Phosphorylation of RNA Helicase A by DNA-Dependent Protein Kinase Is Indispensable for Expression of the *MDR1* Gene Product P-Glycoprotein in Multidrug-Resistant Human Leukemia Cells†

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ABSTRACT: Development of multidrug resistance (MDR) in cancer frequently involves overexpression of the *MDR1* gene product P-glycoprotein (P-gp), a drug transporter which severely impedes the efficacy of chemotherapy. Because intensive efforts to identify therapeutics that reverse MDR by inhibiting the drug transport activity of P-gp have not yet met with success, we have focused on the alternative strategy of targeting *MDR1* promoter activation to knockdown P-gp expression in cancer cells. We recently identified RNA helicase A (RHA) inhibition as a rational strategy to downregulate P-gp in leukemia cells by showing that RHA RNAi knockdown abrogated P-gp expression in MDR variants of human leukemia HL-60 cells. In that report, we also demonstrated that RHA activated the *MDR1* promoter in the MDR variant cells but not in the drug-sensitive counterpart. This led us to hypothesize that P-gp induction by RHA required cooperation with another factor present only in the MDR variants. Here, we identify the RHA cooperating factor as DNA-PK catalytic subunit (cs), and we show that DNA-PKcs resides with RHA at the *MDR1* promoter in a multiprotein complex. Furthermore, targeted DNA-PKcs inhibition abrogated P-gp expression in the MDR variant cells. We demonstrate that constitutive multisite RHA phosphorylation producing retarded migration in SDS−PAGE is catalyzed by DNA-PKcs in the MDR variants, and does not occur in the parental cells, which are DNA-PKcs deficient. The indispensable role played by DNA-PK in P-gp overexpression in MDR leukemia cells in this report identifies targeted DNA-PK inhibition as a rational strategy to reverse drug resistance in cancer.

The multidrug resistance gene *MDR1*[1] encodes the M_r 170,000 ATP-dependent transmembrane transporter P-glycoprotein (P-gp). P-gp is expressed in epithelial cells of the liver, kidney, and intestine and capillary endothelial cells of the brain, ovary, and testis, where it acts as a barrier to the uptake of xenobiotics and promotes their excretion (*1*). P-gp expression produces an MDR phenotype in cancer cells by pumping a variety of structurally unrelated anticancer drugs out of the cells, and the cytoprotective effect of maintaining a diminished intracellular drug concentration is exacerbated in cancer cells that overexpress P-gp (*1*−*6*). Cross-resistance to a broad range of anticancer drugs is frequently developed in many human solid tumors and hematological malignancies prior to or during chemotherapy. Higher doses of cytotoxic drugs fail to improve the pharmacotherapeutic response in resistant tumors. In a recent Southwest Oncology Group study of P-gp overexpression in acute myelogenous leukemia (AML), about a third of the AML patients presented with P-gp-overexpressing disease at diagnosis, and P-gp overexpression was significantly associated with drug-resistant disease (*7*). Thus, MDR has been identified as one of the primary causes of AML therapy failure.

MDR1 promoter-enhancing factor (MEF1) is a nuclear protein that binds to the promoter region of the *MDR1* gene and upregulates P-gp expression (*8*). We recently identified RNA helicase A (RHA) as a component of the MEF1 transcription factor complex bound at the *MDR1* gene promoter in drug-resistant variants of HL-60 acute myelogenous human leukemia cells, i.e., HL-60/Vinc and HL-60/VCR (*9*). RHA, a member of the DEAD/H family of RNA helicases, is a multifunctional cellular protein involved in diverse molecular events including the regulation of transcription (*10*−*15*) and translation (*16*). HL-60 cells do not express P-gp. In contrast, HL-60/Vinc (which was selected from HL-60 cells) and HL-60/VCR (which was selected from HL-60/Vinc cells by exposure to increasing concentrations of the anticancer drug vincristine) are characterized by robust P-gp overexpression and P-gp-mediated multidrug resistance, e.g., they are respectively ∼600-fold and ∼1400-fold less sensitive to vinblastine than HL-60 cells (*8*). Our recent report established that RHA plays an indispensable role in the *MDR1* gene promoter activation and P-gp overexpression in HL-60/Vinc and HL-60/VCR cells, e.g., RNA interference knockdown of RHA decreased P-gp expression in the HL-60/Vinc cells to a barely detectable level (*9*). Furthermore,

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[1] Abbreviations: MDR, multidrug resistance; P-gp, P-glycoprotein; RHA, RNA helicase A; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; DSBs, double-strand breaks; OA, okadaic acid; DMNB, 4,5-dimethoxy-2-nitrobenzaldehyde.

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ectopic RHA expression markedly elevated the P-gp expression level and drug resistance of the HL-60/Vinc cells but did not induce P-gp expression or alter the drug sensitivity of the parental HL-60 cell line (*9*). This suggested that a factor required for the transcriptional upregulation of P-gp expression by RHA in the drug-resistant HL-60 variant cells was missing or inactive in the parental cells. It was therefore intriguing that the migration of RHA extracted from the HL-60/Vinc and HL-60/VCR cells was impeded in SDS−PAGE by M_r ~20,000 compared to RHA extracted from the parental cells, suggestive of differential posttranslational modification with the potential for functional relevance (*9*).

RHA is one of several nuclear proteins that were recently identified as candidate DNA-dependent protein kinase (DNA-PK) substrates based on the ability of purified human DNA-PK (Promega) to phosphorylate the isolated proteins in *in vitro* assays (*17*). DNA-PK is a Ser/Thr protein kinase composed of an ~470 kDa catalytic subunit (DNA-PKcs) and a regulatory heterodimer (Ku70/Ku80) (*18*). The Ku heterodimer, which has high affinity for DNA ends, recognizes and binds the DNA ends and then recruits DNA-PKcs to the DNA, leading to activation of the protein kinase activity of DNA-PKcs. The catalytic domain of DNA-PK consists of 380 amino acids in the C-terminal region of DNA-PKcs. Protein kinases are organized into families based on sequence homology relationships in the catalytic domain. DNA-PK is thus classified as a member of the phosphatidylinositol 3-kinase-like protein kinase (PIKK) family. Apart from recognizing and binding DNA ends through the Ku proteins, DNA-PKcs displays an affinity for other specialized DNA structures that interrupt the standard double helical conformation of DNA (*19*, *20*). Besides Ku70/Ku80 (*21*, *22*), a number of DNA-PKcs-interacting proteins have been reported including Artemis (*23*), XRCC4 (*24*), PARP-1 (*25*), H2AX (*26*, *27*), and p53 (*28*). Furthermore, many of the DNA-PKcs-interacting proteins are the phosphorylation substrates of its kinase activity (*19*). DNA-PKcs plays a critical but incompletely understood role in the repair of DNA double-strand breaks (DSBs) by nonhomologous end joining (NHEJ). DSBs are the most dangerous form of DNA damage arising within the cell, which, if left unrepaired, threaten the integrity of the genome and even survival of the organism. DNA-PKcs is also implicated in other pathways of genome surveillance, e.g., apoptogenic signaling and telomere maintenance (*20*).

