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Histone Deacetylase 1 Phosphorylation Promotes Enzymatic Activity and Complex Formation*

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Accessibility of the genome to DNA-binding transcription factors is regulated by proteins that control the acetylation of amino-terminal lysine residues on nucleosomal histones. Specifically, histone deacetylase (HDAC) proteins repress transcription by deacetylating histones. To date, the only known regulatory mechanism of HDAC1 function is via interaction with associated proteins. Although the control of HDAC1 function by protein interaction and recruitment is well-documented, we were interested in exploring HDAC1 regulation by post-translational modification. Human HDAC1 protein was analyzed by ion trap mass spectrometry, and two phosphorylated serine residues, Ser421 and Ser423, were unambiguously identified. Loss of phosphorylation at Ser421 and Ser423 due to mutation to alanine provided the first step toward characterizing the role of post-translational modifications in regulating HDAC1 activity and complex formation of HDAC1. Deletion of the highly charged carboxyl-terminal region of HDAC1 also increased its deacetylase activity and protein associations, revealing its requirement in maintaining HDAC1 function. Our results reinforce the importance of protein associations in modulating HDAC1 function and provide the first step toward characterizing the role of post-translational modifications in regulating HDAC activity in vivo.

Although 30,000–40,000 genes exist in a human cell, only a fraction of those genes are transcribed into mRNA and ultimately translated into the encoding protein in any given cell type (1, 2). Transcription is regulated at many levels to selectively express only those proteins necessary for proper cell function. Although the role of DNA-binding transcription factors in controlling gene expression is well established, an additional level of regulation has been recently elucidated, the accessibility of genomic DNA to transcription factors (3).

The genome is tightly packed into chromosomes through nucleosomal protein-DNA complexes. Each nucleosome contains ~200 base pairs of double-stranded DNA wrapped twice around a histone protein core (4). Although the carboxyl-terminal region of the histones are involved in forming the nucleosome core, the amino-terminal, lysine-rich tails are located outside of the nucleosome core (5). Various studies revealed a correlation between the acetylation of amino-terminal lysine residues on histones and transcriptional activity in chromatin (3, 6). Therefore, the current model of transcriptional control includes DNA accessibility due to acetylation of histones.

The acetylation state of histones is dependent on the activity of two proteins, histone acetyltransferase and histone deacetylase (HDAC),1 which acetylate and deacetylate histones, respectively. Loss of HDAC activity using small molecule inhibitors correlates with the hyperacetylation of nucleosomal histones (7, 8). Importantly, HDAC proteins in yeast and humans are necessary to achieve the full transcriptional repression and induction of diverse genes (9, 10).

Not surprisingly, HDAC proteins are critical in fundamental cellular events, including cell cycle control, differentiation, and cancer formation (11). A small molecule inhibitor of HDAC function, trichostatin, causes cell cycle arrest in mammalian cells at both G1 and G2 phases, while overexpression of HDAC1 in mouse cells results in reduced growth rate due to lengthening of G0 and M phases (12, 13). Trichostatin induces terminal differentiation of murine erythroleukemia cells (8, 14) and induces apoptosis in neural, lymphoid, and colorectal cancer cell lines (15, 16). Finally, HDAC proteins interact with cellular proteins implicated in cancer development, including the retinoblastoma tumor suppressor (Rb), metastasis-associated protein 2 (MTA2), and nuclear hormone receptors like the retinoic acid receptor (17). As a well studied example, acute promyelocytic leukemia is associated with a fusion protein of retinoic acid receptor α and a promyelocytic leukemia zinc finger gene (promyelocytic leukemia zinc finger-retinoic acid receptor α) that recruits HDAC repression activity to block normal differentiation and lead to leukemia (18, 19). Trichostatin treatment of cells expressing promyelocytic leukemia zinc finger-retinoic acid receptor α derepressed transcription and allowed cells to differentiate normally (18, 19). With this precedent, HDAC inhibitors are being explored as potential drugs for the treatment of certain forms of leukemia (20, 21).

