Molecular Association between ATR and Two Components of the Nucleosome Remodeling and Deacetylating Complex, HDAC2 and CHD4†

Darby R. Schmidt* and Stuart L. Schreiber

Howard Hughes Medical Institute, Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts 02138

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ABSTRACT: Ataxia telangiectasia mutated (ATM)- and Rad3-related protein (ATR) is a phosphatidylinositol-kinase (PIK)-related kinase that has been implicated in the response of human cells to multiple forms of DNA damage and may play a role in the DNA replication checkpoint. The purification of an ATR complex allowed identification of chromodomain-helicase-DNA-binding protein 4 (CHD4) as an ATR-associated protein by tandem mass spectrometric sequencing. CHD4 (also called Mi-2β) is a component of a histone-deacetylase-2 (HDAC2)-containing complex, the nucleosome remodeling and deacetylating (NRD) complex. Endogenous ATR, CHD4, and HDAC2 are shown to coimmunoprecipitate, and ATR and HDAC2 coelute through two biochemical purification steps. Other members of the NRD complex, HDAC1, MTA1, and MTA2, are also detectable in ATR immunoprecipitates. ATR's association with CHD4 and HDAC2 suggests that there may be a linkage between ATR's role in mediating checkpoints induced by DNA damage and chromatin modulation via remodeling and deacetylation.

Eukaryotic cells respond to defects in DNA replication or spindle assembly and to DNA damage by halting cell cycle progression at various checkpoints (*1*, *2*). Stalled DNA replication (*3*), low nucleotide levels (*4*, *5*), strand cross-links, base damage, double-stranded breaks, or ultraviolet (UV)-induced photoadducts can activate checkpoints at the transition from G_1 to S, G_2 to M, or during S phase, causing cell cycle arrest (*6*, *7*).

The PIK-related kinases are a family of large proteins (250−400 kDa) that plays a key role in detection of DNA defects and activation of checkpoints (*8*, *9*). Individuals with ataxia telangiectasia (AT)[1] bear mutations in the PIK-related kinase Ataxia Telangiectasia Mutated (ATM) and show sensitivity to ionizing radiation (IR). These individuals have an abnormally high incidence of cancer (*10*−*13*). Cells from these individuals and from $ATM^{-/-}$ mice have been shown to lack the ability to activate p53 and other checkpoint effectors following treatment with IR (*14*−*17*).

ATM has been the focus of much work because of its clinical relevance and the availability of mutant cell lines. However, ATM may not be the most critical PIK-related kinase involved in DNA damage and replication checkpoints. AT cells, though deficient in the IR-induced G_1/S and G_2/M checkpoints, show no abnormalities when treated with other DNA-damaging agents (methyl methane sulfonate, *cis*-platinum, and UV light) or DNA replication inhibitors (hydoxyurea). Some results indicate that these forms of DNA damage may be mediated through ATR, a closely related member of the PIK-related kinase family. ATR's closest yeast homolog, MEC1, is required for checkpoint responses to multiple forms of DNA damage and replication defects (*18*). Cells that conditionally overexpress a kinase-inactive mutant of ATR exhibit sensitivity toward a wide range of DNA-damaging agents and lack the G_2/M checkpoint following its induction (*19*, *20*). These cells also fail to maintain p53 phosphorylation at serine 15 in response to UV and IR (*21*), which is thought to be a key step in p53 activation. Increased ATR expression has also been implicated as the cause of one form of rhabdomyosarcoma. Cells from this rhabdomyosarcoma show misregulation of p53 and loss of G_1/S arrest after treatment with IR, centrosome amplification, and aneuploidy. (*22*).

ATR contains a domain having kinase activity, and this activity is required for ATR's downstream signaling. The recently identified PIK-related kinases TRRAP (in humans) and Tra1 (in yeast) lack active-site residues normally essential for kinase activity (*23*), yet they maintain a high degree of homology to other family members. Their presence in complexes with components having histone acetylase activity (*24*−*26*) suggests the possibility that other PIK-related kinases function in concert with histone modifying enzymes.

Despite its implication in DNA damage checkpoints, the precise roles of ATR and its molecular mechanisms remain unclear. ATR has been shown to reside in a large molecular mass complex (1−2 MDa) (*20*). We thought that purification of ATR would allow identification of ATR-associated

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* To whom correspondence should be addressed. Phone: (617) 495-8393. Fax: (617) 495-0751. E-mail: schmidt@slsiris.harvard.edu.

