Phosphatase Inhibition Leads to Histone Deacetylases 1 and 2 Phosphorylation and Disruption of Corepressor Interactions*

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The regulation of histone deacetylases (HDACs) by phosphorylation was examined by elevating intracellular phosphorylation in cultured cells with the protein phosphatase inhibitor okadaic acid. After fractionation of extracts from treated versus untreated cells, HDAC 1 and 2 eluted in several peaks of deacetylase activity, assayed using mixed acetylated histones or acetylated histone H4 peptide. Stimulation of cells with okadaic acid led to hyperphosphorylation of HDAC 1 and 2 as well as changes in column elution of both enzymes. Hyperphosphorylated HDAC2 was also observed in cells synchronized with noco- dao acid or taxol, demonstrating regulation of HDAC phosphorylation during mitosis. Phosphorylated HDAC1 and 2 showed a gel mobility retardation that correlated with a small but significant increase in activity, both of which were reversed upon phosphatase treatment in vitro. However, the most pronounced effect of HDAC phosphorylation was to disrupt protein complex formation between HDAC1 and 2 as well as complex formation between HDAC1 and corepressors mSin3A and YY1. In contrast, interactions between HDAC1/2 and RbAp46/48 were unaffected by okadaic acid. These results establish a novel link between HDAC phosphorylation and the control of protein-protein interactions and suggest a mechanism for relief of deacetylase-catalyzed transcriptional repression by phosphorylation-dependent signaling.

Acetylation of nucleosomal histones by histone acetyltransferases generally stimulates transcription, whereas deacetylation of nucleosomes by histone deacetylases (HDACs) is correlated with transcriptional repression. Thus, histone acetylases and deacetylases are potential targets for regulation of chromatin acetylation at targeted promoters by signal transduction pathways. Our previous studies showed that global acetylation of nucleosomal histones changes in response to enhanced phosphorylation induced by inhibition of intracellular phosphatases (1). Therefore, we examined the potential control of mammalian HDACs 1 and 2 by phosphorylation.

Two classes of HDAC in mammalian cells are catalogued based on their homology to yeast catalytic subunits. Class I includes HDACs 1, 2, and 3, enzymes homologous to yeast RPD3 (2–5). Class II includes HDACs 4, 5, 6, and 7, most similar to the yeast histone deacetylase A repressor (6–9). Of these forms, HDAC1 and 2 are expressed ubiquitously (10). Neither of these enzymes is active when expressed in bacteria, but both are active when expressed in insect cells, suggesting that post-translational modifications or other eukaryotic factors are needed for enzymatic activity (11, 12).

HDAC1 and 2 isolated from tissue culture extracts are able to deacetylate free and nucleosome-bound histones as well as histone peptides, but they are unable to deacetylate SV40 minichromosomes, suggesting that other factors must direct enzyme access to acetylated histones in higher ordered chromatin structures (3, 12, 13). In agreement, both are found as catalytic subunits of multiprotein complexes involved in transcriptional repression. Repressor complexes containing HDAC1 and 2 include mammalian Sin3A (mSin3A) and nucleosome-remodeling HDAC (11, 14–17). mSin3A recruits deacetylases to nuclear hormone receptor promoters by interactions with nuclear receptor corepressor and silencing mediator for retinoid and thyroid receptors in the absence of ligand, as well as Mad-Max and Mxi-Max heterodimers, methylated CpG binding protein-1, estrogen receptor and Rpx homeodomain proteins, c-Ski, Sno, Ikaros, Aiolos, tumor suppressor p53, and RE1 silencing transcription factor/neural restrictive silencing factor (15, 18–25). The nucleosome-remodeling HDAC complex functions in part to recruit nucleosome remodeling and deacetylase activities to methylated DNA (26–28). Besides these complexes, HDAC1 and 2 also bind directly to DNA-binding proteins, such as YY1, retinoblastoma protein (pRb), pRb-binding protein 1, Sp1, breast cancer associated susceptibility protein 1, and heterochromatin protein 1 (2, 29–34). These studies indicate that targeted repression involves recruitment of active HDACs to promoter sequences via heterologous DNA-binding protein factors.