The human HDAC proteins are organized into classes based on their similarity to yeast HDAC proteins. Class I HDACs include HDAC1, HDAC2, HDAC3, and HDAC8, which are homologous to the yeast Rpd3 protein (22–26). Class II HDACs are similar to yeast Hda1 and include HDAC4, HDAC5, HDAC6, HDAC7, and HDAC9 (27–29). Finally, the recently

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1 The abbreviations used are: HDAC, histone deacetylase; Rb, retinoblastoma tumor suppressor; RbAg, Rb-associated protein; MTA, metastasis-associated protein; REST, RE1-silencing transcription factor/neuronal restrictive silencing factor; IP, immunoprecipitation; CK1 and CK2, casein kinase 1 and 2, respectively; NRD, nucleosome remodeling and deacetylating; MS, mass spectrometry; DRB, 5,6-dichloro-1-β-ribofuranosylbenzimidazole; RPLC, reverse phase liquid chromatography.
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identified NAD-dependent sirtuin (SIRT) proteins are homologous to the yeast Sir2 protein (30, 31). HDAC function is regulated by two known mechanisms. In the case of class II proteins, HDAC4 and HDAC5 shuttle between the nucleus and the cytoplasm via phosphorylation-dependent interaction with 14-3-3 protein (32–34). With class I proteins, HDAC1 and HDAC2 associate with proteins that modulate their deacetylase activity and recruitment to genomic regions (18, 35). Three characterized protein complexes contain HDAC1 and HDAC2. The Sin3 complex comprises HDAC1, HDAC2, Rbp48, Rbp46, mSin3, and Sin3-associated proteins 18 and 30 and interacts with DNA binding transcription factors, including Mad, Ikaros, REST, and nuclear hormone receptors (36–38). The nucleosome remodeling and deacetylating (NRD or NuRD) complex includes HDAC1, HDAC2, Rbp48, Rbp46, Mi-2 (or CHD), methyl-CpG-binding domain 3, and MTA2. The NRD complex combines deacetylation by HDAC proteins with ATP-dependent nucleosome remodeling to affect transcription (39–41). Recently, the CoREST complex, comprising HDAC1, HDAC2, CoREST, and p110, was identified (42). The identification of a group of repressor and corepressor proteins that bind HDAC proteins suggests a model where HDAC1 and HDAC2 are targeted to certain DNA promoters or chromosomal domains to affect transcription of cellular genes.

Although the control of HDAC1 and HDAC2 function by protein interaction and recruitment is well preceded, we were interested in exploring HDAC1 regulation by post-translational modification. We began our study by analyzing the sequence of HDAC1 by mass spectrometry. Two phosphorylated serines were unambiguously identified at Ser421 and Ser423. Mutagenesis experiments revealed that phosphorylation at Ser421 and Ser423 promoted the enzymatic activity and complex formation of HDAC1. The protein kinase casein kinase 2 (CK2) was shown to phosphorylate HDAC1 in vitro. Disruption of the CK2 consensus sequence directing phosphorylation of Ser421 and Ser423 altered the enzymatic activity and complex formation of HDAC1. Glutamic acid and aspartic acid only partially substituted for the phosphoserines, indicating that the charge and size of phosphate are uniquely suited to maintain HDAC1 function. Finally, our data support a model where the carboxyl-terminal region of HDAC1 is essential for maintaining the protein association and enzymatic activity of HDAC1.

EXPERIMENTAL PROCEDURES

Plasmids—All HDAC1 mammalian expression plasmids were constructed by inserting the HDAC1-FLAG sequence into the NotI/EcoRI sites of pBluescript II KS (22). Point mutants of HDAC1 were created by a two-step PCR amplification using the following primers (mutated bases are underlined): NotI-HDAC1, GGCGATCGGCGGGAGAGGGGAGAGG; EcoRI-HDAC1, CC GGCGAGTCTTCTACATCATACATTTATATACCC. PCR amplification with the algorithm SEQUEST (44), phosphorylated peptides were confidently identified (42). The identification of a group of repressor and corepressor proteins that bind HDAC proteins suggests a model where HDAC1 and HDAC2 are targeted to certain DNA promoters or chromosomal domains to affect transcription of cellular genes.

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**RESULTS**

**HDAC1 Is a Phosphoprotein**—To determine whether HDAC1 is a phosphoprotein, simian virus 40 large T-Ag Jurkat cell lysates were treated with alkaline phosphatase, and HDAC1 was visualized using an HDAC1-specific antibody (Fig. 1A). In the presence of phosphatase, HDAC1 migrated more quickly in a polyacrylamide gel than untreated HDAC1, consistent with the possibility that HDAC1 is phosphorylated.