[1] Abbreviations: ATM, ataxia telangiectasia mutated; ATR, Ataxia telangiectasia mutated- and Rad3-related protein; CHD4, chromodomain-helicase-DNA-binding protein 4; HDAC2, histone deacetylase 2; NRD, the nucleosome remodeling and deacetylating complex; AT, ataxia telangiectasia; IR, ionizing radiation; PMSF, phenylmethanesulfonyl flouride.

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proteins and provide insight into the biochemical functions of the ATR-containing complex. These efforts have identified the chromatin remodeling factor CHD4 (*27*, *28*) as a copurifying protein. The link between CHD4 and HDAC2 led to analysis of the association between ATR and HDAC2. Both were indeed found to coimmunoprecipitate.

MATERIALS AND METHODS

ATR Antibody Generation and ATR Stable Cell Lines. Antibodies were generated to ATR residues 1−19 and residues 814−830 by covalently conjugating these peptides to keyhole limpet hemocyonin (Pierce) and immunizing rabbits. The transformed fibroblasts, derived from GM847, with N-terminal FLAG-ATR stably integrated into the fibroblast chromosomes under a doxycyclin inducible promoter were previously described (*19*). The FLAG-ATR-WT (wild-type) stable cell line, A1, was induced with 1 μg/mL doxycyclin for 24 h prior to harvesting.

Large Scale Immunoprecipitation. HeLa cells (obtained from National Cell Culture Center) and the FLAG-ATR-WT fibroblasts were harvested, washed twice with cold PBS, lysed in 50 mM Tris buffer, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 10% glycerol, 0.5 μg/mL leupeptin, 0.7 μg/mL Pepstatin A, and 0.2 mM PMSF for 30 min at 4 °C with gentle rotating, and centrifuged at 15000*g* for 15 min. Washed cells and lysates could be flash frozen in liquid N_2 with no effect on assays or immunoprecipitations. The final concentration of the lysates was 10−20 mg of protein/mL. Conjugated anti-ATR beads were prepared as previously described (*29*), but briefly, 1.5 mL of sera from immunized rabbits was peptide purified on a Sulfolink column (Pierce), eluted with glycine, pH 2.5, neutralized, bound to protein A beads, and covalently crosslinked with dimethylpimelidate. The beads were quenched with ethanolamine and washed with glycine, phosphate-buffered saline, and then lysis buffer. Five hundred microliters of beads was incubated with 1.5 mL of lysate (30 mgs) for 1 h at 4 °C with gentle rotating. Beads were washed for 5 min three times with 1 mL of 50 mM Tris buffer, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% TritonX-100, 0.5 μg/mL leupeptin, 0.7 μg/mL Pepstatin A, and 0.2 mM PMSF at 4 °C. Proteins were eluted with 500 mM peptide for 1 h at 4 °C with gentle rotating. Protein was then precipitated with 6% trichloroacetic acid/0.04% deoxycholate, separated on SDS−PAGE, and stained with Colloidal blue staining (Novex). The 260 kDa band was excised and sequenced by peptide mass spectrometry.

