and 4 in bottom two panels). Further evidence obtained from experiments using co-transfection of FLAG-HDAC2 and MYC-mSin3, followed by immunoprecipitation with anti-FLAG antibody and Western blot with an anti-MYC antibody, confirmed that phosphorylation of HDAC2 enhances its interaction with some cellular proteins (Fig. 6B, compare lane 2 to lanes 3 and 4).
DISCUSSION

Changes in phosphorylation states control the activity of a wide range of regulatory proteins in the cell. This is indeed the case for many class II histone deacetylases where phosphorylation regulates their subcellular localization and, therefore, their deacetylase enzymatic activities. Recently, it was shown that HDAC1, a class I enzyme, can also be regulated by phosphorylation (30, 31). However, the significance of HDAC1 phosphorylation is ambiguous at this time with one report that phosphorylation promotes enzymatic activity and a contradictory report that phosphorylation did not influence enzymatic activity (30, 31).

While Cai et al. (31) found that at least one phosphorylation site exists between residues 387 and 409 of HDAC1, the study by Pflum et al. (30) indicates that HDAC1 is phosphorylated at Ser$^{421}$ and Ser$^{423}$ (corresponding to Ser$^{422}$ and Ser$^{424}$ of HDAC2). In this current study, using HDAC2 deletions, we found that Ser$^{394}$ (which corresponds to Ser$^{393}$ of HDAC1) is definitely phosphorylated in vivo. Additionally, one or more residues consisting of Ser$^{411}$, Ser$^{422}$, and Ser$^{424}$ of HDAC2 could also be phosphorylated. Since our data suggest that protein kinase CK2 is the major kinase responsible for HDAC2 phosphorylation, and because Ser$^{422}$ and Ser$^{424}$, but not Ser$^{411}$, lie within CK2 recognition sequences, we believe that Ser$^{394}$, Ser$^{422}$, and Ser$^{424}$ constitute the three phosphorylated residues in HDAC2.

Consistent with the reports by Cai et al. (31) and Pflum et al. (30), we found that HDAC1 can be phosphorylated by CK2 and PKA in vitro, but not by PKC. Additionally, we report here that HDAC1 can serve as a substrate for PKG in vitro. However, unlike HDAC1, HDAC2 is phosphorylated in vitro only by CK2 but not by PKA or PKG. Thus, it appears that although there is a remarkable conservation between the HDAC1 and HDAC2 proteins, and that the similar residues of HDAC1 and HDAC2 may be targeted for phosphorylation, the kinases and the mechanisms of phosphorylation may differ between these two different class I enzymes.

CK2 is a serine/threonine protein kinase ubiquitously distributed in both the cytoplasm and nucleus of eukaryotic cells (38). It has been implicated in the regulation of many cellular processes, including DNA replication, basal and inducible transcription, and the regulation of cell growth and
metabolism (39-42). CK2 has a broad range of substrates that include nuclear oncoproteins such as Myc, Myb, Jun, and Fos. It plays an important role in the transduction of extracellular signals to effector proteins in the nucleus and appears to be responsible for phosphorylation of many growth-related and cell-cycle specific proteins (43, 44). Studies have shown that CK2 may contribute to tumorigenesis, and dysregulated expression of CK2 in cells can be oncogenic. In this regard, it is interesting to note that HDACs are also critical in homeostasis, cell cycle control, and cancer development. For example, it is established that HDAC2 interacts with cellular proteins implicated in cancer, such as the Rb, p53, and metastasis-associated protein 2 (MTA2) (22). Future experiments will be directed toward understanding whether phosphorylation, particularly by CK2, might contribute to the overall role of HDACs in cancer.

Many studies have clearly demonstrated that cloned HDAC proteins can repress transcription. For example, a Gal4-HDAC2 fusion protein can effectively repress transcription when targeted to promoters containing Gal4 binding sites (12). However, we do not yet know whether histone deacetylase activity per se is necessary for HDAC2-mediated repression. This is a critical issue because although many mammalian transcription repressors have been shown to interact with HDACs, the role of HDAC activity in the function of these complexes remains largely unknown. Here, we learned that while a direct correlation exists between the degree of HDAC2 phosphorylation and HDAC activity, there is no relationship between HDAC2 phosphorylation and transcriptional repression. An HDAC2 mutant devoid of enzymatic activity still possesses repression activity. This surprising finding, then, suggests that there could be a deacetylase activity-independent repression domain(s) that lies outside of the C-terminal phosphorylated region of HDAC2, and a model in which HDAC-binding proteins effect repression via recruitment of deacetylase activity may not suffice in all cases.