In this report, we establish that constitutive multisite phosphorylation of RHA is maintained by DNA-PK in HL-60 drug-resistant variant but not parental cells, and that DNA-PK-catalyzed RHA phosphorylation is indispensable for *MDR1* promoter activation and P-gp overexpression in the drug-resistant cells. These results constitute the first evidence that DNA-PK targeted therapeutics may be effective against P-gp/MDR drug resistance mechanisms in human cancer.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies, and Plasmids. The human acute myelogenous leukemia cell line HL-60, and its P-gp-mediated multidrug-resistant derivative HL-60/Vinc, the *MDR1* promoter −198/+43-*firefly* luciferase reporter plasmid construct pMDR1-luc, and its mutant that has a deletion in the CAAT-like element, as well as P-gp antibody and RHA antibody have been described previously (*8*, *9*). Other antibodies used are anti-GST antibody (Amersham Biosciences), PhosphoSerine antibody Q5 (Qiagen), and DNA-PKcs Ab-2 (Lab Vision).

To maintain the MDR phenotype, HL-60/Vinc cells were cultured in growth media containing 0.1 μg/mL vincristine. Sf21 insect cells were maintained in Sf-900 II SFM media (Invitrogen) at 27 °C.

The donor plasmid containing the His-tagged full-length human RHA-coding sequence (Accession Number NM_001357), pFastBAC1/His-RHA, and the plasmid containing the GST-human RHA N-terminal fragment (aa 1−318)-coding sequence were kindly provided by Dr. F. Grosse (Institut fur Molekulare Biotechnologie, Germany). The human DNA-PKcs (Accession Number NM_006904) expression vector, pCMV6/DNA-PKcs, was a gift from Dr. K. Meek (Michigan State University, East Lansing, MI).

Preparation of Nuclear Extracts. The nuclear extracts from HL-60 and HL-60/Vinc cells were prepared essentially as described (*29*). Briefly, cells were washed with ice-cold phosphate-buffered saline, suspended in hypotonic buffer, and lysed in a Dounce homogenizer. Nuclear proteins were extracted from the isolated nuclei by addition of high salt buffer and dialyzed against 500× volumes of the dialysis buffer (20 mM Hepes, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, and 20% glycerol) at 4 °C. Concentration of the nuclear proteins was determined by the method of Bradford.

Western Blotting. Cells were harvested and lysed in Nonidet P-40 buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 50 mM NaF, 0.5 mM Na_3VO_4 plus protease inhibitor cocktail (Sigma). Equal microgram amounts of protein were subjected to SDS−PAGE and then transferred onto Immobilon polyvinylidene difluoride membrane (Millipore). Membranes were incubated with the indicated antibodies. Secondary antibodies were anti-mouse Ig, anti-rabbit Ig, or anti-goat Ig conjugated to horseradish peroxidase (Amersham Biosciences). Immunoreactive protein bands were visualized by using the ECL kit (Pierce).

Immunoprecipitation. Cell lysates were prepared in RIPA buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, 50 mM NaF, 0.5 mM Na_3VO_4 plus protease inhibitor cocktail (Sigma). Five-hundred microgram quantities of the proteins were precleared using preimmune serum and protein A agarose (Invitrogen) and incubated with 5 μL of anti-RHA antibody or preimmune serum at 4 °C with constant rotation. The immune complexes were collected on the protein A agarose beads by centrifugation, washed three times with the RIPA buffer, and resuspended in 30 μL of the SDS sample buffer. The protein samples were heated to 95 °C and fractionated by SDS−PAGE, followed by in-gel phosphoprotein staining or Western blotting.

Phosphoprotein Staining. Fluorescent staining of SDS−polyacrylamide gels using Pro-Q Diamond phosphoprotein gel stain (Molecular Probes) was performed based on the manufacturer's instruction. Briefly, the gels were fixed in 50% methanol, 10% acetic acid overnight, washed three times in distilled and deionized water for 10 min per wash, incubated in Pro-Q Diamond phosphoprotein gel stain for 90 min, and then destained with three washes in Pro-Q

Diamond destaining solution for 30 min per wash. Fluorescence images were acquired on a fluorescent image scanning unit FMBIO II (Hitachi Genetic Systems MiraiBio Inc. Division) with excitation set at 532 nm and emission at 585 nm. Following image acquisition, the same gels were stained for total protein with SYPRO Ruby protein gel stain (Molecular Probes), followed by two washes in 10% ethanol, 7% acetic acid. The Pro-Q Diamond stain is washed away during the staining procedure for total protein. The gel was then scanned on FMBIO II with excitation set at 532 nm and emission at 605 nm. PeppermintStick phosphoprotein molecular weight standards (Molecular Probes) were used as negative and positive controls for the phosphoprotein and total protein staining.

Expression and Purification of Recombinant RHA. The Bac-to-Bac baculovirus expression system (Invitrogen) was used to generate recombinant baculovirus for the expression of the His-tagged full-length RHA according to the manufacturer's instructions. In brief, the pFastBAC1/His-RHA was transformed into DH10Bac cells that contain the bacmid and a helper plasmid. The recombinant bacmid was isolated and used to infect Sf21 insect cells. The infected Sf21 cells were lysed and His-tagged RHA transgene products were purified using the Ni-NTA Purification System (Invitrogen) as indicated in the manufacturer's protocol. Purity of the His-RHA protein was determined by SYPRO Ruby protein gel stain, and identity was verified by Western blotting with the anti-RHA antibody.

The N-terminal fragment of RHA (aa 1−318) was expressed in *Escherichia coli* as a GST fusion protein. The GST-RHA (aa 1−318) fusion protein was purified according to the manufacturer's instructions (Amersham Biosciences), and the purity was determined using the SYPRO Ruby protein gel stain and identity was verified by Western blotting with the anti-GST antibody.