Immunoprecipitations. Anti-HDAC2-conjugated beads were prepared as previously described (*29*). ATR and HDAC2 immunoprecipitations were performed as above with conjugated beads, with the exception that beads were incubated with only 0.5 mL (5−10 mg) of lysate, incubation times were 2 h, and wash times were 30 s. Twenty microliters of beads was used for each immunoprecipitation. The ATR immunoprecipitations were performed with an antibody to an internal peptide. CHD4 and preimmune immunoprecipitations were carried out with 4 μL of CHD4 and preimmune serum [obtained from Prof. Wang, prepared as previously described (*30*)] and incubated with 0.5 mL (5−10 mg) of HeLa lysate prepared as above. After 1 h, 20 μL of protein A (GibcoBRL) was added, and after another hour, washes were performed as for ATR and HDAC2 immunoprecipitations. Samples were eluted from beads with 50 mM Tris, pH 7.5, 1% SDS for 10 min at room temperature with shaking. Samples were run out on SDS−PAGE gels, transferred to immobilon P, and blotted with the appropriate antibodies. Samples were boiled in 2-mercaptoethanol containing loading buffer and loaded on a 5% SDS−PAGE gels for CHD4 and ATR blots, but for HDAC blots, samples were not boiled, were loaded in buffer lacking 2-mercaptoethanol to avoid interference of any antibody heavy chain with the HDAC2 signal, and were loaded on 10% SDS−PAGE gels. Primary antibodies were used at the following concentrations: CHD4 at 1/200 dilution of sera, HDAC2 at 1/1000 dilution of sera, ATR (N-terminal antibody was used) at 1/1000 dilution of sera, HDAC1 at 1/1000 dilution of sera (*31*), MTA1 at 1/500 dilution of sera, and MTA2 at 1/1000 dilution. MTA1 antibody was an anti-peptide antibody provided by Professor Nicolson. (*32*) MTA2 was provided by Professor Reinberg. (*33*) The PP2A antibody (Calbiochem) was used at a 1/2000 dilution. Bands were visualized with an enhanced chemiluminescent (ECL) detection protocol using an anti-Rabbit antibody conjugated to horseradish peroxidase as the secondary antibody. Input lysate loaded on the SDS−PAGE gel represents (15 μL of lysate) 3% of total lysate used. One-fourth of each immunoprecipitate was loaded on the SDS−PAGE gel for detection. Immunoprecipitations preblocked with peptide were incubated with 20 μg of peptide for 15 min at 4 °C with gentle shaking before addition of lysate.

Histone Deacetylase Assays and Treatment with Ionizing Radiationt. Assays were performed as previously described (*29*). Immunoprecipitates were prepared as described above. One-fourth of the immunoprecipitate was resuspended in 50 μL of wash buffer and incubated with 2 μg of ^{3}H-acetylated histones for 2 h at 37 °C with shaking. The solution was acidified with 0.1 M HCl and 0.16 M acetic acid and extracted with 0.6 mL of ethyl acetate. Free acetic acid was quantitated on a scintillation counter.

A549 cells were obtained from ATTC and grown to 40−70% confluence in Dulbecco's modified Eagle's media supplemented with 10% fetal bovine serum. Low cell density and early passage cells were critical to these experiments. Cells were treated with 10 Gy of ionizing radiation from a ^{137}Cs source emitting at a dose of 2.5 Gy/min and harvested 1 h later. Cells were lysed, and ATR immunprecipitations were carried out as above except all buffers contained phosphatase inhibitors (25 mM sodium flouride, 0.1 mM sodium orthovanadate, and 25 mM β-glycerophosphate).

Biochemical Purification. HeLa cells (3 g) were lysed in 40 mL of lysis buffer described above and precipitated in 5% increments with ammonium sulfate using the solid method as described (*34*). Fractions were redissolved in wash buffer described above. They were dialyzed against 4 L of 50 mM Tris buffer, pH 7.5, 150 mM NaCl, and 1 mM EDTA for 24 h. The precipitate from the 20−25% ammonium sulfate fraction was centrifuged at 15000*g* for 15 min at 4 °C, passed through a 0.2 μM filter, and loaded on a Pharmacia Biotech FPLC Mono S HR 5/5 column equilibrated with 50 mM Tris buffer, pH 7.5, 50 mM NaCl, 1 mM EDTA, and 0.03% Triton X-100. A gradient from 50 mM to 1 M NaCl was run over the column, and ATR eluted between 360 and 550 mM NaCl. Fractions were pooled, and

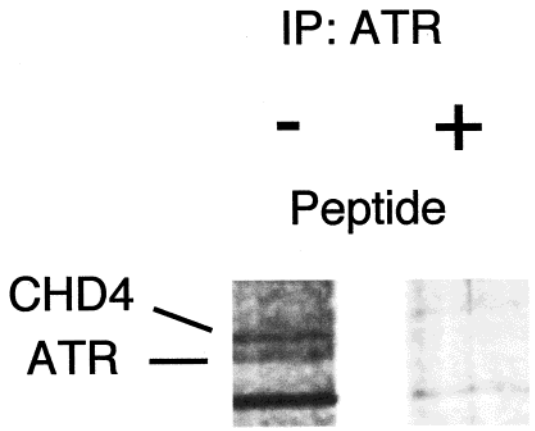

FIGURE 1: Silver stain gel of ATR immunoprecipitate with an antibody to an internal epitope from HeLa lysate without (lane 1) or with (lane 2) peptide preblocking of the antibody. ATR immunoprecipitations precipitated an ATR (250 kDa) and a 260 kDa band which was identified as CHD4 by tandem mass spectroscopy while the immunoprecipitation preblocked with peptide did not precipitate either band. ATR and CHD4 bands are indicated.

salt was reduced to 150 mM by dilution and concentration on Amicon concentrators. Samples were then immunoprecipitated as described above.

