Inhibition of Phosphoinositide 3-Kinase Related Kinases by the Radiosensitizing Agent Wortmannin

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ABSTRACT

Members of the phosphatidylinositol-3 kinase related kinase (PIKK) family function in both cell cycle progression and DNA damage-induced cell cycle checkpoints. The fungal metabolite, wortmannin, is an effective radiosensitizer that irreversibly inhibits certain members of the PIKK family. Based on their roles in DNA damage responses, several PIKKs, DNA-dependent protein kinase (DNA-PK), ataxia telangiectasia mutated (ATM) and the ataxia- and Rad3-related protein (ATR), are potential targets for the radiosensitizing effect of wortmannin. In this report, we demonstrate that wortmannin is a relatively potent inhibitor of DNA-PK (IC50, 16 nM) and ATM (IC50, 150 nM) activities, whereas ATR activity is significantly less sensitive to this drug (IC50, 1.8 μM). In intact A549 lung adenocarcinoma cells, wortmannin inhibited both DNA-PK and ATM at concentrations that correlated closely with those required for radiosensitization. Furthermore, pretreatment of A549 cells with wortmannin resulted in radioresistant DNA synthesis, a characteristic abnormality of ATM-deficient cells. These results identify wortmannin as an inhibitor of ATM activity and suggest that ATM and DNA-PK are relevant targets for the radiosensitizing effect of this drug in cancer cells.

INTRODUCTION

Cell cycle checkpoints ensure that critical events, such as DNA replication and chromosome segregation, are completed in a timely and accurate fashion during each eukaryotic cell cycle. In addition, certain checkpoints are activated by environmental insults that result in DNA damage, such as ionizing or UV radiation. Checkpoint activation by unreplicated or damaged DNA triggers a signal transduction cascade that orchestrates a variety of cellular responses, including cell cycle arrest, DNA repair, and apoptotic death (1, 2). The importance of cell cycle checkpoints in the maintenance of genomic stability is highlighted by the severe clinical manifestations resulting from germ-line mutations in the ATM gene (2, 3). Patients with A-T exhibit progressive cerebellar degeneration, immunodeficiency, radiation hypersensitivity, and a dramatically increased incidence of various cancers, particularly lymphomas. Cells derived from A-T patients exhibit characteristic defects in G1, S, and G2 checkpoints after exposure to ionizing radiation. These cell cycle checkpoint defects presumably underlie the radiation hypersensitivity and the high mutational rates displayed by A-T cells (3).

The COOH-terminal region of the ATM polypeptide contains a domain bearing significant sequence homology to those of mammalian and yeast PI-3Ks. Recent studies have uncovered a novel family of eukaryotic signaling proteins that share this region of similarity to the PI-3K-related catalytic domain. This family of PIKKs plays key roles in the regulation of eukaryotic cell cycle progression and cell cycle checkpoint functions (1, 3). The ATM protein is one of four known mammalian PIKK family members. ATR is most closely related to ATM and shares some checkpoint control functions with ATM (4). DNA-PKcs has also been linked genetically to DNA damage response pathways (1–3). Together with its associated Ku70 and Ku80 subunits, DNA-PKcs associates directly with free ends of double-stranded DNA, suggesting that this kinase plays a proximal role in the cellular response to DNA strand breaks. In contrast to the other mammalian PIKKs, the mTOR appears to participate in mitogenic signal transduction, rather than in DNA damage-induced checkpoint functions.

In light of the radiosensitive phenotypes exhibited by cells defective in ATM, ATR, or DNA-PK, a logical prediction is that a small molecule inhibitor of one or more of these kinases might enhance the cytotoxic effects of ionizing radiation or DNA-damaging cancer chemotherapeutic agents. Supporting evidence for this prediction stems from recent observations that the sterol-like fungal metabolite, wortmannin, is a highly effective radiosensitizer (5–7). At low nanomolar concentrations, wortmannin irreversibly inhibits the lipid kinase activities of certain mammalian PI-3Ks by covalent modification of a critical lysine residue in their phosphotransferase domains (8, 9). The presence of the PI-3K-related catalytic domain in the PIKK family members suggested that these kinases may also be sensitive to inhibition by wortmannin. Indeed, recent findings indicate that the protein kinase activities of DNA-PK and mTOR are inhibited by nanomolar concentrations of wortmannin (10, 11). The effect of wortmannin on the catalytic activities of ATM and ATR and the possible contribution of ATM and/or ATR inhibition to wortmannin-induced radiosensitization remain unclear.