Although such findings have established the importance of HDAC recruitment to DNA through specific DNA-binding complexes, little is understood about how the assembly or deacetylase activity of these complexes is regulated. Phosphorylation is a prominent post-translational mechanism for controlling enzyme activities, localization, and protein interactions. Recent studies have begun to reveal mechanisms for regulating activities of HDAC complexes by signal-dependent phosphorylation. For example, phosphorylation of pRb by cyclin D/cdk4 negatively regulates the interaction of pRb complexes with HDACs (35), and phosphorylation of the MEF2 transcription factor by calmodulin-dependent kinase inhibits interaction with HDACs 4 and 5 (36). In addition, HDAC4 and 5 are phosphorylated at three sites that control their cytoplasmic localization and interaction with 14-3-3 proteins (37, 38). Most recently, HDAC1

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¶ The abbreviations used are: HDAC(s), histone deacetylase(s); mSin3A, mammalian Sin3A; OA, okadaic acid; PP1 and PP2A, protein phosphatase 1 and 2A, respectively; Rb, retinoblastoma; TSA, trichostatin A.
has been reported to be a phosphoprotein, and mutation of identified sites reduces enzymatic activity (39, 40).

In this study we have extended such observations by examining alterations in HDAC1 and 2 activities and complex formation after induction of phosphorylation. Here we show that HDAC1 and 2 arebasally phosphorylated in resting cells and hyperphosphorylated in response to inhibition of protein phosphatase by okadaic acid (OA). Phosphorylation of HDAC2 is also observed in mitotically arrested cells and appears to be regulated by protein phosphatase 1 (PP1). Reversal of hyperphosphorylation in vitro leads to a small but significant reduction in deacetylase activity, indicating that phosphorylation positively regulates HDAC specific activity. Importantly, treatment of cells with OA disrupts complex formation between HDAC1 and HDAC2, and between HDAC1 and mSin3A or YY1. These findings indicate that phosphorylation-dependent mechanisms disrupt HDAC and repressor complexes, suggesting potential control of targeted transcriptional repression by phosphorylation.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment—K562 human erythroleukemia cells and MCF10A human breast epithelial cells were obtained from the American Type Culture Collection. WM35 human melanoma cells were a kind gift from Dr. Meenhard Herlyn, Wistar Institute. K562 cells were grown in Spinner cultures at 37 °C, 5% CO2, at a density of 5 × 106 cells/ml in RPMI (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) and 100 μg/ml streptomycin and penicillin. MCF10A and WM35 cells were grown on 10-cm plates in 10% fetal calf serum, Dulbecco’s modified Eagle’s medium (Invitrogen), and 100 μg/ml streptomycin and penicillin. Cells were treated with the indicated concentrations of OA (Alexis) dissolved in Me2SO, and control treatments received the same volume of Me2SO. Other cell treatments used cycloheximide at a final concentration of 100 μg/ml, taxol at a final concentration of 400 ng/ml, nocodazole at 500 ng/ml, or thymidine at 2 mM (Sigma). The acetylation state of K562 histones under these conditions was described previously (1).