RESULTS

Purification of an ATR Complex. ATR was immunoprecipitated from HeLa lysate using three different antibodies. (1) N-terminal (AA 1−19) and (2) internal (AA 814−830) anti-peptide antibodies were purified, conjugated to protein A beads, and used to precipitate endogenous ATR from HeLa cells. (3) An anti-FLAG antibody was used to precipitate ATR from fibroblasts with a chromosomally integrated construct encoding the epitope-tagged FLAG-ATR-WT protein. The beads were washed and then eluted with the corresponding peptides. Only the immunoprecipitation using the internal peptide antibody gave a detectable and specific band. A 260 kDa band that migrated slightly slower than ATR on a 7% SDS−PAGE gel was detected in the internal peptide antibody immunoprecipitate, but not in an immunoprecipitate preblocked with peptide (Figure 1). Tandem mass spectrometric sequencing of the 260 kDa band produced two peptides that were identical in mass to the sequences VGGNIEVLGFNAR and GAADVEKVEEK found in the protein CHD4. CHD4, originally identified as an autoantigen in the disease dermatomyositis, was recently identified by several groups as a component of the nucleosome remodeling and deacetylating (NRD) complex, which contains the histone deacetylases HDAC1/2 (*30*, *35*, *36*)

Coprecipitation of Endogenous and Recombinant ATR, CHD4, and HDAC2. ATR was immunoprecipitated from HeLa extracts using conjugated anti-ATR beads, the beads were washed, and the retained proteins were analyzed by Western blotting. The ATR antibody beads immunoprecipitated proteins that were reactive with CHD4 and HDAC2 antibodies. No bands were seen when the immunoprecipitation was preblocked with the ATR peptide antigen (Figure 2). When ATR immunoprecipitates were immunoblotted for PP2A, an abundant protein not expected to be part of this complex, no bands were seen even after long exposure.

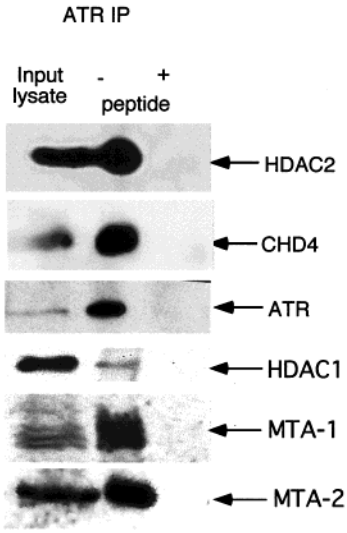

FIGURE 2: Western analysis of ATR immunoprecipitates from HeLa cell lysate with antibodies to HDAC2, CHD4, ATR, HDAC1, MTA1, and MTA2. HeLa lysates (lane 1) and immunoprecipitates without (lane2) and with (lane 3) preblocking with peptide are shown. Comparison of protein quantities in the immunoprecipitates and the lysate used for the immunoprecipitation show that the ATR immunoprecipitate was significantly enriched for CHD4, HDAC2, MTA1, and MTA2. No ATR, HDAC2, CHD4, HDAC1, MTA1, or MTA2 were detected in the ATR immunoprecipitate which was preblocked with peptide.

Immunoprecipitation of CHD4 allowed detection of HDAC2 and a faint band of ATR while no ATR or HDAC2 were present in immunoprecipitates of preimmune serum. Because the amount of ATR detected in the CHD4 immune complex was low under the conditions used and the background signal was too high with the rabbit polyclonal ATR antibody, an epitope FLAG-tagged ATR-WT was used in an attempt to strengthen the signal from ATR. The A1 cell line, derived from GM847 and stably expressing a doxycyclin inducible FLAG-ATR-WT protein, was induced for 24 h and lysed. The lysate was immunoprecipitated with CHD4 and preimmune sera. An epitope reactive band that comigrated with ATR was detected more readily than endogenous ATR in CHD4 immune complexes from stable cell line expressing FLAG-ATR-WT. Preimmune serum did not precipitate the band (Figure 3).