To further define the mechanism whereby wortmannin sensitizes cells to killing by ionizing radiation, we have determined the effects of this drug on the protein kinase activities of DNA-PK, ATM, and ATR. We demonstrate that DNA-PK and ATM kinase activities are inhibited by wortmannin at nanomolar concentrations, whereas the kinase activity of ATR is significantly more resistant to this drug. Inhibition of the protein kinase activities of all three PIKKs by wortmannin is highly correlated with the covalent binding of the drug to these proteins. Interestingly, the concentrations of wortmannin that inhibit ATM and DNA-PK, but not ATR, activities in intact A549 lung adenocarcinoma cells also radiosensitize these cells. Furthermore, treatment of A549 cells with wortmannin and radiation results in radioresistant DNA synthesis, a hallmark abnormality of ATM-deficient cells. Collectively, these results support the idea that pharmacological inhibitors of the DNA damage-responsive PIKKs may effectively sensitize tumor cells to killing by ionizing radiation or chemotherapeutic agents.

MATERIALS AND METHODS

Cell Culture and Antibodies. The A549 lung adenocarcinoma cell line was maintained in RPMI 1640 (Life Technologies, Inc.) containing 10% fetal...
bovine serum. A fibroblast cell line, GM02052, derived from an A-T patient, was obtained from the Coriell Institute for Medical Research. The GM02052 cell line was maintained in minimal essential medium (Life Technologies, Inc.) supplemented with 15 mm HEPES, 20% fetal bovine serum, and 2X nonessential and 2X essential amino acids (Life Technologies, Inc.). Wortmannin (Sigma Chemical Co.) was dissolved in DMSO to yield a 20 mM stock solution, which was aliquoted and stored at −80°C. The drug was diluted into either RPMI 1640 or aqueous buffer immediately prior to drug treatment of either cells or immunopurified proteins, respectively. Cells were pretreated with wortmannin for 1 h at 37°C prior to the preparation of cell extracts for immunoprecipitations with PIKK-specific antibodies. We observed that the inhibitory effects of wortmannin on the test kinases reached steady state within 10 min of addition of drug to intact cells or to the immunopurified proteins (data not shown). Wortmannin-specific monoclonal antibodies were prepared in the Mayo Department of Immunology Monoclonal Antibody Facility by immunization of BALB/c mice with wortmannin conjugated to ovalbumin. Rabbit polyclonal antiserum specific for ATR and antibodies specific for ATM (Ab-3) and DNA-PK (Ab-1) were obtained from Oncogene Research-Calbiochem.

Immune Complex Kinase Assays. A549 cells were cultured in 100-mm tissue culture dishes and were harvested for assays during exponential growth. The cells were washed twice with PBS and then scraped on ice to 0.25 ml of lysis buffer (20 mM HEPES, 0.15 M NaCl, 1.5 mM MgCl₂, and 1 mM EGTA, pH 7.4) containing 1 mM DTT, 10 μg/ml aprotinin, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 20 mM microcystin, and 0.2% Tween 20. Lysates were cleared of insoluble material by centrifugation, and equivalent amounts of protein (0.5 mg) were incubated on ice for 2 h with ATM-specific antibodies. The immune complexes were precipitated with protein A-Sepharose beads, and the resulting immunoprecipitates were washed twice in lysis buffer, once in high-salt buffer (0.1 M Tris-HCl, pH 7.4, containing 0.6 M NaCl), and once in kinase base buffer (10 mM HEPES, 50 mM NaCl, and 10 mM MgCl₂, pH 7.4). In the indicated experiments, samples were incubated for 30 min at room temperature with kinase base buffer containing various concentrations of wortmannin. The kinase reaction mix was then added to yield final concentrations of 10 mM HEPES, 50 mM NaCl, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM DTT, 10 μM [γ-32P] ATP (specific activity, 5 Ci/mmol; ICN), and 25 ng/μl recombinant PHAS-I (Stratagene) in a total volume of 40 μl. Kinase reactions were incubated for 20 min at 30°C. In preliminary studies, linear reaction conditions were established for phosphorylation of exogenous substrate by ATM, as well as DNA-PK and ATR. The reactions were terminated with equal volume of 30% acetic acid, and duplicate aliquots were spotted onto P-81 phosphocellulose papers (Whatman). After 4–5 min washes in 1% phosphoric acid containing 10 mM sodium PP, the radioactivity retained on the paper was measured by liquid scintillation counting.