To verify that HDAC1 is phosphorylated, T-Ag Jurkat cell lysates were labeled in vivo with [32P]orthophosphate, and HDAC1 was immunoprecipitated with an HDAC1-specific antibody. Immunoprecipitated HDAC1, which migrated by SDS-PAGE identically to HDAC1 from lysates (data not shown), was labeled with [32P], indicating that the protein is phosphorylated in vivo (Fig. 1B). These results are consistent with previous experiments indicating that HDAC1 is post-translationally modified by phosphorylation (48).

**Determination of Phosphorylated Residues**—To identify residues on HDAC1 that are phosphorylated, HDAC1 was purified from T-Ag Jurkat cells using K-trap, an affinity resin comprising modified trapoxin, an irreversible inhibitor of HDAC, attached to Affi-Gel 10 (Fig. 2A; Ref. 22). After separation by SDS-PAGE, the purified HDAC1 was excised from the gel (Fig. 2B) and analyzed by mass spectrometry. Two phosphorylated serine residues were unambiguously identified, Ser421 and Ser423 (Fig. 2C). Interestingly, every phosphorylated peptide observed was doubly phosphorylated at Ser421 and Ser423, no peptide detected contained only one of the two identified phosphorylated serines.

The phosphorylated serines are located in the highly charged, carboxyl-terminal tail of HDAC1, removed from the catalytic site (Fig. 2C). A sequence comparison of the class I HDAC proteins, HDAC1, HDAC2, HDAC3, and HDAC8, indicates that only HDAC1 and HDAC2 contain serines in equivalent sequence positions. HDAC3 contains two potential CK2 consensus sites; however, a lack of sequence conservation in the C-terminal region of HDAC3 makes comparison with HDAC1 and HDAC2 difficult. HDAC8 does not contain the carboxyl-terminal charged tail, where the serine residues are located. For comparison, the yeast RPD3 protein contains a truncated carboxyl-terminal tail; however, no homologous serines exist in the equivalent HDAC1 Ser421 and Ser423 positions (not shown). The fact that the phosphorylated serines identified are located in the carboxyl-terminal charge tail of HDAC1 is consistent with previous results indicating that HDAC1 is phosphorylated between residues 387 and 482 (48).

**Phosphorylation Promotes Enzymatic Activity**—To determine whether phosphorylation influences enzymatic activity, Ser421 and Ser423 were independently and simultaneously mutated to alanine. Mutant HDAC1 proteins fused to a FLAG epitope tag were expressed in T-Ag Jurkat cells, which contain only small amounts of endogenous HDAC2 (49). The HDAC1 fusion protein was immunoprecipitated with α-FLAG affinity resin, and enzymatic activity was determined in vitro. Western blot experiments confirmed that only the FLAG-tagged HDAC1 protein bound to the α-FLAG affinity resin, and not detectable quantities of endogenous HDAC1 protein (data not shown). Under conditions where equal amounts of HDAC1 or mutant proteins were immunoprecipitated, mutant proteins displayed significantly diminished deacetylase activity compared with the wild-type protein (Fig. 3A). HDAC1 S421A, S423A, and S421A/S423A mutants demonstrated 33.0 ± 4.2, 25.3 ± 2.9, and 22.9 ± 3.1% activity compared with wild-type, respectively. The minimal activity displayed by the mutants was lost upon treatment with trichostatin A, an inhibitor of HDAC activity, indicating that the residual activity may be the result of partially active enzyme or a small amount of co-immunoprecipitated, endogenous HDAC1 activity (data not shown; Ref. 50).

To confirm that phosphorylation of HDAC1 promotes enzymatic activity in vivo, luciferase assays were performed. HDAC1 and mutants were expressed in T-Ag Jurkat cells as fusion proteins to Gal4 and VP16, which activate transcription of the luciferase gene under the control of four tandem Gal4 DNA binding sites. Where Gal4–VP16 activated the transcription of luciferase, the HDAC1-Gal4–VP16 fusion almost entirely repressed transcription due to deacetylase activity of HDAC1 (Fig. 3B), consistent with previous studies (49). The HDAC1 S421A, S423A, and S421A/S423A mutant fusion proteins activated transcription of luciferase under conditions where the wild-type HDAC1 fusion protein did not (Fig. 3B), consistent with the fact that the mutants display diminished deacetylase activities in vitro. Interestingly, the phosphorylation mutants activated the expression of luciferase to a greater extent than the HDAC1 H141A mutant (Fig. 3B), which is mutated at a conserved catalytic histidine residue (49).