Histone Isolation and Substrate Preparation—Histones were prepared as deacetylase substrates by in vivo radiolabeling for 16 h in the presence of 5 mM sodium butyrate and 100 μCi/ml [3H]acetic acid. Histones were isolated from 2.5 × 107 cells following the method of Johns (42). Briefly, cells were harvested by centrifugation (2,000 × g, 5 min, 4 °C), washed in ice-cold phosphate-buffered saline, and resuspended in 1 ml of buffer A (20 mM HEPES (pH 7.2), 1% sodium deoxycholate, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 100 μg/ml Na3VO4, 1 mM Na3VO4, 100 μM phenylmethylsulfon fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 μg/ml pepstatin A, and 1 mM benzamidine). Lysates were prepared by 20 strokes in a tight fitting Dounce homogenizer and three 20-s pulses of sonication. Pellets were collected by centrifugation (55,000 × g, 15 min), and supernatants were loaded at 0.5 ml/min onto a Mono Q HR5/5 column (Amersham Biosciences) preequilibrated in column buffer. Proteins were eluted with a linear gradient of 0.1—4 M NaCl in column buffer and collected in 180-ml fractions. Conductivity was measured in every fifth fraction to verify consistency in gradients among different runs.

HDAC Assays—Deacetylase assays were carried out using 30 μl of fractionated extract or immunoprecipitated material and 10 μg of 15,000–20,000 dpm H-acetylated mixed histones or 100,000 dpm H-acetylated histone H4 peptide in a final volume of 50 μl. Reactions were carried out for 2 h at 37 °C and stopped by the addition of 10 μl of 0.1 M acetic acid and 700 μl of ethyl acetate. Samples were vortexed and centrifuged (17,000 × g, 5 min), and the organic layer containing released [3H]acetate was removed and counted. Some reactions were inhibited by preincubation in 500 mM trichostatin-A (TSA) (Wako) for 10 min at 4 °C before the deacetylase assays. Reaction time courses confirmed linearity of deacetylase initial rate measurements (data not shown).

Immunocytochemistry—Antibodies to HDAC1 (H-51, rabbit polyclonal), HDAC2 (H-52, rabbit polyclonal), mSin3A (K-20, rabbit polyclonal) and YY1 (H-10, mouse monoclonal) were purchased from Santa Cruz Biotechnology. Antibodies to Rhap66/48 were a kind gift from Dr. Alain Verrault. Immunoprecipitations were carried out with 1 × 107 cells that were harvested, washed, and lysed in RIPA buffer (100 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 100 mM phenylmethylsulfon fluoride, 100 μM Na3VO4, 1 mM OA, and protease inhibitors). Lysates were clarified by centrifugation (17,000 × g, 15 min), and immune complexes were formed by the addition of 1–2 μg of antibody and 10 μl of protein A-Sepharose (Amersham Biosciences) to the supernatants, followed by incubation at 4 °C for 16 h. Immune complexes were isolated by centrifugation and washed three times with 1 ml of RIPA buffer and twice with 1 ml of 10 mM HEPES (pH 8.0). Low bisacyclamide gels (acylation/bisacyclamide 29:0.1) were used to enhance the gel mobility retardation resulting from phosphorylation. For Western blotting, proteins were transferred to Immobilon-P (Millipore), blocked for 1 h in 3% nonfat milk, and incubated with primary antibodies (1:1,000 dilution) followed by a 1-h incubation with goat anti-rabbit IgG coupled to horseradish peroxidase (1:10,000 dilution) (Jackson Laboratories). After extensive washing, blots were visualized by enhanced chemiluminescence (Amersham Biosciences).

Phosphatase Treatment—Fractions were desalted using 1-ml Sephadex G-50 columns, or alternatively, immunoprecipitated material was exchanged into 10 mM HEPES (pH 8.0) plus protease inhibitors. Dephosphorylation reactions contained calf alkaline intestinal phosphatase (Promega), λ-phosphatase (Invitrogen), PP1, or PP2A (Promega) at 5, 20, 0.5, and 0.1 units/reaction, respectively, and were carried out for 2 h at 30 °C. Control reactions contained the same phosphatases that had been inactivated by heating at 90 °C for 15 min. MnCl2 or MgCl2, which were inhibitory to deacetylase activity, were omitted from reactions. Immunoprecipitations were washed twice with 1 ml of RIPA buffer and twice with 1 ml of 10 mM HEPES (pH 8.0) before deacetylase activity assays.