Shortly after the discovery of HDAC1 and HDAC2, a number of studies indicated that association with cellular proteins that can modulate their activity or recruit them to DNA is one of the chief regulatory mechanisms for these two proteins. HDAC2 is always found present with HDAC1 and together they exist in two major complexes called the Sin3 complex and the
NuRD/NRD/Mi2 complex (13, 14, 16-18, 20, 21). It is generally thought that by interacting with mSin3 it provides a common platform for a large number of repressors to indirectly interact with HDAC1/2, and the interaction with Mi2 provides HDAC1/2 a functional link with an ATP-dependent chromatin remodeling activity. Our observations that hypophosphorylated HDAC2 disrupts interaction with mSin3 and Mi2, but only modestly affects formation of HDAC1/2 complex, suggest that the HDAC2-interacting domain is probably similar for mSin3 and Mi2 but distinct from HDAC1. Also, it is conceivable that phosphorylation of HDAC2 alters its structural conformation to favor interaction with some cellular proteins, such as mSin3 and Mi2, but has no influence on its interaction with other proteins. In future work, it will be important to determine whether phosphorylation of HDAC2 alters its interaction with other proteins in addition to Mi2 and mSin3.

Perhaps the most interesting finding from this current study is that in addition to HDAC1 and HDAC2, we have found that the remaining class I enzymes, HDAC3 and HDAC8, can also be phosphorylated *in vitro*. While CK2, but not PKA or PKG, phosphorylates HDAC3, HDAC8 is phosphorylated by PKA, but not CK2 or PKG. Since the C-terminal domains in HDAC1 and HDAC2 are not conserved in HDAC3 or HDAC8, it is obvious that the phosphorylation sites in HDAC3/8 are different from those in HDAC1/2. We believe a next step in elucidating how posttranslational modification regulates class I HDACs is to determine if HDAC3 and HDAC8 are phosphorylated *in vivo*, and if so, which residues are involved and what kinase(s) is responsible. Current studies in our laboratory are focused upon these important issues.
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While this manuscript was under final preparation, we learned that Ahn and colleagues reported that phosphatase inhibition leads to HDAC1/2 phosphorylation and disrupts corepressor interactions (45). The data presented in this current manuscript fits well with this recent development.
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The abbreviations used are: HDAC, histone deacetylase; HAT, histone acetyltransferase; CaMK, calcium/calmodulin-dependent protein kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; PVDF, polyvinylidene difluoride.
REFERENCES


LEGENDS

Fig. 1. **In vivo phosphorylation of HDAC2.**  

**A**, HeLa cells were labeled with 0.5 mCi/ml $^{32}$P orthophosphate for 6 h before harvest and subsequently immunoprecipitated with an anti-HDAC2 antibody. Immunoprecipitates were separated in an 8% SDS-polyacrylamide gel and visualized by autoradiography. "Mock IP" indicates a negative control in which the reaction was carried out identically but without any antibody. The positions of molecular weight markers are indicated on the left.  

**B**, Two-dimensional phosphoamino acid analysis. Immunoprecipitated, labeled HDAC2 was excised from the gel and subjected to partial acid hydrolysis and analyzed by cellulose thin-layer chromatography. PS, PT, and PY indicate the position of phosphoserine, phosphothreonine, and phosphotyrosine, respectively.

Fig. 2. **Identification of phosphorylation sites within HDAC2.**  

**A**, Plasmids expressing FLAG-HDAC2 or various mutants were transfected into HeLa cells. After labeling with $^{32}$P orthophosphate, an extract was prepared and FLAG-tagged proteins were immunoprecipitated with an anti-FLAG antibody followed by analysis on SDS-polyacrylamide gels. Western blots were performed prior to autoradiography to show expression of various proteins.  