**Acidic Residues Partially Substitute for Phosphorylated Serine in HDAC1**—Glutamic acid and aspartic acid have been previously used as mimics of phosphorylated serine residues (51). To test whether glutamic acid or aspartic acid substitutes for phosphoserine in HDAC1, Ser421 and Ser423 were individ-
Physically inhibits enzymatic activity. Rather, the data suggest that the carboxyl-terminal region is critical for maintaining enzymatic activity (50).

**HDAC1 Is a Substrate of CK2 in Vitro and in Vivo**—Ser421 and Ser423 of HDAC1 are located in a consensus site for the protein kinase CK2 (Figs. 2C and 6A). To determine whether CK2 is capable of phosphorylating Ser421 and Ser423 in vitro, phosphatase-treated lysates were incubated with recombinant CK2 catalytic domain, and HDAC1 was separated and assessed for changes in migration through the polyacrylamide gel. As shown in Fig. 6B, HDAC1 incubated with CK2 migrated more slowly on a polyacrylamide gel than phosphatase-treated HDAC1 or HDAC1 incubated with casein kinase 1 (compare lane 3 with lanes 2 and 4), suggesting that HDAC1 is a substrate for CK2 but not CK1. These results are consistent with previous work demonstrating that human HDAC1 is phosphorylated in vitro by CK2 (48).

To determine whether HDAC1 is a substrate of CK2 in vivo, the CK2 consensus sites that potentially direct phosphorylation of Ser421 and Ser423 in HDAC1 were disrupted individually and simultaneously by replacing the critical glutamic acids with alanine (Fig. 6A). If disruption of the CK2 sites results in reduced phosphorylation, we would expect the mutants to display reduced deacetylase activity compared with wild type. The enzymatic activities of the mutants were assessed and are shown in Fig. 6C. Consistent with the prediction, all CK2 consensus site mutants displayed reduced deacetylase activity compared with wild-type HDAC1. The double mutant (E424A/E425A) displayed the greatest reduction in activity.
E426A), disrupting phosphorylation of Ser\(^{421}\) and Ser\(^{423}\), displayed 30.9 ± 1.6% activity of wild-type HDAC1, while the E426A mutant, directing phosphorylation of Ser\(^{423}\) only, demonstrated 48.4 ± 7.2% of wild-type activity. Mutation of the glutamic acid directing Ser\(^{421}\) phosphorylation (E424A) was not as effective in reducing the enzymatic activity of HDAC1, maintaining 78.5 ± 5.4% of wild-type HDAC1 activity. As a critical control, mutation of a nearby glutamic acid not directing CK2 phosphorylation (E425A) displayed 108 ± 4.3% of wild-type activity, indicating that the loss of a negatively
reduced enzymatic activity compared with wild-type HDAC1 suggests that HDAC1 is a substrate for CK2 \textit{in vivo}.

To provide further evidence that CK2 phosphorylates HDAC1 \textit{in vivo}, T-Ag Jurkat cells were incubated in the presence of DRB, a specific small molecule inhibitor of CK2 enzymatic activity, and the migration of endogenous HDAC1 in a polyacrylamide gel was assessed. Interestingly, the migration of HDAC1 through a polyacrylamide gel was independent of whether CK2 activity was inhibited \textit{in vivo} (Fig. 6D, compare lanes 1 and 3). In contrast, the migration of IxBo, a known CK2 substrate, was influenced by incubation with DRB (52). Because mutagenesis studies demonstrated that HDAC1 is a substrate of CK2 \textit{in vivo}, these results raise the possibility that phosphorylation of HDAC1 is not dynamic during a 45-min incubation.

Finally, to probe the ability of CK2 to interact with HDAC1 \textit{in vivo}, endogenous HDAC1 and CK2 were immunoprecipitated from T-Ag Jurkat lysates. As shown in Fig. 6E, HDAC1 and CK2 co-immunoprecipitated, indicating that the proteins interact \textit{in vivo}. These combined data strongly argue that CK2 phosphorylates HDAC1 \textit{in vivo}.