To investigate the interaction between ATR and HDAC2 further, anti-HDAC2 antibody raised against a C-terminal peptide (*29*) was purified, conjugated to protein A beads, and used to immunoprecipitate HDAC2 from HeLa lysate. ATR was detectable in these precipitates, but not in immunoprecipitates that were preblocked with the HDAC2 peptide (Figure 4).

The ratio of ATR to HDAC2 in the HDAC2 immunoprecipitate was found to be less than in the ATR immunoprecipitate. Some possible explanations for this result are that the HDAC2−antibody interaction may weaken the ATR−HDAC2 interaction, some ATR may not be associated with HDAC2, or HDAC2−CHD4 (NRD) complexes may be a module used by multiple complexes. CHD4 (*37*−*39*) and HDAC2 (*40*−*42*) have been identified in other complexes, so CHD4−HDAC2 may be bound to many other proteins with only a fraction of the population associated with the less abundant ATR. Therefore, HDAC2 immunoprecipita-

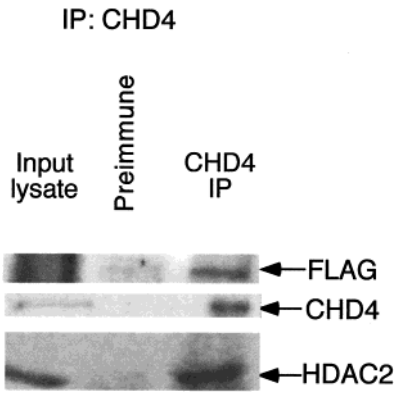

FIGURE 3: Western analysis of CHD4 immunoprecipitates from A1 cells, a line derived from GM847 cells, which expresses a stably integrated doxycyclin inducible copy of FLAG-ATR, with antibodies to FLAG, CHD4, and HDAC2. Input lysates (lane 1), preimmune antibody (lane 2), and CHD4 antibody immunoprecipitates (lane 3) are shown. ATR was induced for 24 h with 1 μg/mL doxycyclin. The CHD4 immunoprecipitate shows a FLAG reactive band that comigrates with ATR and was not present in the immunoprecipitate of preimmune serum.

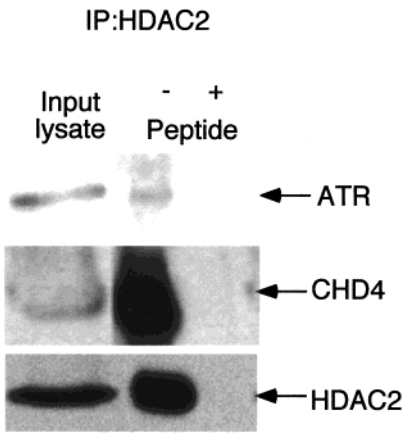

FIGURE 4: Western analysis of HDAC2 immunoprecipitates from HeLa cell lysate with antibodies to ATR, CHD4, and HDAC2. HeLa lysates (lane 1) and immunoprecipitates without (lane2) and with (lane 3) preblocking with peptide are shown. The HDAC2 immunoprecipitate contains an ATR reactive band which was not present in HDAC2 immunoprecipitate preblocked with peptide. However, only a small fraction of the ATR in the input lysate was immunoprecipitated with HDAC2 while CHD4 and HDAC2 proteins were enriched over the amount of protein detectable in the lysate used for the immunoprecipitation.

tions might precipitate a variety of complexes, only a small number of which are the ATR complex. Thus, the proportion of ATR to HDAC2 would change depending on which protein is immunoprecipitated.

The inability of the ATR N-terminal antibody to immunoprecipitate CHD4 and HDAC2 was confirmed by immunoblotting. This antibody may interfere with complex stability or block the binding surface of CHD4 and/or HDAC2. The FLAG-ATR immunoprecipitates were also immunoblotted for CHD4 and HDAC2. A small amount of each protein coprecipitated. The FLAG tag is N-terminal and may not precipitate the NRD components for the same reasons mentioned above with the N-terminal antibody. Additionally, recombinant, overexpressed ATR may not fold well or may not be incorporated efficiently into complexes. This is not surprising given ATR's large size and low abundance.