For measurements of DNA-PK activity, cells were lysed as described above, with the exception that the lysates were sonicated prior to clearing. Cell extracts (0.75 mg protein) were immunoprecipitated with anti-DNA-PK antibodies. The immune complexes were washed twice with lysis buffer and twice with kinase base buffer prior to the kinase reaction. The kinase reaction conditions were identical to those described above, except that the reaction time was 15 min and the substrate was a p53-derived peptide (Promega), added to a final concentration of 250 ng/μl in the kinase reaction. After addition of an equal volume of 30% acetic acid, the soluble material was spotted onto P-81 phosphocellulose paper. The paper was then washed four times (5 min per wash) with 15% acetic acid containing 10 mM sodium PP, prior to liquid scintillation counting.

The ATR kinase assay has been described recently.4 The assay conditions are similar to those described for ATM above, except that cellular extracts were prepared in 0.5% NP40-containing lysis buffer. The histone H1 kinase assay for Cdc2 has also been described (12).

Western Blotting. Cells were harvested by scraping into lysis buffer containing 0.5% NP40. The cleared extracts (2 mg protein) were immunoprecipitated with the appropriate antiserum and protein A-Sepharose. Immunoprecipitates were washed twice in lysis buffer and twice in kinase buffer before incubation with the indicated concentrations of wortmannin. Samples were subjected to SDS-PAGE and transferred to Immobilon-P membranes (Millipore) prior to immunoblotting. Wortmannin-bound proteins were detected by probing the membrane for 12 h at 4°C with 0.3 μg Wm7.1 monoclonal antibody per ml of Tris-buffered saline containing 0.02% Tween 20 (TBST) and 5% nonfat dried milk. After washing in TBST, membranes were incubated with a secondary polyclonal rabbit anti-mouse IgG antibody (Pierce). Blots were developed with horseradish peroxidase coupled to protein A and the Enhanced Chemiluminescence reagent (Amersham). Finally, the blots were stripped and reprobed with the indicated PIKK-specific antibodies.

Clonogenic Assay. The effect of wortmannin on the radiosensitivity of A549 cells was assessed with a clonogenic assay. A549 cells in log-phase growth were harvested, resuspended in fresh growth medium, and plated in triplicate in 60-mm dishes at cell concentrations estimated to yield 20–100 colonies/dish after treatment. Four h after plating, cells were irradiated with a 137Cs source at a dose rate of 6.4 Gy/min. Wortmannin was added to the indicated samples immediately after irradiation. The final concentration of the drug solvent did not exceed 0.1% (v/v), and this solvent concentration had no effect on either the clonogenicity or radiosensitivity of the A549 cells (data not shown). Cells were cultured for 2 weeks prior to fixation and staining with Coomasie Blue. Colonies with >50 cells were scored.

Cell Cycle Analysis. A549 cells were synchronized at the G1-S border by culturing for 18 h in 5 μg/ml aphidicholin (Sigma). Dishes were washed once with PBS, and fresh medium was added. At 3.5 h after release from the aphidicolin block, the S-phase-enriched cells were treated with wortmannin. Thirty min later, cells were irradiated with 0 or 5 Gy as described above. After an additional 30 min, the drug-containing medium was replaced with fresh culture medium. Twenty-four h later, cells were harvested by trypsinization and fixed in PBS containing 70% ethanol; the samples were stored at −20°C. The fixed cells were resuspended in PBS containing 20 μg/ml propidium iodide and 100 μg/ml boiled RNaseA and were incubated for 30 min at 37°C prior to flow cytometric analysis on a Becton Dickinson FACScan. Twenty-thousand un gated events were collected. Cell cycle distribution was determined with the ModFit software package (Verity) after excluding doublets and clumps by gating on the DNA pulse height versus pulse area displays.

Radioresistant DNA Synthesis. A549 cells in exponential growth were harvested and plated in 96-well plates (10,000 cells/well in 0.1 ml of standard growth medium). Each treatment condition was tested in six replicate wells. After 18 h, the cells were irradiated as described above. The cells were treated as indicated with wortmannin and then incubated for 20 min at 37°C. The fixed cells were pulsed for 40 min with 2 μCi [methyl-3H] thymidine (specific activity, 5 Ci/mmol; Amersham). Cells were harvested by trypsinization, transferred onto glass filters, and lysed in distilled water. Filter-bound radioactivity was determined by scintillation counting.

Statistics. All statistical analyses were performed with the Sigma Plot 4.0 (SPSS) software package. The concentrations resulting in half-maximal inhibition (IC₅₀) for the various kinases were calculated by fitting the data to the Hill 4-parameter equation, using a least-squares regression, and then solving the equations for a relative activity of 0.5. The radiation dose-response curves were fit with the linear-quadratic equation: ln(S) = −αD − βD², where S is the surviving fraction, D is the dose of radiation, and α and β are constants (13). The SERs for each experiment were calculated as the ratio of radiation doses that resulted in 10% survival of the cells in the absence or presence of wortmannin. A paired, two-tailed Student t test was used to determine the statistical difference between the calculated SER at each dose of wortmannin and the nondrug-treated controls.