RESULTS

OA Alters the Elution and Activity Profile of Fractionated Deacetylases—After treating K562 cells for 2 h with 1 μM OA, clarified cell extracts were fractionated by anion exchange chromatography, and fractions were assayed for rate of deacetylation of [3H]acetylated histones. The histone deacetylase eluted in four peaks (P3–P6) from control extracts and four peaks (P10–P13) from OA-treated extracts (Fig. 1A). The reproducible variation in elution of peaks between control and OA-treated cells suggested that inhibiting intracellular phosphatase activity alters the pI of deacetylase complexes through changes in protein composition and/or covalent modification. Furthermore, integrating the total amount of deacetylase activity over the whole profile showed a 30% increase in the total amount of deacetylase activity from OA-treated extracts compared with controls.
Fractions were next assayed for deacetylase activity using an $^3$H-acetylated peptide substrate corresponding to residues 2–19 of histone H4. The peptide substrate revealed differences in activity between cell treatment conditions that were not observed using histone proteins (Fig. 1). For example, fraction 8 from control and OA-treated samples (P1 and P7, respectively) showed little difference in deacetylase activity toward acetylated histones but significantly increased activity toward the acetylated peptide in OA-treated samples, suggesting that histone H4 is a preferred substrate for OA-sensitive deacetylases. Use of the peptide substrate also revealed new peaks corresponding to P2 in control fractions and P8 and P9 in OA fractions (Fig. 1B).

Comparison of activity profiles in Fig. 1, A and B, showed that for the most part, similar deacetylase peaks were detected with both protein and peptide substrates but with enhanced activity toward the H4 peptide. To confirm that these meas-

**Fig. 1. Resolution of histone deacetylase activities.** K562 cells were treated with 1 μM OA or Me$_2$SO (DMSO) carrier for 2 h prior to harvesting and Mono Q chromatography. Even numbered fractions were assayed for deacetylase activity from control or OA-treated extracts. Activities were detected by release of counts from $^3$H-acetylated histones (A) or $^3$H-acetylated histone H4 peptide substrate (B). Rates of deacetylation are reported as dpm released after 2 h at 37°C. Three fractions around peaks of activity from B were pooled and analyzed in subsequent experiments, indicated as P1–P6 for control peaks and P7–P13 for OA-treated activities. Identical salt gradients used for experiments in A and B are indicated. Elution of each peak was comparable between samples, as verified by conductivity measurements. Similar activity profiles were observed in five independent experiments.

**Fig. 2. Fractionated deacetylase activities are sensitive to TSA.** P1–P13 were desalted into 10 mM HEPES (pH 8.0) and treated without or with 500 nM TSA for 10 min prior to measuring deacetylase activity with $^3$H-acetylated H4 peptide substrate.
urements reflected deacetylase activities; each peak was tested for inhibition by the deacetylase inhibitor TSA (Fig. 2). The activities in P2–P6 and P8–P13 were inhibited by at least 10-fold with TSA, indicating that each peak contains TSA-sensitive deacetylases. Peaks P1 and P7 were inhibited only 2-fold, indicating that the enhanced release of [3H]acetate in P7 compared with P1 is partly the result of TSA-sensitive deacetylase activity and partly different enzymes. Peak P12 showed higher activity after desalting (compare Fig. 1B with Fig. 2), which most likely reflects salt inhibition of deacetylase activity in this pool.

**HDAC1 and 2 Are Components of the Fractionated Deacetylases**—HDAC1 and 2, which represent major deacetylase forms, were detected by Western blotting in all peaks except P1 and P7 (Fig. 3). This implies that HDAC1 and 2 are found in distinct complexes separable by ion exchange chromatography. Importantly, in peaks P2–P6 and P8–P13 the HDAC1:HDAC2 ratios were constant, and the total reactivities of HDAC1 + HDAC2 were proportional to the deacetylase activities in each pool, measured in Fig. 2. Fractions outside of the major activity peaks showed little or no reactivity with anti-HDAC1 or 2 (data not shown).