Phosphatase Treatment. Nuclear extracts and recombinant RHA were subjected to dephosphorylation, where indicated, with calf intestinal alkaline phosphatase (New England BioLabs). The phosphatase-treated samples were then fractionated by SDS−PAGE and immunoblotted with the indicated antibodies.

Phosphatase Inhibitor or DNA-PK Inhibitor Treatment. HL-60/Vinc cells were incubated with the indicated concentration of okadaic acid (OA) (Sigma), a Ser/Thr protein phosphatase inhibitor, for 24 h, or 4,5-dimethoxy-2-nitrobenzaldehyde (DMNB), a selective inhibitor of DNA-PK (Calbiochem). The cells were harvested and lysed in the Nonidet P-40 buffer. The lysates were then subjected to SDS−PAGE and Western blotting with RHA antibody.

Dual Luciferase Reporter Gene Assay. For the promoter enhancement study, HL-60/Vinc cells (5×10^5)/well in six-well plates were transiently cotransfected with 0.5 μg of pMDR1-luc or pMDR1(del)-luc reporter construct and 1.5 μg of pDNA-PKcs expression vector in 1 mL of serum-free medium using DMRIE-C transfection reagent (Invitrogen). The pRL-SV40 vector (0.03 μg/well), which contains the *Renilla* luciferase gene driven by the early SV40 enhancer/promoter, was included in each transfection and served as an internal control for transfection efficiency. Where needed, the total amount of DNA per well was adjusted to 2.03 μg by addition of an empty vector pcDNA3. The cell/DNA mixture was incubated at 37 °C for 5 h and then supplemented with 4 mL of standard growth medium. The cells were harvested at 48 h posttransfection and assayed for the luciferase activities with a dual luciferase reporter assay system (Promega) in a TD-20/20 luminometer (Turner Designs). Transfections were performed in triplicate at least twice. Firefly luciferase light units were normalized against *Renilla* luciferase and expressed as fold induction of the normalized luciferase activities observed with pMDR1-luc, which was set at 1.0. The promoter-less pGL3-Basic vector was used for background luciferase activity, which was subtracted from each normalized *firefly* luciferase unit obtained with the promoter-containing constructs. For the DNA-PK inhibition experiment, similar procedures as described above were taken except that pMDR1-luc (1.0 μg) and pRL-SV40 were used in the transfection, and DMNB was added at a final concentration of 100 μM 5 h posttransfection. The DMNB-treated sample was expressed as percentage of the DMSO-treated control, which was set at 100%.

RESULTS

Endogenous RHA in the Drug-Resistant Cancer Cells (HL-60/Vinc) Is Phosphorylated. We recently established a stringent requirement for RHA in the constitutive activation of the *MDR1* gene promoter and consequent stable P-gp overexpression that produce the profoundly MDR phenotype of the drug-resistant HL-60/Vinc cell line (*9*). Furthermore, enforced RHA expression further increased the P-gp expression level in HL-60/Vinc cells but did not induce P-gp in the drug-sensitive parental HL-60 cell line (which does not express P-gp at a level detectable by Western blotting analysis of the cell lysate). This indicated that P-gp induction by RHA required cooperation with another factor(s). Interestingly, we also found that these two cell lines have distinct forms of RHA, with the protein band of higher molecular mass being present only in HL-60/Vinc. In the present study, we sought to identify the factor responsible for the strongly retarded migration of RHA extracted from HL-60/Vinc cells, which corresponded to an increased apparent molecular mass of M_r ~20,000 *versus* RHA extracted from HL-60 cells, to determine whether posttranslational RHA modification accounted for its capacity to activate the *MDR1* gene promoter in HL-60/Vinc but not HL-60 cells.

Alkaline phosphatase efficiently and indiscriminately dephosphorylates phospho residues in proteins. To assess whether the apparent mass difference in the RHA species in HL-60 *versus* HL-60/Vinc cells was due to phosphorylation, we prepared nuclear extracts (NE) from HL-60 and HL-60/Vinc cells, and incubated the HL-60/Vinc NE with alkaline phosphatase. The results of Western blotting analysis of the NE with RHA antibody are shown in Figure 1A. The HL-60 RHA species migrated at M_r ~127,000 (lane 1), while the HL-60/Vinc RHA species migrated primarily at M_r ~150,000, with a faint band comigrating with the HL-60 RHA species (lane 2). Furthermore, the heavy sample loading in lane 1 clearly indicates the absence of the M_r ~150,000 RHA species in the HL-60 NE. Treatment of the HL-60/Vinc NE with 0.5 activity unit of purified alkaline phosphatase for 2 h at 30 °C partially converted the M_r ~150,000 RHA species into the M_r ~127,000 RHA species, producing RHA bands of equivalent intensity at these positions (lane 3). Near-complete conversion to the M_r ~127,000 RHA

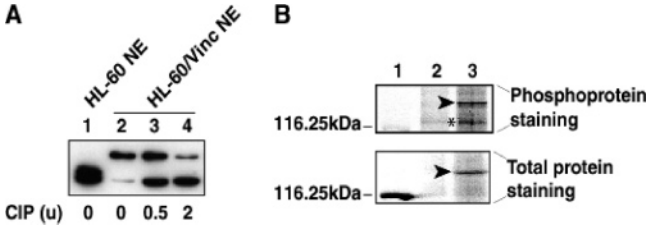

FIGURE 1: Endogenous RHA in the drug-resistant human leukemia cell line (HL-60/Vinc) is phosphorylated. (A) Nuclear extracts (NE) prepared from the drug-sensitive parental cancer cell line HL-60 and the drug-resistant counterpart HL-60/Vinc were left untreated or treated with the indicated amounts of alkaline phosphatase for 2 h at 30 °C and resolved by SDS−7.5% PAGE, followed by Western blotting using α-RHA antibody. (B) Cell lysate prepared from HL-60/Vinc cells was immunoprecipitated with preimmune serum (negative control) (lane 2) or α-RHA antibody (lane 3). The immunoprecipitates were subjected to SDS−7.5% PAGE and in-gel phosphoprotein staining (upper panel). Following fluorescence image acquisition, the same gel was stained for total protein (lower panel) and scanned. Lane 1 contains the molecular weight standards consisting of phosphoproteins and nonphosphoproteins with only the 116.25 kDa nonphosphoprotein presented. Arrowheads, M_r ~150,000 RHA species. Asterisk, nonspecific band.