Immunoprecipitation of Other Components of the NRD Complex. Immunopreciptitations of ATR were also able to coimmunprecipitate HDAC1 (Figure 1), which is a member of an HDAC2 complex, although at lower quantities than expected. ATR immunoprecipitations were unable to precipitate HDAC3, 4, or 6 (data not shown). ATR immunoprecipitates were immunoblotted for MTA1 (*32*) and MTA2 (Figure 1), which have been shown to be part of the NRD complex (*30*, *43*). A triplet of bands reactive with the MTA1 antibody was enriched in the ATR imunnoprecipitate, but was not present in the immunoprecipitates preblocked with peptide competition. A protein band the size of MTA2 was observed in MTA2 immunoblots of ATR immunoprecipitates, but was not seen in immunoprecipitates preblocked with peptide. ATR immunoprecipitates were also blotted for CHD3, and no coprecipitating band was detected. We cannot conclude, however, that this protein is absent from the complex, because the anti-CHD3 antibody reacts only weakly with CHD3, and CHD3 is of very low abundance in HeLa cells.

Histone Deacetylase Activity of the ATR Complex. To determine whether the HDAC2 associated with ATR was enzymatically active, ATR immunoprecipitates were tested for their ability to deacetylate free histones in vitro. Immunoprecipitations were carried out as described above, and the immunoprecipitates were incubated with ^{3}H-labeled hyperacetylated HeLa histones. The histone deacetylase activity was measured by quantitating the radioactivity released via the purification and quantitation of free ^{3}H-acetic acid. ATR immunoprecipitates showed histone deacetylase activity that was 50% of that found in HDAC2 immunoprecipitates. The activity of ATR immunoprecipitate was 5.5 times greater than immunoprecipitates preblocked with peptide (Figure 5A). An immunoblot of the immunoprecipitates showed that the histone deacetylase activity correlates with the amount of HDAC2 immunoprecipitated.

Since ATR is involved in the cellular response to DNA damage, we tried to determine if the histone deacetylase activity of the complex is altered by DNA damage. A549 cells were treated with increasing doses of IR and harvested at various times. A small change in the histone deacetylase activity associated with ATR was detected by this assay when cells were treated with 10Gy of IR and harvested an hour later. Five separate experiments all showed a decrease in histone deacetylase activity upon treatment with IR in ATR immunoprecipitates. The percent decrease in activity upon treatment with IR compared to untreated cells was $41 \pm 13\%$. The variability in the value of the decrease may arise from differences in enzyme and substrate concentrations between experiments.

Copurification of ATR and HDAC2. Biochemical purification was used to assess qualitatively the strength of the ATR−HDAC2 and ATR−CHD4 interactions. Ammonium sulfate precipitation was used to eliminate much of the HDAC2 and CHD4 while yielding a large quantity of ATR. Since some results indicate (*37*, *38*, *42*) that HDAC2−CHD4 is a module used by different proteins, this step allowed us to separate ATR-associated HDAC2−CHD4 from HDAC2−CHD4 associated with other complexes. Following precipitation and dialysis to remove ammonium sulfate, ATR was found to coimmunoprecipitate with HDAC2. However, CHD4 was not detectable in ATR immunoprecipitates.