\textbf{Phosphorylation Influences HDAC1 Complex Formation—} HDAC1 function is regulated by association with cellular proteins. Not only are associated proteins able to recruit HDAC to specific DNA sequences for targeted deacetylation and transcriptional repression (35), but biochemically purified HDAC1 also displays higher deacetylase activity when in complex with associated proteins (40, 49). These previous results suggest that phosphorylation might affect deacetylase activity by disrupting complex formation.

To determine whether phosphorylation affects the protein interactions of HDAC1, immunoprecipitated FLAG-tagged HDAC1 and mutant proteins were probed for binding to HDAC1-associated proteins (Fig. 7). Phosphorylation site mutants did not interact with associated proteins as effectively as wild-type HDAC1. Where equivalent amounts of FLAG-tagged protein were immunoprecipitated, the S421A, S423A, and S421A/S423A mutants ineffectively co-immunoprecipitated proteins from HDAC1-containing complexes compared with wild-type HDAC1, including RbAp48 found in the Sin and NRD complexes, MTA-2 from the NRD complex, mSin3A from the Sin complex, and CoREST from the CoREST complex (Fig. 7A, compare lanes under \textit{α-FLAG IP}).

Co-immunoprecipitation of HDAC1 by RbAp48, MTA2, and mSin3A verified that HDAC1 binding was reduced with the S421A, S423A, and S421A/S423A mutants (Fig. 7A, compare lanes under \textit{α-RhAp48}, \textit{α-MTA2 IP}, and \textit{α-mSin3A IP}). As expected, MTA-2 from the NRD complex did not co-immunoprecipitate with Sin3 from the Sin complex (Fig. 7A, compare \textit{α-MTA2} and \textit{α-mSin3A IP}; Ref. 40). RhAp48 and mSin3A were also associated in the presence or absence of HDAC1, consistent with previous results demonstrating that they interact directly (Fig. 7A, compare \textit{α-RhAp48} and \textit{α-mSin3A IP}; Ref. 38). Interestingly, MTA-2 and RhAp48 were associated in the presence or absence of the HDAC1-FLAG fusion protein, although they did not interact directly in glutathione \textit{S}-transferase pull-down experiments (Fig. 3A, compare \textit{α-MTA2} and \textit{α-RhAp48}; Ref. 40). Perhaps endogenous HDAC1 or another NRD complex member is involved in MTA2/RhAp48 association.

To confirm that reduced phosphorylation at Ser^{421} and Ser^{423} disrupts complex formation, HDAC1 mutants with CK2 consensus site alterations were probed for protein interactions. In this case, the ability of the mutants to bind associated proteins was roughly correlated with observed enzymatic activity (compare Fig. 6C with Fig. 7B). Where the E424A mutant, which disrupts phosphorylation of Ser^{421}, displayed charged residue is not responsible for the reduced deacetylase activity. In all cases, the CK2 consensus site mutation reduced activity to a lesser extent than the alanine mutation. However, the fact that all CK2 consensus site mutants demonstrated...
78.5 ± 5.4% of wild-type HDAC1 activity, it immunoprecipitated RbAp48 and mSin3A effectively compared with wild-type HDAC1. In the case of E426A or E424A/E426A, which displayed 48.4 ± 7.2 and 30.9 ± 1.6% activity of wild-type HDAC1, respectively, they poorly immunoprecipitated RbAp48 and Sin3A compared with HDAC1 wild type. In addition, deletion of the HDAC1 carboxyl-terminal tail that reduced enzymatic activity to 22.1 ± 2.3% disrupted interaction with associated proteins (data not shown). These results suggest that the enzymatic activity of HDAC1 phosphorylation site mutants may be related to their ability to interact with associated proteins.

**DISCUSSION**

Studies with human, mouse, and maize HDAC proteins demonstrated that histone deacetylases are phosphoproteins (32, 48, 53). To identify phosphorylation sites on HDAC1, K-trap purified human HDAC1 protein was analyzed by mass spectrometry, and two phosphorylated serine residues, Ser421 and Ser423, were unambiguously identified. Disruption of phosphorylation at Ser421 and Ser423 by alanine mutation or mutation of the CK2 consensus sites reduced HDAC1 enzymatic activity and perturbed its binding to interacting proteins.