**B**, Schematic drawing of plasmids used in transient transfections. For simplicity, the FLAG portions of the fusion proteins and the other areas of each plasmids are not shown here. The ability of each fusion protein to be phosphorylated *in vivo* is indicated (+ or -).  

**C**, The C-terminal regions of HDAC1 and HDAC2 from mice and humans. The amino acid sequence of mouse and human HDAC1/2 are aligned and amino acid positions are boxed where there is identity among HDAC1 and HDAC2 in both species. Asterisks indicate potential phosphorylation sites in HDAC2 and numbers above each asterisk correspond to residues in HDAC2.
Fig. 3. **In vitro phosphorylation of class I HDACs with purified kinases.** Bacterially expressed, purified, GST-tagged (A, B, C) or FLAG-tagged (D) HDAC proteins were used as substrates for **in vitro** phosphorylation. The proteins were resolved by SDS-polyacrylamide gels and the $^{32}$P-radiolabel was visualized by autoradiography. GST-H2B was used as a positive control. The positions of molecular weight markers are indicated on the left. The positions of the most heavily phosphorylated proteins are indicated by an arrow (top panels). Coomassie blue stains were performed prior to autoradiography to visualize the locations and amounts of the different fusion proteins. Arrows in bottom panels indicate bands corresponding to each fusion protein.

Fig. 4. **Inhibition of HDAC2 phosphorylation by CK2 inhibitors.** Bacterially expressed, purified FLAG-HDAC2 proteins were incubated with a HeLa cell nuclear extract in the presence of [$\gamma$-$^{32}$P]-ATP or -GTP. Different amounts of CK2 inhibitors were added to each **in vitro** phosphorylation reaction as indicated. Western blots were performed prior to autoradiography to show approximately equal loading of HDAC2 proteins in each lane.

Fig. 5. **HDAC2 phosphorylation is critical for enzymatic activity but not for transcriptional repression.** A, HeLa cells were transfected with various constructs expressing Gal4-HDAC2, as indicated, plus a pGal4E1B-luc reporter. Transcriptional repression was determined by luciferase activity. All transfections were normalized to equal amounts of DNA with parental expression vectors. Data shown are the average results ± SD from three separate transfections. B, HeLa cells were transfected with various constructs expressing FLAG-HDAC2, as indicated, and FLAG-immunoprecipitates were assayed for histone deacetylase activity. Each experiment was performed in triplicate and data shown are the mean ± SD.
Fig. 6. **HDAC2 phosphorylation is critical for protein complex formations.**  

A, Expression plasmids for FLAG-HDAC2 or mutants were transfected into HeLa cells. Two days later, cells were harvested and extracts prepared for immunoprecipitation with an anti-FLAG antibody. Immunocomplexes were separated on an 8% SDS-polyacrylamide gel followed by Western blotting with indicated antibodies. B, A Western blot was performed on anti-FLAG immunoprecipitates using an anti-MYC antibody (top panel). Immunoprecipitated products were obtained from HeLa whole cell extracts transfected with plasmids expressing the indicated FLAG-tagged proteins. Separate Western blots were performed on the same extracts to show equivalent expression of the MYC-mSin3 proteins and to confirm approximately equal immunoprecipitation of the FLAG-fusion proteins (middle and bottom panels).
Table 1. Potential phosphorylation sites in HDAC1/2

<table>
<thead>
<tr>
<th>kinase</th>
<th>consensus</th>
<th>potential sites in HDAC1</th>
<th>potential sites in HDAC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK2</td>
<td>X-S/T-X-X-D/E</td>
<td>393, 421, 423, 445, 460, 465</td>
<td>394, 422, 424, 480</td>
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<tr>
<td>PKA</td>
<td>R-X1-2-S/T-X</td>
<td>406, 434, 445</td>
<td>407, 411</td>
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<td>PKC</td>
<td>X-S/T-X-R/K</td>
<td>410</td>
<td>407, 411</td>
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<td>PKG</td>
<td>(R/K)2-3-X-S/T-X</td>
<td>406, 434</td>
<td>407, 454</td>
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</table>