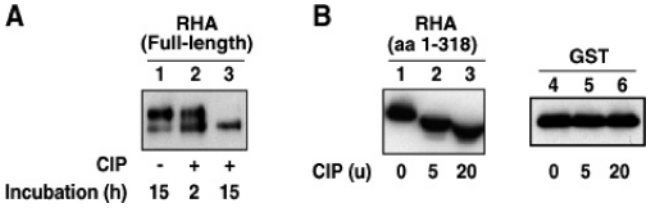

FIGURE 2: Recombinant RHA is a phosphoprotein. (A) Recombinant His-tagged full-length RHA was left untreated or treated with alkaline phosphatase CIP (20 u) for the indicated time and subjected to SDS−7.5% PAGE, followed by Western blotting with the antibody recognizing phosphorylated serines. (B) Recombinant GST-tagged N-terminal RHA fragment (aa 1−318) or GST (negative control) was incubated without or with the indicated amounts of CIP at 30 °C for 2 h, resolved by SDS−7.5% PAGE and SDS−10% PAGE respectively, and immunoblotted with α-GST antibody.

species was achieved in an HL-60/Vinc NE sample that was treated with an increased amount of phosphatase (2 units) (lane 4). These results indicate that RHA is constitutively phosphorylated in HL-60/Vinc cells at sites that are dephosphorylated in HL-60 cells. Furthermore, the M_r ~20,000 retardation of migration in SDS−PAGE produced by RHA phosphorylation strongly suggested that the differential phosphorylation of RHA in the drug resistant cells occurs at multiple sites.

For direct visualization of the HL-60/Vinc phospho-RHA species, we employed a phosphoprotein staining method developed by Molecular Probes. In these experiments, immunoprecipitates were extracted from the HL-60/Vinc cell lysate with RHA antibody (Figure 1B, lane 3) or preimmune serum as a negative control (lane 2). After separation by SDS−PAGE, the samples were in-gel-stained for phosphoprotein (Figure 1B, upper panel) and restained to visualize total protein (lower panel). In the molecular marker lane (lane 1), the nonphosphoprotein molecular weight standard at M_r 116,250 illustrates the principle that a readily detected dephosphoprotein (lower panel) is not detected with the phosphoprotein stain (upper panel) (other nonphospho/phospho markers were outside the boundaries of the gel section shown). The HL-60/Vinc band indicated by arrowhead is identified as the RHA species based on its immunoprecipitation by RHA antibody (lane 3) and not by preimmune serum (lane 2) and migration position of M_r ~150,000. The RHA species is clearly identified as phosphoprotein in the analysis by its strong detection with the phosphoprotein stain (upper panel, lane 3). Corroborating the HL-60/Vinc RHA protein content by Western analysis in Figure 1A, lane 2, only the M_r ~150,000 RHA species was detected by protein staining (lower panel, lane 3). The band indicated by asterisk (upper panel, lane 3) is considered to be a nonspecific band as it is also visible in the immunoprecipitate by preimmune serum (upper panel, lane 2).

Recombinant Human RHA Is a Phosphoprotein in Insect Cells. Since the extent of endogenous human RHA phosphorylation in HL-60 cells was markedly affected by the cell context, i.e., multisite constitutive RHA phosphorylation creating a slower migrating species in a drug-resistant variant was absent in the parental line, we next investigated recombinant human RHA, reasoning that a defined transgene product would facilitate determination of whether the *MDR1* gene promoter activation by RHA is controlled by the phosphorylation state of the helicase. Because RHA has an ancient evolutionary origin (*30*), e.g., human RHA (Accession Number Q08211) has 50% amino-acid sequence identity and 69% similarity to the *Drosophila* homologue RNA helicase Mle (Accession Number P24785), we chose an insect cell expression system for the analysis, reasoning that crucial phosphoregulatory sites in RHA would likely be conserved from insects to humans, as would the protein kinases that phosphorylate those sites.

We expressed His-tagged full-length human RHA in Sf21 cells with the baculovirus expression system, recovered recombinant RHA in purified form by nickel affinity chromatography, treated the purified helicase with alkaline phosphatase, and examined its phosphorylation state by Western blotting with a phospho-Ser selective antibody. In the absence of alkaline phosphatase treatment, recombinant RHA migrated in SDS−PAGE as a major phospho-Ser species at M_r ~150,000 and a minor phospho-Ser species at M_r ~140,000, i.e., the predicted migration position of the polypeptide transgene product (Figure 2A, lane 1). Immunodetection of the M_r ~150,000 phospho-Ser RHA species was corroborated by the disappearance of this species in an RHA sample that was incubated with alkaline phosphatase for 15 h at 30 °C (Figure 2A, lane 3). Furthermore, the equivalent intensities of the faster and slower migrating phosphorylated RHA species in the sample treated with alkaline phosphatase for 2 h at 30 °C is indicative of a gradual conversion of the slower migrating species to the faster migrating species by dephosphorylation (Figure 2A, lane 2). In addition, immunodetection of phospho-Ser in the faster migrating RHA species was corroborated by the diminution of the band intensity with prolonged alkaline phosphatase treatment (Figure 2A, lane 2 *versus* lane 3).

It is not surprising that the precise migration positions of human RHA differ in human *versus* insect cells, given the phylogenetic differences in posttranslational processing and other regulatory controls in these cell contexts. The conservation of multisite human RHA phosphorylation amounting to SDS−PAGE migration shifts of 10,000 to 20,000 in insect *versus* human HL-60/Vinc cells (Figure 1A and Figure 2A)

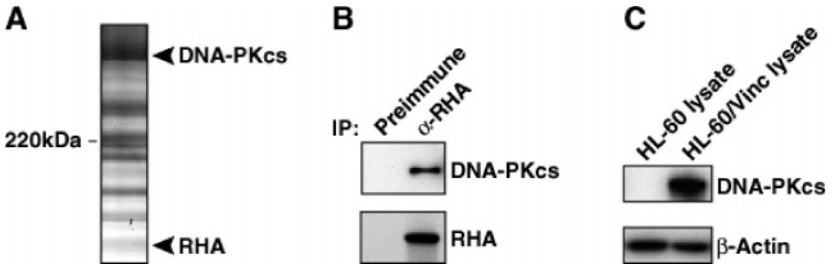

FIGURE 3: RHA is associated with DNA-PKcs in HL-60/Vinc cells. (A) CAAT-like element-containing oligonucleotides coupled to CNBr-activated Sepharose 4B were used to affinity purify the drug-resistant leukemia cell nuclear extracts which were preincubated with a 20-fold molar excess of ds CAAT-deleted and GC-box-like oligonucleotides, as well as poly(dI-dC)·(dI-dC) to remove nonspecific DNA-binding proteins. The CAAT-like element bound proteins were resolved by SDS−5−15% PAGE and visualized by silver staining. Protein identification was performed by mass spectrometry. Arrowheads, the identified proteins. (B) HL-60/Vinc cell lysates were immunoprecipitated with preimmune serum (negative control) or α-RHA antibody and immunoblotted with α-DNA-PKcs or α-RHA antibodies. IP, immunoprecipitation. The RHA species migrated at M_r ∼150,000. (C) Immunoblots prepared from cell lysates of HL-60 and HL-60/Vinc cancer cells were probed with antibodies against DNA-PKcs or β-actin (loading control).

is strongly suggestive of a conserved mechanism of RHA phosphoregulation.