In Western blots, increased reactivity of both HDAC1 and 2 was observed in the OA-treated fractions (peaks P8–P13) compared with controls (peaks P2–P6), although cell numbers and extract volumes were equal between experiments. It was unlikely that a 2-h treatment with OA increased HDAC expression, therefore we investigated the possibility that OA affected the solubility of HDACs and their subsequent recovery from cell lysates. Immunoblots in Fig. 4 show recovery of HDAC1 and 2 from control versus OA-treated cells after lysis, Dounce homogenation, sonication, and centrifugation. Although significant amounts of HDAC1 and 2 remained insoluble in control cell extracts (Fig. 4A), both enzymes were completely recovered in soluble pools after treatment of cells with OA (Fig. 4B). The insoluble pool of HDACs in controls most likely represents enzyme that remained tightly bound to chromatin or nuclear matrix proteins, as reported previously (40). Thus, the increased deacetylase activity observed in fractionated extracts of OA-treated cells was at least partly the result of increased solubility and recovery, indicating reduced interactions of HDACs with chromatin or matrix.

**Phosphorylation of HDAC1 and HDAC2**—After OA treat-
HDAC are indicated as unphosphorylated (0), basally phosphorylated HDAC1 and HDAC2. Differentially phosphorylated forms of each HDAC are indicated as unphosphorylated (0), basally phosphorylated (P), or hyperphosphorylated (PP).

Varying cell cycle conditions were examined for regulated HDAC phosphorylation in the absence of OA. K562 cells were synchronized at the G2/S boundary by double thymidine arrest or in prometaphase with the microtubules poisons nocodazole and taxol. Mitotic arrest resulted in hyperphosphorylation of HDAC2, although no change in HDAC1 mobility was observed (Fig. 6D, lanes 3 and 4). No effect on mobility of either enzyme was observed in thymidine-treated cells arrested in G1/S (Fig. 6D, lane 2). Thus, spindle checkpoint activation provides a physiological stimulus that leads to significant hyperphosphorylation of HDAC2.

The OA concentration range used in these experiments is known to inhibit both PP1 and PP2A (40, 41), thus it was likely that either or both enzymes are involved in HDAC regulation. To test sensitivities to specific phosphatases, HDAC1 and 2 were immunoprecipitated from control or OA-treated cells and treated with PP1, PP2A, or λ-phosphatase for 30 min (Fig. 7). The shift to faster mobility of HDAC1 after OA treatment was reversed by λ-phosphatase and PP1 (Fig. 7, lanes 6 and 7). PP2A had no effect on HDAC1 (Fig. 7, lane 8), although positive controls confirmed activity of the enzyme toward a known substrate, phosphoflagrarin (data not shown). The mobility of HDAC2 from control cells was increased by λ-phosphatase, although neither PP1 nor PP2A affected basal phosphorylation (Fig. 7, lanes 1–4). In contrast, the shift to slower mobility of HDAC2 caused by OA-dependent hyperphosphorylation was reversed by both PP1 and λ-phosphatase, but not PP2A (Fig. 7, lanes 5–8). Together, the differential sensitivities to individual phosphatases indicate that hyperphosphorylation of HDAC1 and 2 is most likely physiologically regulated by PP1 and that the site(s) of basal phosphorylation in HDAC2 differ from the OA-induced site(s) occupied in the hyperphosphorylated state.

**Phosphorylation Affects HDAC Activity**—We next examined the effects of HDAC phosphorylation on deacetylase specific activity, using Mono Q peaks from fractionated control and OA-treated cells. Peaks P1–P13 (as indicated in Fig. 3) were desalted and buffer exchanged to remove β-glycerophosphate, treated with a mixture of PP1, λ-phosphatase, and calf intestinal phosphatase, and assayed for deacetylase activity using 3H-acetylated H4 peptide as substrate (Fig. 8). Negative controls were treated with buffer alone or the same phosphatase mixture after heat inactivation. Overall, peaks P1–P6 from the control profile showed little or no sensitivity to phosphatase.