One possible result of mutagenesis is the nonspecific global unfolding of the HDAC1 mutants. Three experiments refute this possibility and argue that phosphorylation specifically results in altered HDAC1 activity. First, all HDAC1 mutants expressed were not degraded in our experiments, indicating that they are folded sufficiently to prevent protease digestion in vivo. Second, mutagenesis of the negatively charged Glu425, a residue in the vicinity of the CK2 consensus sites but not involved in direct phosphorylation, did not affect deacetylase activity or complex formation (Figs. 6C and 7B). The E425A control experiments indicate that loss of phosphoserine, and not the neutralization of any negatively charged residue in the region, affects deacetylase activity and complex formation of HDAC1. Finally, mutation of Ser421 and Ser423 to glutamic acid only partially substituted for phosphorylation, suggesting that phosphoserine is uniquely suited to maintain HDAC1 activity. Our data suggest that HDAC1 specifically requires phosphorylation at Ser421 and Ser423 to allow proper activity.

Although HDAC2 contains equivalent serines at HDAC1 Ser421 and Ser423, the other class I HDACs do not (Fig. 2C). The lack of conservation of Ser421 and Ser423 among the class I HDACs may reflect the differences in regulation of these proteins. For example, HDAC2 is found in all characterized HDAC1-containing complexes, indicating that HDAC1 and HDAC2 are similarly regulated in vivo via a protein association mechanism (54). Consistent with the possibility that HDAC2 is phosphorylated, phosphatase treatment alters the migration of HDAC2 through a polyacrylamide gel (data not shown). The fact that HDAC2 contains Ser421 and Ser423 and phosphorylation at Ser421 and Ser423 promotes complex formation of HDAC1 suggests that HDAC2 may interact with associated proteins in the same manner as HDAC1. On the other hand, HDAC3 is known to bind HDAC4, HDAC5, HDAC7, and the nuclear hormone receptor corepressor; however, multiprotein complexes containing HDAC3 are not well characterized (32, 55, 56). Although HDAC3 contains two CK2 consensus sequences in its short C-terminal region, the lack of conservation of HDAC3 in the C-terminal region compared with HDAC1 and HDAC2 may indicate that HDAC3 does not function through the same complexes as HDAC1 and HDAC2 but rather through HDAC3-specific complexes. Regulation of HDAC8, the newest addition to the class I HDACs, has not yet been characterized. The fact that HDAC8 does not contain the carboxyl-terminal region suggests that it may be regulated through a distinct mechanism.

HDAC1 function is regulated by associated proteins, which govern enzymatic activity and specific DNA sequence recruitment (18, 40). Co-immunoprecipitation experiments demonstrated that the interaction of HDAC1 with associated proteins is phosphorylation-dependent. Interestingly, the ability of HDAC1 to interact with associated proteins was roughly correlated with HDAC1 enzymatic activity; when HDAC1 phosphorylation site mutants bound poorly to interacting proteins, including RbAp48, MTA-2, Sin3A, and CoREST, they also displayed reduced enzymatic activity (compare Fig. 6C with Fig. 7B). In addition, the HDAC1 H141A mutant, which has reduced enzymatic activity but still interacts with RbAp48 and Sin3A, was not as effective in activating luciferase gene expression as the phosphorylation site mutants (Fig. 3B; Ref. 49). Although more studies are needed to assign a causative role for associated proteins in modulating deacetylase activity, our results reinforce the importance of protein association in regulating HDAC1 function in vivo.

Glutathione S-transferase protein pull-down experiments with mouse HDAC1 deletion mutants identified the 51-amino
acid amino-terminal region of HDAC1 as mediating interaction with RhaP48 and Sin3A (50). Our results reveal that the carboxyl-terminal region of HDAC1 is also required to maintain enzymatic activity. The structure of HDLP, an HDAC homolog from hyperthermophilic bacterium Aquifex aeolicus, does not include the carboxyl-terminal region of HDAC1, but the 51-amino-terminal amino acids of HDLP are partially solvent exposed and available for protein interactions (57). Taken together, our results support a model where a large structural domain composed of the amino-terminal and carboxyl-terminal tails of HDAC1 cooperate to allow protein association.