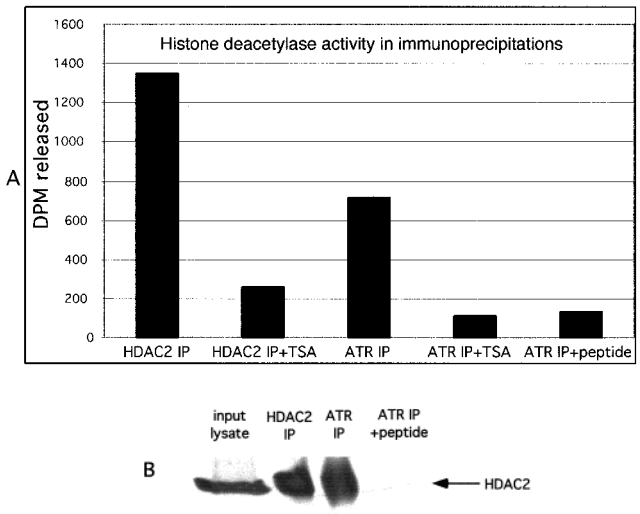

FIGURE 5: ATR immunoprecipitates contain histone deacetylase activity. ATR and HDAC2 were immunoprecipitated from HeLa lysate and assayed for histone deacetylase activity in the presence or absence of 300 nM trichostatin A (TSA). An ATR immunoprecipitate in the presence of peptide competition was also assayed for histone deacetylase activity. The ATR immunoprecipitate contained 50% of the histone deacetylase activity present in HDAC2 assays and was 5.5-fold greater than ATR immunoprecipitates preblocked with peptide. This activity was inhibited by the histone deacetylase inhibitor trichostatin A. (B) Western analysis of immunoprecipitates with antibody to HDAC2. HeLa lysate (lane 1), HDAC2 (lane 2), and ATR without (lane 3) and with (lane 4) peptide competition are shown. HDAC2 immunoprecipitate contained a greater quantity of HDAC2 consistent with its greater histone deacetylase activity.

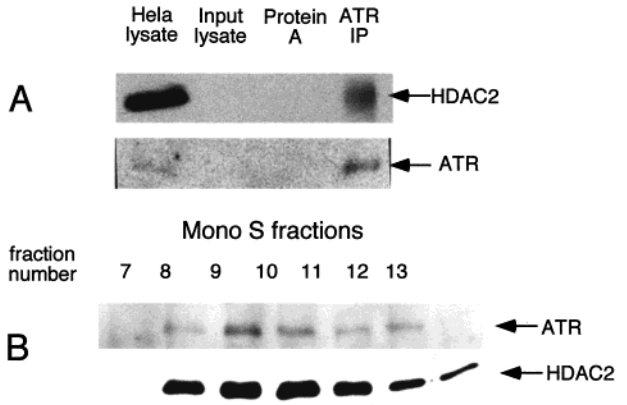

FIGURE 6: ATR and HDAC2 copurify after ammonium sulfate precipitation and Mono S chromatography. **(A)** Western analysis with HDAC2 and ATR antibodies of immunoprecipitate of ATR from protein samples after ammonium sulfate precipitation and Mono S column purification. ATR immunoprecipitate contained HDAC2 after ammonium sulfate and Mono S column purification of ATR. The ATR immunoprecipitation showed enrichment for HDAC2 over the amount of HDAC2 detectable in the purified protein fractions (input lysate). No HDAC2 was detectable in protein A beads incubated with lysate. (B) Fractions from the Mono S column were immunoblotted for ATR and HDAC2. ATR and HDAC2 show similar elution profiles on a Mono S column after ammonium sulfate precipitation purification.

Further purification of the ATR complex on a Mono S column indicated a cofractionation of HDAC2 and ATR, with both eluting at the same salt conditions (360−550 mM NaCl) (Figure 6B). Dilution to reduce salt followed by immunoprecipitation using ATR antibodies showed that HDAC2 remained associated with ATR after the Mono S column (Figure 6A). CHD4 could not be detected in ATR or HDAC2 immunoprecipitates, suggesting that the ATR−CHD4 interaction was disrupted by the purification conditions used or that ATR−CHD4 and ATR−HDAC2 are independent complexes. Thus, the ATR−HDAC2 complex is a robust biochemical entity that withstands ammonium sulfate precipitation and anion exchange chromatography.

DISCUSSION

We provide evidence here that the DNA-damage checkpoint protein ATR is present in a complex that includes the chromatin remodeling factor CHD4 and/or the histone deacetylase HDAC2. The endogenous versions of these proteins coprecipitate efficiently. Other members of the NRD complex HDAC1, MTA-1, and MTA-2 are also detectable in ATR immunoprecipitates. The robust ATR−HDAC2 interaction persisted through two biochemical purification steps.

Many molecular checkpoint events have been shown to be downstream of PIK-related kinases, but the roles of PIK-related kinases still remain elusive (*18*). This work raises the possibility that ATR and other family members may play broader roles by participating in complexes that remodel chromatin and/or alter the acetylation state of histones or other proteins.

The use of acetylation to modulate activity is most prominently seen with histone proteins. Generally, acetylated histones promote accessible and transcriptionally active chromatin while deacetylated histones promote inaccessible, silent chromatin. Histone acetyltransferases (HATs) have been identified as components of transcriptional activator complexes. Histone deacetylases (HDACs) associate with proteins involved in transcriptional repression such as the nuclear receptor corepressor (NCoR) and the repressor Sin3. Interestingly, while HDACs can deacetylate free histones, they seem to have limited ability to deacetylate histones that are compacted into nucleosomes. The recent identification of CHD4 and HDAC2 as members of the nucleosome remodeling and deacetylating (NRD) complex and the ability of this complex to increase deacetylation of compacted DNA in vitro (*30*, *35*, *36*) has led to speculation that HDACs need remodeling factors to improve access to histone acetyl groups.