Interestingly, these experimental results are consistent with the results of a bioinformatics analysis that we conducted with the web-based tool DISPHOS (disorder-enhanced phosphorylation predictor, core.ist.temple.edu) (*31*). Those results predicted that human RHA may have as many as 25 phospho-Ser acceptor sites, while only one Thr and one Tyr residue were predicted as candidate phosphoacceptor sites (data not shown).

Recombinant Human RHA Is a Phosphoprotein in Bacteria. The N-terminal sequence of human RHA (aa 1−318) encodes two regulatory elements, which are tandem double-stranded RNA binding domain (dsRBD) modules. The dsRBD is an ∼70 residue module conserved across the phylogenetic spectrum from prokaryotes, e.g., *E. coli* ribonuclease III (RNase III) to human RHA and other dsRNA-interacting human gene products (*32*). *E. coli* RNase III is stimulated several-fold by phosphorylation, and two of the phosphorylation sites identified thus far are Ser residues in the dsRBD module of the prokaryotic ribonuclease (*33*). The dsRBD region of human RHA has been reported to contain a binding site for the general transcription coactivator p300/CBP (*10*), but it is not known if this region contains phosphorylation sites. We therefore investigated whether the N-terminal dsRBD domain of human RHA contained phospho-acceptor sites recognized by prokaryotic protein kinases (*34*). Recognition of phosphorylation sites in human RHA by prokaryotic protein kinases would suggest stringent conservation of the sites and hence strong potential for regulatory function.

To investigate whether human RHA, like *E. coli* RNase III, contains phosphorylation sites in the dsRBD region that are recognized by prokaryotic protein kinases, we expressed the GST-tagged N-terminal RHA fragment (aa 1−318) in *E. coli* and analyzed its phosphorylation state by alkaline phosphatase treatment. Treatment with alkaline phosphatase increased the mobility of the GST-tagged N-terminal RHA fragment in the gel in a dose-dependent manner as visualized by probing with anti-GST antibody (Figure 2B, left panel), but had no effect on the mobility of the control GST construct (Figure 2B, right panel). Our result showing that the mobility of GST did not change after the phosphatase treatment not only illustrates that the GST-RHA band shift is due to the dephosphorylation of the RHA moiety in the fusion protein, but also excludes the existence of proteolytic degradation.

The different mobilities of the RHA dsRBD region, i.e., untreated control M_r ∼62,000 (lane 1), 5 units of alkaline phosphatase M_r ∼58,000 (lane 2), and 20 units of alkaline phosphatase M_r ∼54,000 (lane 3), indicate that the dsRBD region of human RHA is phosphorylated at multiple sites by prokaryotic protein kinases. Furthermore, the phosphorylation-dependent retardation of migration amounted to M_r ∼8000, similar to the migration shift of M_r ∼10,000 observed for full-length human RHA in insect cells. Taken together with the stimulation of prokaryotic RNase III activity by dsRBD phosphorylation, this strongly suggests a stringently conserved mechanism of phospho-regulation in human RHA.

RHA Is Associated with the Catalytic Subunit of DNA-PK (DNA-PKcs) in HL-60/Vinc Cells. Using a DNA-affinity chromatography approach, we previously isolated a multiprotein complex bound to the CAAT-like sequence of the human *MDR1* gene promoter (*9*). Mass spectrometry analysis of the proteins and database search using the ProFound online search engine (prowl.rockefeller.edu) identified several candidate regulatory proteins bound to the promoter (unpublished observations), one of which, RHA, has been characterized as indispensable for promoter activation and P-gp expression (*9*).

We now report that DNA-PKcs is also a member of the promoter-bound multiprotein complex (Figure 3A, arrowhead) (*9*). To corroborate the DNA affinity chromatography-based evidence that a multiprotein complex containing RHA and DNA-PKcs is constitutively expressed in the drug-resistant variant HL-60/Vinc, co-immunoprecipitation experiments were done. When RHA antibody was used to immunoprecipitate endogenous RHA from the HL-60/Vinc cell lysate, Western blotting analysis of the precipitate revealed that DNA-PKcs coprecipitated with M_r ∼150,000 RHA, i.e., the phospho-RHA species (Figure 3B, lane 2); neither RHA nor DNA-PKcs was detected in the negative control, i.e., by Western blotting analysis of the preimmune serum precipitate (lane 1).

As a test of the hypothesis that enforced RHA expression elevated the P-gp expression level and drug resistance of HL-60/Vinc cells, but had no such effect in HL-60 cells because the drug-sensitive parental cells lacked a factor required for the response, we measured the relative expres-