Our results also suggest that Ser\(^{421}\) and Ser\(^{423}\) are constitutively phosphorylated and buried when properly organized. Specifically, phosphatase-treated HDAC1 maintained the same enzymatic activity as wild-type HDAC1, despite the fact that unphosphorylated Ser\(^{421}\) and Ser\(^{423}\) inactivated HDAC1 (data not shown; Ref. 48). We hypothesize that Ser\(^{421}\) and Ser\(^{423}\) are inaccessible to the phosphatase enzyme because they are buried, either in the HDAC1 globular structure or at the interface with an associated protein. The marked change in HDAC1 migratory activity when phosphatase-treated, as shown in Fig. 1, may be caused by the removal of phosphate at solvent-accessible sites but not at Ser\(^{421}\) and Ser\(^{423}\). Consistent with this hypothesis is the fact that CK2 phosphorylation of HDAC1 in Fig. 6B resulted in a decreased migratory shift compared with that in Fig. 1. In addition, incubation of cells with DRB, an inhibitor of CK2 activity, did not alter the migration of HDAC1 through a polyacrylamide gel, despite the fact that HDAC1 is a substrate for CK2 in vitro and in vivo. These data suggest that HDAC1 is not dynamically phosphorylated during a 45-min time scale. The observation by mass spectrometry that HDAC1 was always doubly phosphorylated and the fact that HDAC1 migrates as a single band by PAGE is consistent with the possibility that HDAC1 is constitutively phosphorylated at Ser\(^{421}\) and Ser\(^{423}\). Although more studies are necessary to determine under what conditions HDAC1 becomes dephosphorylated in vitro, these results support a model where Ser\(^{421}\) and Ser\(^{423}\) are constitutively phosphorylated and inaccessible to phosphatase enzymes. Perhaps Ser\(^{421}\) and Ser\(^{423}\) phosphorylation is essential for organizing the large structural domain that mediates protein interactions. The fact that the mutants were stable in the presence of endogenous proteases suggests that the domain organization necessary to support protein association may be subtle.

Consistent with the proposed structural model is the fact that Ser\(^{421}\) and Ser\(^{423}\) are substrates for CK2. CK2 is involved in diverse cellular events, such as transcription, proliferation, and development, and is essential for viability in numerous organisms, including Saccharomyces cerevisiae (58). CK2 is an acidophilic, requiring one or more acidic residues positioned downstream from the target serine, including glutamic acid, aspartic acid, phosphoserine, and phosphothreonine, for recognition and phosphorylation. For this reason, CK2 is thought to be involved in a mechanism of hierarchical phosphorylation, where one phosphorylation event leads to subsequent protein modifications (59). Importantly, CK2 is constitutively active due to the proper positioning of an activation arm, and CK2 activity is predominantly regulated by expression levels. The high basal activity of CK2 would ensure that HDAC1 is constitutively phosphorylated and able to bind associated proteins and deacetylase.

The physiological significance of phosphorylation at Ser\(^{421}\) and Ser\(^{423}\) by CK2 remains unclear. As suggested, CK2 phosphorylation may serve predominantly a structural role in organizing the catalytic and protein interaction sites on HDAC1. Another possibility is that CK2 phosphorylation of HDAC1 leads to additional post-translational modifications that are critical for HDAC1 function. In fact, hierarchical phosphorylation events often occur in highly charged regions, analogous to the highly charged, carboxyl-terminal tail of HDAC1 and involve kinases with acidicotropic or basotropic substrate specificities, such as CK2 (60). Perhaps phosphorylation of HDAC1 facilitates its further modification by phosphorylation or acetylation, leading to a fully active protein.

HDAC1 and CK2 are thought to be involved in cancer development. CK2 concentrations are increased in rapidly proliferating tumors, including those in breast, prostate, and lung (58). Although the relationship between heightened CK2 activity and proliferating tumors is still unclear, CK2 phosphorylates a number of proteins possibly involved in tumor proliferation, such as Myc and p53 (58). We speculate that the enhanced CK2 concentrations in proliferating tumors may result in enhanced HDAC1 activity or an increased fraction of functionally active HDAC1. In the same way that unnaturally increased HDAC1 recruitment by retinoic acid receptor 1-promyelocytic leukemia zinc finger protein to DNA target sites in acute promyelocytic leukemia is linked to leukemia development, in this case by inhibiting the differentiation of promyelocytes into later lineages, enhanced HDAC1 activity in tumors may be involved in maintaining a proliferating state, characteristic of an early lineage cell type. In fact, the MTA-2 protein, associated with metastatic tumors, is an essential component of the HDAC1-containing NRD complex (40). Elucidating how CK2 modulates HDAC1 function in vivo is the first step toward understanding the role of HDAC1 and CK2 in cancer development.

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