White shadowed numbers indicate potential phosphorylation sites identified by mutational analyses.
Figure 1

A

MW (KDa)
150
100
75
50
37.5

mock IP
IP α-HDAC2

B

pH 3.5

PS
PT
PY

pH 1.9
Figure 2B

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</tr>
<tr>
<td>1-398 (S394A)</td>
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<tr>
<td>1-488 (S394A)</td>
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<tr>
<td>1-414 (S394/411A)</td>
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<tr>
<td>1-488 (S394/411/422/424A)</td>
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<td>** **</td>
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</tbody>
</table>
Figure 2C

379  V  Q  M  Q  A  I  P  E  D  A  I  P  E  E  S  G  D  E  D  E  D  D  P  D  K  R  I  S  I  C  hHDAC1
379  V  Q  M  Q  A  I  P  E  D  A  I  P  E  E  S  G  D  E  D  E  D  E  D  P  D  K  R  I  S  I  C  mHDAC1
380  V  Q  M  Q  A  I  P  E  D  A  V  H  E  D  S  G  D  E  D  G  E  D  P  D  K  R  I  S  I  R  hHDAC2
380  V  Q  M  Q  A  I  P  E  D  A  V  H  E  D  S  G  D  E  D  G  E  D  P  D  K  R  I  S  I  R  mHDAC2

394  *

411  *

422  *

424  *

409  S  S  D  K  R  I  A  C  E  E  E  F  S  D  S  D  E  E  G  E  G  G  R  K  N  S  S  S  N  F  K  mHDAC1
410  A  S  D  K  R  I  A  C  D  E  E  F  S  D  S  E  D  E  E  G  E  G  G  R  R  N  V  A  D  H  K  hHDAC2
410  A  S  D  K  R  I  A  C  D  E  E  F  S  D  S  E  D  E  E  G  E  G  G  R  R  N  V  A  D  H  K  mHDAC2

439  K  -  A  K  R  V  K  T  E  D  E  K  E  K  D  P  E  E  E  E  K  K  E  V  T  E  E  E  K  T  K  E  mHDAC1

468  -  -  -  E  K  P  E  A  K  G  V  K  E  E  V  K  L  A  hHDAC1
468  -  -  -  E  K  P  E  A  K  G  V  K  E  E  V  K  L  A  mHDAC1
470  N  S  G  E  K  T  D  T  K  G  T  K  S  E  Q  L  S  N  P  hHDAC2
470  N  S  G  E  K  T  D  P  K  G  A  K  S  E  Q  L  S  N  P  mHDAC2
Figure 3

A

CK2

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<th>GST-H2B</th>
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<th>GST-HDAC3</th>
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B

PKA

<table>
<thead>
<tr>
<th>MW (KDa)</th>
<th>GST-H2B</th>
<th>GST-HDAC1</th>
<th>GST-HDAC2</th>
<th>GST-HDAC3</th>
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</tbody>
</table>

Coomassie

1 2 3 4 5
Figure 3C
Figure 3D
Figure 4A

HDAC2

apigenin (μM) 0 0.3 1.2 5 20

WB α-HDAC2

MW (KDa) 250 150 100 75 50 37.5

100 μM DRB
40 μM apigenin
**Figure 4B**

[Image of a Western Blot with apigenin concentrations on the x-axis and HDAC2 and WB α-HDAC2 bands on the y-axis, showing the effect of ATP and GTP on HDAC2 activity.]
Figure 5A

relative luciferase activity

Gal4  Gal4-HDAC2  Gal4-1-488 (S394A)  Gal4-1-488 (S394/411/422/424A)  Gal4-1-380
Figure 5B

The graph shows the histone deacetylase activity (cpm) for different samples:

- Vector
- HDAC2 (1-488)
- 1-488 (S394A)
- 1-488 (S394/411/422/424A)
- 1-380

The activity is highest for HDAC2 (1-488), followed by 1-488 (S394A) and 1-380, with the lowest activity in the Vector control.
Figure 6
Regulation of histone deacetylase-2 by protein kinase CK2
Shih-Chang Tsai and Edward Seto

J. Biol. Chem. published online June 24, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M204149200

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