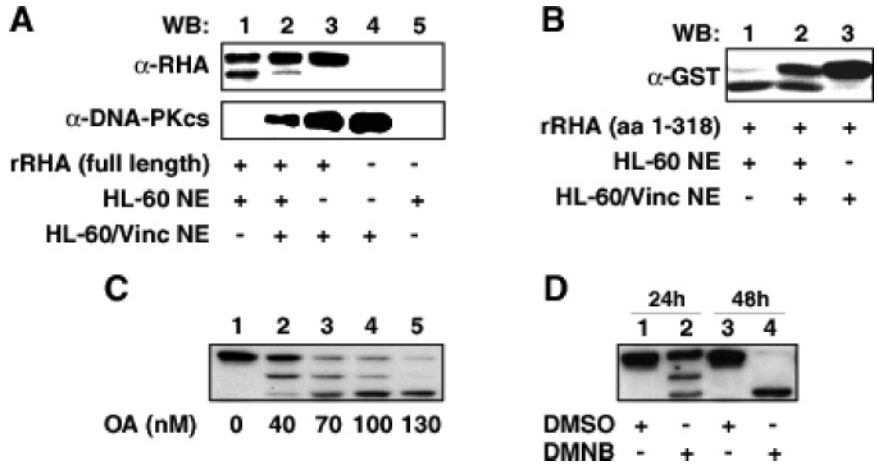

FIGURE 4: DNA-PKcs is the kinase of RHA phosphorylation *in vivo*. (A) and (B) Recombinant RHA (rRHA) (full length and GST-tagged N-terminal fragment aa 1−318) were incubated with HL-60 nuclear extract (NE) (lacking DNA-PKcs) and/or HL-60/Vinc NE. The mobility changes of the recombinant RHA on SDS−7.5% PAGE were detected by Western blotting with α-RHA or α-DNA-PKcs antibody (A) or α-GST antibody (B). (C) and (D) HL-60/Vinc cancer cells were treated with the indicated concentration of the protein phosphatase inhibitor okadaic acid (OA), which inactivates DNA-PKcs, for 24 h (C) or with DMSO (negative control) or the selective DNA-PK inhibitor (DMNB) (final concentration: 100 μM) for 24 h or 48 h (D). The cell lysates were prepared and the mobility changes of RHA on SDS−7.5% PAGE were detected by Western blotting with α-RHA antibody. WB, Western blotting.

sion levels of DNA-PKcs in the HL60/Vinc and HL-60 cell lines. The Western blotting analysis results in Figure 3C revealed copious expression of DNA-PKcs in the HL-60/Vinc cells whereas DNA-PKcs expression was beneath the detection limit in HL-60 cells. Given the association of RHA and DNA-PKcs in HL-60/Vinc cells (Figure 3B) and the fact that HL-60 cells are deficient in the protein kinase activity which sustained the expression of the M_r ~150,000 phospho-RHA species in HL-60/Vinc cells (Figure 1A), this further suggests that DNA-PKcs might be the RHA kinase that produces the M_r ~150,000 phospho-RHA species.

RHA Is Phosphorylated by DNA-PKcs. To test the prediction that phosphorylation by DNA-PKcs produces the M_r ~150,000 phospho-RHA species in HL-60/Vinc cells, we examined whether phosphorylated recombinant human RHA purified from insect cells would be dephosphorylated by endogenous phosphatases when incubated with HL-60 nuclear extract (NE) and would resist dephosphorylation in the HL-60/Vinc NE. Recombinant full-length RHA purified from insect cells was incubated with HL-60 NE and HL-60/Vinc NE overnight at 4 °C. Western blot analysis of the incubated mixtures was conducted with RHA and DNA-PKcs antibodies. The amount of recombinant RHA chosen for incubation with the nuclear extracts rendered the relative level of endogenous RHA beneath the Western blot analysis detection limit, allowing straightforward analysis of the recombinant RHA species (Figure 4A, lanes 4 and 5).

As shown in lane 1 of the upper panel in Figure 4A, the band intensities of the M_r ~150,000 recombinant phospho-RHA species and the M_r ~135,000 RHA species were equivalent after incubation in the HL-60 NE, whereas incubation in a 1:1 mixture of the HL-60 and HL-60/Vinc NEs strongly protected against conversion to the M_r ~135,000 RHA species (lane 2), and complete protection against conversion to the M_r ~135,000 RHA species was afforded by incubation in the HL-60/Vinc NE (lane 3). The DNA-PKcs expression profile in the NE is shown in the lower panel. As observed with HL-60 cell lysate (Figure 3C), DNA-PKcs was undetectable in HL-60 NE (Figure 4A, lane 5). Similar results were obtained with the recombinant N-terminal human RHA fragment produced in *E. coli*, implicating the dsRBD region as a major locus of differential RHA phosphorylation in HL-60/Vinc versus HL-60 cells (Figure 4B).

To test the correlative evidence that phosphorylation by DNA-PKcs is responsible for constitutive expression of the slower migrating phospho-RHA species in HL-60/Vinc cells, inhibitors of DNA-PK activity were used. DNA-PK is activated by protein phosphatase 5-catalyzed dephosphorylation. Protein phosphatase 5 is an okadaic acid-sensitive protein Ser/Thr phosphatase, and okadaic acid treatment decreases the kinase activity of DNA-PKcs in cells (*35*, *36*). We reasoned that if the slower migrating form of RHA was due to phosphorylation by any of the multitude of protein kinases that are not stimulated by a dephosphorylation mechanism, then okadaic acid should protect the slower migrating RHA species against conversion to the faster migrating species. On the other hand, if okadaic acid accelerated conversion to the faster migrating species, this would narrow the candidate protein kinases involved to DNA-PKcs or another protein kinase activated by an okadaic acid-sensitive Ser/Thr dephosphorylation mechanism. Inclusion of okadaic acid (OA) in the HL-60/Vinc cell culture at a range of concentrations between 40 nM and 130 nM resulted in a progressive dephosphorylation of RHA (Figure 4C). It is noteworthy that the endogenous RHA, like the recombinant dsRBD RHA fragment (Figure 2B), also exhibited two dephosphorylated species of distinct M_r, reflecting stepwise dephosphorylation, indicative of constitutive multisite RHA phosphorylation in the drug-resistant cells. In contrast, OA treatment effects on RHA mobility were negligible in the drug-sensitive, DNA-PKcs deficient (Figure 3C) HL-60 cell line (data not shown).

The vanillin derivative 4,5-dimethoxy-6-nitrobenzaldehyde (DMNB) was recently identified as a selective DNA-PK inhibitor. DMNB inhibited DNA-PK with an IC_{50} of 15 μM in *in vitro* assays of the purified kinase, and DMNB did not inhibit other critical PIKKs, e.g., ATM and ATR (*37*). Treatment of the HL-60/Vinc cells with 100 μM DMNB resulted in time-dependent RHA dephosphorylation (Figure

4D). At 24 h, the DMNB-treated cells expressed two dephosphorylated RHA species with the same M_r as those produced by okadaic acid treatment, i.e., M_r ~135,000 and M_r ~127,000, and only the faster dephosphorylated RHA species was expressed in the cells after 48 h of DMNB treatment. Thus, stepwise RHA dephosphorylation to the fast migrating species in HL-60/Vinc cells was induced by an inhibitor of DNA-PK allosteric activation (okadaic acid) and an inhibitor of DNA-PK kinase activity (DMNB), implicating DNA-PK in the constitutive *MDR1* promoter activation and P-gp overexpression in the drug-resistant HL-60/Vinc cells.