FIG. 5. HDAC1 and HDAC2 are phosphoproteins. HDAC1 and 2 were immunoprecipitated from control or OA-treated cells and treated with buffer (B, lanes 1 and 4); a mixture of PP1, PP2A, and λ-phosphatase (PP, lanes 2 and 5); or heat-inactivated phosphatases (hPP, lanes 3 and 6). Each reaction was probed by Western blotting with antibodies to HDAC1 and HDAC2. Differentially phosphorylated forms of each HDAC are indicated as unphosphorylated (0), basally phosphorylated (P), or hyperphosphorylated (PP).

FIG. 6. Characteristics of HDAC2 hyperphosphorylation. In all experiments, cells were harvested in RIPA buffer, separated by SDSPAGE, and immunoblots were probed with anti-HDAC2 antibodies. A, hyperphosphorylation is rapid and reversible, shown by treatment of K562 cells with cycloheximide 30 min before treatment with Me2SO (lane 1) or OA for 2 h (lane 2). B, hyperphosphorylation is rapid and reversible, shown by cells treated with Me2SO (lane 1) or OA for 1 h (lane 2), 2 h (lane 3), or 3 h (lane 4) before harvesting. Cells treated with Me2SO (lane 5) or OA (lane 6) for 3 h were then washed, replaced in fresh medium, and allowed to recover for a further 24 h. C, HDAC2 hyperphosphorylation is also observable in MCF10A and WM35 cells treated with 1 μM OA for 2 h. D, mitotic arrest results in hyperphosphorylation of HDAC2. Cells were untreated (lane 1), treated with 2 mM thymidine for 16 h to inhibit DNA synthesis and arrest cells in G1/S-phase (lane 2), or treated with 500 ng/ml nocodazole (lane 3) or 400 ng/ml taxol (lane 4) for 24 h to disrupt microtubule function and arrest cells in prometaphase. Blots were probed for HDAC1 (upper panel) and HDAC2 (lower panel).

FIG. 7. Sensitivity of HDAC1 and HDAC2 to different phosphatases. Immunoprecipitates (IP) of HDAC1 or HDAC2 from control or OA-treated cells were divided and treated for 30 min at 30 °C with buffer control (lanes 1 and 5), λ-phosphatase (lanes 2 and 6), PP1 (lanes 3 and 7), or PP2A (lanes 4 and 8) catalytic subunits, and reactions were probed by Western blotting (WB) with anti-HDAC1 and anti-HDAC2.
treatment, with the exception of P5, which was slightly inhibited (Fig. 8). This shows that loss of basal phosphorylation had little effect on deacetylase activity. In contrast, peaks P7–P13 from OA-treated extracts each showed small but significantly reduced activities after phosphatase treatment, with the greatest decrease in P11 and P12. Assuming that P7–P13 from OA-treated cells are related to P1–P6 from control cells, the result suggests that hyperphosphorylation of HDACs measurably alters their activities in addition to their chromatographic behavior.

Sensitivities of HDAC1 and 2 to dephosphorylation were also assayed after immunoprecipitation from total cell lysates (Fig. 9A). Because HDAC solubility was increased by OA, ratios of cellular extract to antibody were adjusted to immunoprecipitate equivalent amounts of HDAC1 and 2 from control versus OA-treated samples. Each immunoprecipitate was divided and treated with buffer or phosphatase mixture. Little change in activity was observed after phosphatase treatment of immunoprecipitated HDACs compared with the fractionated peaks, possibly reflecting associated factors that regulate phosphatase sensitivity which are lost during the immunoprecipitation.