RESULTS

Recent studies have defined methodologies that allow determinations of the protein kinase activities of three of the mammalian PIKKs, mTOR, ATR, and DNA-PK, in immune complex kinase assays (1, 11, 14). However, a sensitive, reproducible assay for the in vitro catalytic activity of ATM, isolated from nontransfected cells, has not been described. We had observed previously that the serine-threonine-rich polypeptide PHAS-I was a suitable substrate for measurements of the

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Fig. 1. Phosphorylation of PHAS-I by immunoprecipitated ATM. In A, A549 cell extracts were immunoprecipitated with preimmune serum (Pre) or with ATM-specific antibodies. The indicated sample was pretreated for 30 min at room temperature with 1 μM wortmannin (Wm). Immune complex kinase reactions were done as described in “Materials and Methods,” and the reaction products were separated by SDS-PAGE. The numbers below the sample lanes indicate fold increase in 32P incorporation into the PHAS-I substrate (arrowhead), as measured with a Molecular Dynamics Phosphorimager System and ImageQuant software. In B, ATM was immunoprecipitated from detergent extracts of A549 cells or from ATM-null fibroblasts (GM02052). Immune complex kinase assays were done with [γ-32P]ATP and PHAS-I as the substrate. Incorporation of radioactivity into PHAS-I was quantitated with a filter-binding assay (see “Materials and Methods”). Results shown represent the means of two independent experiments; bars, SD. C, effect of high-salt treatment on ATM and DNA-PK activities. ATM and DNA-PK were immunoprecipitated from A549 cell extracts, and the immunoprecipitates were washed with either kinase buffer (•) or high-salt buffer (□). The washed immunoprecipitates were then assayed for kinase activity toward PHAS-I. For both the ATM and DNA-PK kinase assays, the relative activity was defined as the ratio of 32P incorporation into the PHAS-I substrate for the sample washed in high-salt buffer over the parallel sample washed in kinase buffer only. The results are presented as the means from two independent experiments; bars, SD. D, immunoblot analysis of immunoprecipitated PIKKs. Detergent extracts from A549 cells were immunoprecipitated (IP) with pre-immune antiserum (Pre) or with ATM-, DNA-PK-, or ATR-specific antibodies. The immunoprecipitates were washed according to the standard procedure for each PIKK (see “Materials and Methods”). Three sets of immunoprecipitated proteins were separated by SDS-PAGE, transferred to individual membranes, and immunoblotted (IB) in parallel with the indicated antibodies.

**in vitro** kinase activities of mTOR (11) and ATR. Consequently, we reasoned that PHAS-I might also serve as an **in vitro** substrate for the kinase activity of ATM. Detergent extracts from A549 lung adenocarcinoma cells were immunoprecipitated with anti-ATM antibodies, and kinase reactions were done in the presence of Mn2+ [γ-32P]ATP, and recombinant PHAS-I. These initial studies revealed that anti-ATM immunoprecipitates phosphorylated PHAS-I to a significantly higher level than the background activity present in the control (pre-immune serum) immunoprecipitates (Fig. 1a). We have not yet observed a reproducible alteration in the **in vitro** kinase activity of ATM after treatment of A549 cells with radiation (data not shown).

To confirm that ATM, rather than a coprecipitating protein kinase, was responsible for the phosphorylation of PHAS-I, we performed two additional sets of control experiments. Extracts prepared from an ATM-negative fibroblast cell line (GM02052) were immunoprecipitated with anti-ATM antibodies, and PHAS-I phosphorylating activity was determined with a filter binding assay (see “Materials and Methods”). The PHAS-I-phosphorylating activity present in anti-ATM immunoprecipitates from the A-T fibroblasts did not differ significantly from the background (Fig. 1b). In contrast, the ATM immunoprecipitates from A549 cells phosphorylated PHAS-I to an approximately 25-fold higher level than that catalyzed by the control antibody immunoprecipitates.