Enforced Expression of DNA-PKcs Enhances the Transcriptional Activity of the MDR1 Promoter via the CAAT-like Element, and Inhibition of DNA-PK Abrogates P-gp Expression. To assess the effect of DNA-PKcs on *MDR1* gene expression, we performed a functional study in the HL-60/Vinc cells. The luciferase reporter gene construct containing the *MDR1* promoter (pMDR1-luc) was transiently cotransfected with the DNA-PKcs expression vector (pDNA-PKcs) or an empty vector (pcDNA3). Luciferase activities were measured with a luminometer. As shown in Figure 5A, an ~1.8-fold increase in the transcriptional activity of the reporter gene was observed by DNA-PKcs expression. To test whether this increase was achieved through the CAAT-like element in the *MDR1* promoter to which the MEF1 multiprotein complex including RHA and DNA-PKcs is bound, the activity of the promoter/luciferase construct containing the CAAT deletion in the promoter (pMDR1-(del)-luc) was determined following cotransfection with the DNA-PKcs expression vector. The CAAT deletion abolished the induction by DNA-PKcs (Figure 5A), indicating that the CAAT element is required for DNA-PK activation of the *MDR1* gene.

Since DNA-PKcs enhances *MDR1* promoter activation, we expected that the promoter activity of the pMDR1-luc construct would be reduced by treating the cells with the DNA-PK inhibitor DMNB. DMNB treatment produced ~80% reduction in the *MDR1* promoter activity (Figure 5B). We then examined the expression level of P-gp, the *MDR1* gene product, by Western blot analysis of lysates prepared from HL-60/Vinc cells treated with DMNB. The results in Figure 5C show that DMNB treatment produced an ~2-fold decrease in P-gp expression at 15 h. After 24 h of DMNB treatment, P-gp expression plummeted to a negligible level, and this persisted for at least 48 h. Taken together, the results in this report reveal that maintenance of the P-gp/MDR drug resistance phenotype in acute myelogenous leukemia stringently requires DNA-PK activity in the MEF-1 transcription factor complex. Most importantly, the observations in this report are the first to identify targeted DNA-PK therapy with small-molecule inhibitors as a rational strategy to reverse P-gp-mediated multidrug resistance in cancer.

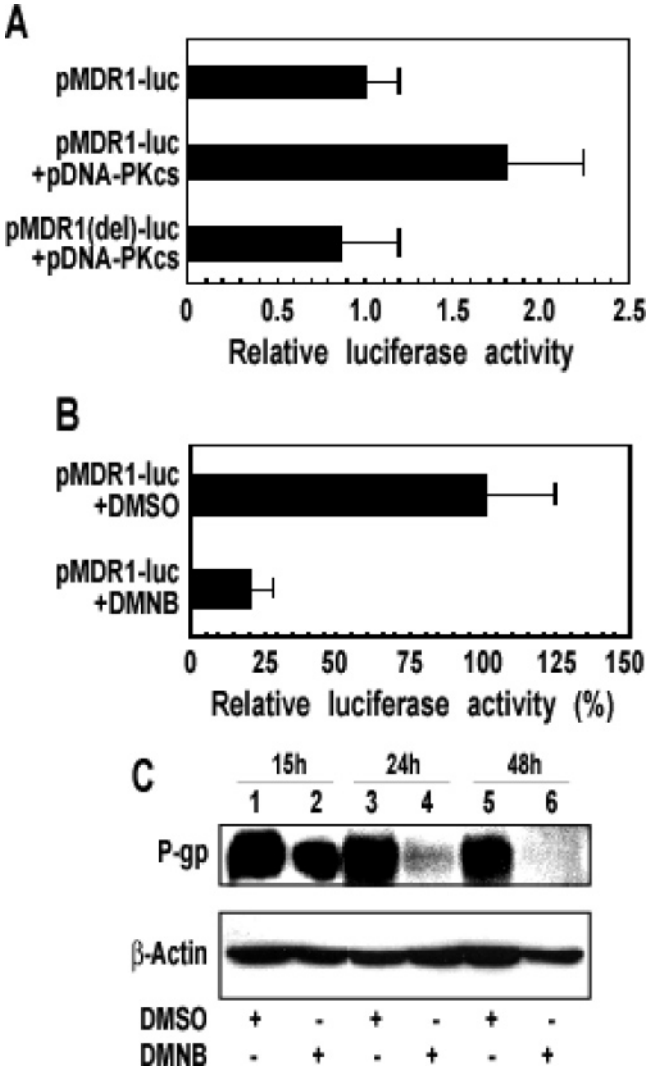

FIGURE 5: DNA-PKcs regulates the *MDR1* gene expression. (A) Luciferase reporter gene construct (0.5 μg) driven by the wild-type *MDR1* gene promoter (pMDR1-luc) or by the mutant promoter containing the CAAT sequence deletion (pMDR1(del)-luc) was cotransfected with or without 1.5 μg of the DNA-PKcs expression vector (pDNA-PKcs) into HL-60/Vinc cancer cells by using the DMRIE-C transfection reagent. The mean activity unit from pMDR1-luc transfection was set at 1.0, and others were expressed as an activity relative to that of pMDR1-luc. (B) pMDR1-luc (1.0 μg) was transfected into HL-60/Vinc using DMRIE-C transfection reagent, and the cells were treated with the DNA-PK inhibitor DMNB or DMSO at 5 h posttransfection. The DMNB-treated sample was expressed as a percentage of the DMSO-treated one, which was set at 100%. Bars, SD. (C) HL-60/Vinc cells were treated with DMSO or DMNB (final concentration: 100 μM) for the indicated time. The cell lysates were subjected to SDS−7.5% PAGE, followed by Western blotting with the antibody against P-gp or β-actin (loading control).