**HDAC-Protein Interactions Are Disrupted by Phosphorylation**—Our results demonstrated copurification of HDAC1 and 2 on anion exchange chromatography (Fig. 3). Interactions between HDAC1 and 2 have also been reported by others (12). Effects of phosphorylation between these enzymes was examined by immunoprecipitating each enzyme individually, then probing enzymes by Western blotting (Fig. 9B). Immunoprecipitation of HDAC1 showed coprecipitation of HDAC2 from control but not OA-treated cell extracts (Fig. 9B). Similarly, immunoprecipitation of HDAC2 led to coprecipitation of HDAC1 in control extracts but not after OA treatment (Fig. 9B). These results indicate that HDAC1 and 2 normally associate with each other and that OA treatment disrupts this interaction.

We next addressed whether interactions between HDACs and other associated proteins might also be regulated by OA. Mammalian Sin3A and YY1 both associate with HDACs in complexes that are independent of each other (44). mSin3A appears to be part of a HDAC corepressor complex that is recruited to specific promoters through interactions with DNA-binding proteins (14), whereas YY1 directly binds DNA and is thought to recruit HDACs as part of its repressor function (2). mSin3A, HDAC1, and HDAC2 were immunoprecipitated from control versus OA-treated cells, separated by SDS-PAGE, and probed by Western blotting for coprecipitation of each protein (Fig. 10). Association between HDAC1 and mSin3A was observed in control cells (Fig. 10A, lanes 3 and 5) but was reduced in cells treated with OA (Fig. 10A, lanes 4, 7, and 8). Interactions between mSin3A and HDAC2 were weak or insignificant (Fig. 10A, lanes 3, 4, 7, and 8), suggesting that the majority of HDAC2 exists outside mSin3A complexes in this cell line. We also probed immunoprecipitates for the presence of RbAp46/48, which is reported in most HDAC complexes. RbAp46/48 was reduced in mSin3A immunoprecipitates after treatment with OA, correlating with the loss of HDAC1 (Fig. 10A, lanes 3 and 4). However, little change in RbAp46/48 association with HDACs 1 and 2 was observed after OA treatment (Fig. 10A, lanes 5–8), indicating that the OA-induced disruption shows specificity for HDAC-mSin3A interactions.

Similarly, immunoprecipitation of YY1 showed weak coimmunoprecipitation with HDAC1 or 2 in control cells and disruption of these interactions after treatment with OA (Fig. 10B). We did not detect YY1 in immunoprecipitations of either HDAC under control conditions, which indicates that the majority of HDAC1 and 2 exists in complexes that do not include
Nevertheless, the results show that both mSin3A and YY1 interactions with HDACs are regulated in response to OA.

Both mSin3A and YY1 could be targets of OA-induced phosphorylation, therefore we examined whether mSin3A and YY1 were also phosphorylated after treatment. mSin3A and YY1 were immunoprecipitated from control or OA-treated cells and treated with a phosphatase mixture (Fig. 11). The shift to slower mobility of both mSin3A and YY1 after treatment with OA was reversed upon phosphatase treatment (Fig. 11, A and B, lanes 4–6). A smaller gel shift of mSin3A in control cells was also sensitive to phosphatase (Fig. 11A, lanes 1–3). Thus, mSin3A or YY1 are also phosphorylated under the conditions that disrupt association with HDACs.

**DISCUSSION**

Histone acetylation has been shown to influence the transcriptional potential of genes by disrupting the chromatin structure and/or targeting transcription factor binding which in turn, regulates the recruitment of the RNA polymerase machinery. Although the acetylation of histones by type A histone acetyl transferases and deacetylation by HDACs has been substantiated, relatively little is known about how their specific activity or recruitment is regulated. Our study shows that HDACs 1 and 2 are rapidly hyperphosphorylated after treatment of cells with OA, most likely through inhibition of PP1 (43). This suggests that HDACs may be downstream targets of signaling pathways, providing a novel mechanism for acute control of transcription through changes in chromatin post-translational modification. HDAC phosphorylation is independent of protein synthesis and occurs reversibly, consistent with what would be expected during signaling responses. Importantly, the accumulation of hyperphosphorylated forms of HDAC2 during mitotic arrest demonstrates that the event is not unique to OA treatment and suggests that regulated HDAC2 phosphorylation occurs during the cell cycle in a manner undescribed previously. Conceivably, increased deacetylase activity toward histone or non-histone substrates or release from chromatin might mediate mitotic transcriptional silencing or chromosome condensation.