Acetylation is not, however, limited to histones. Acetylation of the transcriptional factors p53 (*44*−*46*) and GATA-1 (*47*, *48*) has also been shown to occur in vivo, and this acetylation increases the activity of these proteins. The acetylation of p53, in fact, occurs following treatment with UV and IR (*44*, *45*), indicating that the DNA damage responses involve acetylation.

Several lines of evidence have introduced the idea that chromatin status is an important factor in DNA damage checkpoints. In yeast, there are several examples in which chromatin structure affects DNA damage checkpoints. Ku70, a detector of double-stranded breaks in humans, was shown in yeast to interact with the silencing factor Sir4 by two-hybrid analysis (*49*). Sir2, 3, and 4 mutants show defects in illegitimate recombination and DNA end-joining. Recent work in yeast has shown that Ku70, Sir3, and Sir4 can delocalize from telomeres upon DNA damage and relocalize to regions of double stranded breaks in a MEC1-dependent fashion (Mec1p is a yeast homologue of ATR) (*50*, *51*), indicating that relocalization of chromatin regulating enzymes

might occur upon activation of DNA damage checkpoints. In yeast, DNA damage was recently shown to repress the firing of late origins of replication in S phase (*52*, *53*). Mutations in the DNA damage checkpoint genes MEC1 and RAD53 cause constitutive firing of late origins of replication, indicating that checkpoint genes may control the accessibility of DNA to the replication machinery at late firing origins of replication.

DNA damage checkpoints are places in the cell cycle at which a delay occurs in the presence of DNA damage. The possibility that chromatin modulation is an integral part of the mechanism to effect these delays is supported by the fact that remodeling and acetylation factors appear to play an important role in regulating normal cell cycle progression. Two human chromatin remodeling factors, hBRM and BRG-1, are phosphorylated and excluded from the condensed chromatin during mitosis. (*54*) These events coincide with a decrease in their remodeling ability (*55*). The histone acetylase CBP in humans is phosphorylated at G_1/S, which increases its histone acetyltransferase activity (*56*). Increased acetylation activity of CBP is also induced by the viral oncogene E1A, indicating that the acetylase activity of CBP may be an important mechanism for driving cells into S phase. Ikaros, a DNA-binding protein involved in lymphoid development, was shown to interact with CHD3/4 and HDAC1/2. The Ikaros complex becomes localized to heterochromatin upon T cell stimulation and entry into S phase (*38*, *57*). Decreased levels of active Ikaros lead to misregulation of S phase entry.

The above evidence leads us to consider several possible roles for ATR. CHD4 contains two chromodomains (*58*) that have been shown to localize other proteins to condensed DNA (*59*). One model is that CHD4's presence in the complex indicates that ATR is associated with heterochromatin. Early work on DNA damage focused on how repair could occur in DNA condensed in nucleosomes and some studies indicate that repair enzymes have difficulty accessing condensed DNA (*60*, *61*). In this model, ATR could be the guardian of heterochromatin, sampling DNA to check for damage in densely compacted DNA by unfolding and refolding heterochromatin. A second possible role for the ATR−CHD4−HDAC2 complex(es) could be in downstream signaling. DNA damage or replication halts could signal the ATR−HDAC2−CHD4 complex(es) to alter its repressive activity toward certain transcripts involved in DNA repair and checkpoints or toward origins of replication. This could occur through changes in activity, complex composition, or localization. A third possibility suggested by others (*49*) (*62*−*64*) is a model where silencing factors localize to regions of damage and turn off or prevent transcription to avoid interference of transcriptional machinery with DNA repair.

PIK-related kinases are key elements of DNA damage and replication checkpoints, but their functions are still unclear. This work suggests that chromatin remodeling and deacetylation have a role in the function of the PIK-related kinase ATR. A detailed knowledge of the biochemical function of ATR and other PIK-related kinases is essential for a more comprehensive understanding of cell cycle checkpoints.

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