DISCUSSION

We previously showed that ectopic expression of RHA enhances the activity of the *MDR1* gene promoter, elevates the P-gp expression level, and increases the level of drug resistance in the drug-resistant human leukemia cell line HL-60/Vinc but not in the drug-sensitive parental cell line HL-60. We also found that RHA protein with different molecular masses, migrating at M_r ~127,000 and ~150,000, respectively, on SDS−PAGE, is present in the two cell lines. In this report, we demonstrate that this mass difference results from phosphorylation of RHA in HL-60/Vinc cells as revealed by the phosphoprotein staining approach and the dephosphorylation analysis using alkaline phosphatase. RHA phosphorylation by purified human DNA-PK (Promega) in *in vitro* kinase assays has been reported (*17*, *38*). However, it is unknown whether RHA is in fact an endogenous substrate of DNA-PK. We demonstrate that RHA is a phosphorylation target of DNA-PK in HL-60/Vinc cells by

showing that (1) DNA-PKcs was copurified with RHA by DNA-affinity chromatography and co-immunoprecipitated using RHA antibody, (2) HL-60 lacks DNA-PKcs and does not express the phosphorylated ~150,000 RHA species, (3) phosphorylated recombinant RHA was dephosphorylated after incubation with HL-60 nuclear extracts which lack DNA-PKcs, but not with HL-60/Vinc nuclear extracts which contain DNA-PKcs, and (4) selective DNA-PK inhibition blocked RHA phosphorylation. Importantly, we demonstrate that DNA-PK-catalyzed RHA phosphorylation enhances the transcription of the *MDR1* gene through the CAAT-like element of the *MDR1* gene promoter, which provides a locus for targeted therapy reversal of drug resistance in cancer (see below). Our findings extend the range of DNA-PK cellular functions to the regulation of P-gp expression.

We also demonstrate that multisite RHA phosphorylation is conserved in protein kinase proteomes across the phylogenetic spectrum from prokaryotes to insects to humans, strongly implicating DNA-PK-catalyzed multisite phosphorylation as critical to the regulation of RHA function. Indeed, multisite phosphorylation of a protein has been found in many human cellular proteins and is recognized as an important mechanism for regulating protein function (*39*). RHA is a multifunctional protein and interacts with a number of critical proteins, such as the general coactivator CREB-binding protein (CBP)/p300 (*10*), RNA polymerase II (*12*), histone γ−H2AX (*38*), the breast cancer-specific tumor suppressor protein BRCA1 (*11*), Werner syndrome helicase (*40*), and the oncoprotein EWS-FLI1 (*14*). The N-terminus of RHA protein contains two copies of a double-stranded RNA-binding domain, the central part bears the helicase catalytic motif and binds ATP, and the C-terminus contains a binding site for single-stranded nucleic acids. Using the predictor of natural disorder regions (PONDR, www.pondr.com), a number of disordered regions in the RHA protein were identified (data not shown). Disordered regions are defined as entire proteins or regions of proteins that lack a fixed tertiary structure. As disordered regions are recognized to be commonly responsible for important protein functions such as transcriptional regulation, translation, and cellular signal transduction (*41*, *42*), they have recently drawn increasing interest. Disorder in and around the potential phosphorylation target site is thought to be a prerequisite for phosphorylation (*31*). Thus, given the multiple functions of RHA and the importance of multisite phosphorylation and protein disorder, it is of great interest to determine how the multisite phosphorylation, especially that in the disordered regions of RHA, affects its DNA/RNA/protein recognition and binding specificity/affinity (enhancing or attenuating) and how the effects subsequently regulate the cellular events in which RHA is involved.

A significant finding in the present report is that we identified DMNB, a selective small-molecule DNA-PK inhibitor, as a modulator of *MDR1* gene expression. DMNB treatment of the drug-resistant leukemia cells led to robust reduction in activity of the *MDR1* promoter and abrogation of the gene product P-gp. P-gp confers cancer cell drug resistance by reducing intracellular drug accumulation, which severely impairs the efficacy of chemotherapy (*6*). Thus, we propose that DNA-PK inhibitors may serve as potent inhibitors of P-gp expression. In fact, many DNA-PK inhibitors have been developed and evaluated for their ability to potentiate the cytotoxicity of the anticancer drugs and enhance the efficacy of radiotherapy, likely via inhibition of DNA repair requiring DNA-PK (*19*, *37*, *43*, *44*). Combination therapies consisting of DNA-PK inhibitors and cytotoxic agents may prove an effective strategy for targeting P-gp-induced multidrug-resistant tumor cells. Conceptually, preventing the transcriptional activation of the *MDR1* gene to knockdown production of the gene product P-gp may be superior to blocking the drug transport function of P-gp. However, due to limited knowledge of the mechanism involved in the transcriptional activation of the gene, intensive efforts to identify therapeutics that reverse multidrug resistance have focused on blocking P-gp transporter, which have not yet met with success. Thus, our study offers an innovative strategy for cancer therapy by which new modalities could be established via validating RHA and DNA-PK as targets with "druggable" active sites to knockdown P-gp production to improve the efficacy and lessen the toxicity of cancer chemotherapy.

While DNA-PKcs is undetectable in the parental HL-60 cancer cell, it is expressed in the drug-resistant counterpart HL-60/Vinc. We propose that DNA-PKcs expression is induced during the process of drug selection to establish the drug-resistant cell line. The balance between kinases and phosphatases controls the net phosphorylation state of a phosphorylatable protein. Therefore, induction of DNA-PKcs results in hyperphosphorylation of RHA in the drug-resistant cancer cell, and in turn activation of the *MDR1* gene and development of the MDR phenotype.

The protein phosphatase inhibitor okadaic acid (OA) is a naturally occurring toxin (*45*, *46*), which can inhibit most members of the phosphoprotein phosphatase PPP family such as PP1 and PP2A, as well as the recently cloned PP4, PP5, PP6, and PP7. Because DNA-PK activity is reported to be controlled by a PP2A-like enzyme (*35*) and PP5 phosphatase (*36*) and four phosphorylation sites in the catalytic subunit of DNA-PK have been identified in okadaic acid-treated human cells (*47*), OA was used in the present study to investigate whether inhibition of DNA-PK activity would lead to RHA dephosphorylation. The result showed that RHA from OA-treated HL-60/Vinc cancer cells was dephosphorylated, which suggests that the DNA-PK phosphorylation state is regulated by OA-sensitive PPP family member(s) which in turn regulates the phosphorylation state of RHA. The result also implies that OA-sensitive phosphatases do not directly dephosphorylate RHA since, if they did, inhibition of these phosphatases by OA would prevent instead of promote dephosphorylation of RHA. Therefore, other phosphatase(s) (*48*, *49*) might be involved in direct dephosphorylation of RHA. Given the multifunctional tasks of RHA, such as enhancing *MDR1* gene expression (*9*), identification of the phosphatase(s) directly dephosphorylating RHA may provide another molecule target for therapeutic application or a probe for the study of cellular regulation that involves RHA.

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