Chromatographic separation revealed elution of HDAC1 and 2 within the same fractions implies that both exist within the same complexes. The elutions of these complexes change in response to OA, suggesting phosphorylation control of deacetylase-protein interactions.

HDAC1 and 2 show changes in gel mobility upon OA treatment which are sensitive to phosphatase treatment *in vitro*. Based on the changes in chromatographic elutions after OA treatment, it seems conceivable that phosphorylation could change the composition of HDAC complexes or otherwise modify deacetylase activities. Dephosphorylation of HDACs led to small but significant decreases in deacetylase specific activities in OA-treated fractions which were not observed in control fractions. However, HDAC1 or 2 immunoprecipitated directly from lysates showed little sensitivity to phosphatase, suggest-
ing that the phosphatase-sensitive activity may depend on higher order complexation.

Importantly, OA disrupted interactions of HDACs 1 and 2 normally occurring in control isolates. This was observed at the level of chromatin/matrix association and solubilization, interactions between HDAC1 and 2, and interactions between HDAC1/2 and corepressors. The disruption of HDAC1 and 2 suggested that other HDAC-protein interactions might be regulated by phosphorylation. Both mSin3A and YY1 interact with HDACs 1 and 2 independently, in a manner disrupted by OA. Their exclusion from the same complexes might reflect interactions with the same regions in HDACs, and phosphorylation of such regions could explain why both interactions are disrupted. Interestingly, both mSin3A and YY1 are also targets for OA-induced phosphorylation. A simple hypothesis suggests that many phosphorylation events contribute to the disruption of protein interactions. Further experiments are needed to identify the sites of phosphorylation on the HDACs, mSin3A, and YY1, to determine the individual significance of each site. It will also be informative to determine whether HDAC phosphorylation disrupts interactions with other repressor DNA-binding proteins as well as the nucleosome-remodeling HDAC complex or affects cellular localization.

What is the significance of disrupting HDAC protein interactions by phosphorylation? It is currently unknown whether relief of histone deacetylation and transcriptional repression is regulated by recruitment of histone acetyltransferase complexes versus regulated dissociation of the deacetylase complexes. We hypothesize that optimally, transcriptional activation would require both mechanisms (Fig. 12). Our results support a model in which transcriptional repression can be reversed by phosphorylation of HDAC complexes and disruption of corepressor interactions followed by dissociation from chromatin. Furthermore, dephosphorylation of HDAC complexes, possibly by PP1, may regulate establishment of repressive complexes at promoters during cell cycle-regulated gene inactivation. This model for HDAC1/2 phosphorylation contrasts with the known function of class II HDAC phosphorylation which occurs in a domain not found in class I HDACs and mediates nucleocytoplasmic localization via interactions with 14-3-3 (45). Such mechanisms seem amenable to acute control of early-immediate response genes directly downstream of signaling pathways.

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Fig. 12. A model for removal of HDAC mediated transcriptional repression by phosphorylation. Transcription from nucleosome-bound DNA is repressed by the recruitment of HDAC complexes with mSin3A or YY1, RbAp46/48, various DNA-binding proteins (DBP), and unknown factors (?). Stimulation of intracellular phosphorylation in response to OA or mitotic arrest, and possibly signaling pathways, results in the disruption of HDAC complexes which may involve phosphorylation of HDAC1 and 2. HDAC-mediated transcriptional repression can be reestablished by dephosphorylation (by PP1 and/or other phosphatases) and association of these repressor components.
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