Subsequently, we addressed the possibility that the abundantly expressed PIKK family member, DNA-PK, might be contributing to the PHAS-I kinase activity present in anti-ATM immunoprecipitates. Earlier biochemical studies demonstrated that exposure of the native DNA-PK heterotrimer to high ionic strength buffer inhibited DNA-PK activity by causing the dissociation of the Ku subunits from the catalytic subunit (15). If the DNA-PK holoenzyme contributes to the PHAS-I phosphorylation by ATM immunoprecipitates, then this contaminating activity should be reduced by treatment with buffer containing 0.6 M NaCl. Our assay for DNA-PK activity yielded an average signal to background phosphorylation ratio of 4.7. As shown in Fig. 1c, the kinase activity of immunoprecipitated DNA-PK was decreased by 50% after a single wash with high-salt buffer, whereas the protein kinase activity found in ATM immunoprecipitates was completely resistant to this treatment. Moreover, immunoblots of the anti-ATM immunoprecipitates prepared under the standard assay conditions (i.e., with the high-salt wash) revealed no detectable contamination by DNA-PK or ATR (Fig. 1d). The anti-DNA-PK and anti-ATR immunoprecipitates used in the present studies were similarly free of contamination by immunologically detectable levels of ATM or other PIKK family members. Collectively, these results support the conclusion that the PHAS-I-phosphorylating activity present in anti-ATM immunoprecipitates is mediated by ATM itself, rather than by a contaminating protein kinase.

Previous results indicated that the catalytic activities of the PIKK family members, DNA-PK and mTOR, were inhibited by nanomolar concentrations of wortmannin (10, 11). However, the relevant protein
target(s) for wortmannin-mediated radiosensitization remains unclear. As a first step toward the identification of such target proteins, we compared the sensitivities of the known DNA damage-responsive PIKKs (DNA-PK, ATM, and ATR) to in vitro inhibition by wortmannin. The wortmannin sensitivities of the individual PIKKs varied over a relatively wide range, with IC_{50} values of 16 nM for DNA-PK, 150 nM for ATM, and 1.8 µM for ATR (Fig. 2). It should be noted that the phosphorylation of PHAS-I by immunoprecipitated ATR averaged 3.3-fold over the background measured in control immunoprecipitates. Hence, the relative resistance of ATR to wortmannin is not explained by a major difference in the level of PHAS-I phosphorylation activity between the immunoprecipitates of the three PIKKs.

Wortmannin irreversibly inhibits the lipid kinase activity of PI-3K by covalently modifying a critical lysine residue in the catalytic domain (9). The presence of a homologous lysine residue in the catalytic domains of ATM, DNA-PK, and ATR suggests that these PIKKs may also react covalently with wortmannin. We reasoned that an antibody directed against wortmannin-bound proteins would allow direct comparisons of the drug binding to the PIKK catalytic domains with the inhibition of PIKK catalytic activities in immune complex kinase assays. Therefore, we developed a monoclonal antibody, Wm7.1, which selectively recognizes wortmannin-derivatized proteins under immunoblotting conditions. After treatment with wortmannin, immunoprecipitates containing ATM, DNA-PK, or ATR each displayed a Wm7.1-reactive band that comigrated with the respective PIKK family member. The wortmannin concentrations that resulted in binding of Wm7.1 correspond closely to those required for inhibition of the protein kinase activities of ATM, ATR, and DNA-PK (Fig. 2, insets). The striking correlation between drug binding and inhibition of catalytic activity substantiates the conclusion that the immunoprecipitated PIKKs are direct, wortmannin-sensitive effectors of PHAS-I phosphorylation in these immune complex kinase assays.

The irreversible nature of the PIKK-inhibitory activity of wortmannin also permitted evaluations of the effects of this drug on the catalytic activities of ATM, ATR, and DNA-PK in intact cells. Treatment of A549 cells with wortmannin inhibited both ATM and DNA-PK activities, with IC_{50} values of 5.8 and 3.6 µM, respectively (Fig. 3, a and b). The catalytic activities of both PIKKs were nearly abolished by pretreatment of the cells with 30 µM wortmannin. Once again, the inhibitory effect of wortmannin on the catalytic activities of these proteins correlated with the binding of the drug to the immunoprecipitated PIKK (Fig. 3, a and b, insets). As predicted by the results obtained after treatment of immunopurified ATR with wortmannin, the kinase activity of ATR in intact cells was significantly more resistant to this drug (Fig. 3c). Half-maximal inhibition of ATR kinase activity in intact cells was not achieved by treatment with 100 µM wortmannin. Similarly, the Wm7.1 antibody failed to detect any wortmannin-bound ATR in A549 cells exposed to less than 100 µM wortmannin (Fig. 3c, inset). To further test the specificity of the protein kinase inhibition by wortmannin, we treated A549 cells with wortmannin and then immunoprecipitated Cdc2 complexes from the cellular extracts. The histone H1 kinase activity of Cdc2 was not affected by exposure of A549 cells to 30 µM wortmannin (data not shown).

Wortmannin enhances the sensitivity of a number of human tumor cell lines to radiation (6, 7). We examined the impact of wortmannin treatment on the radiosensitivity of A549 lung adenocarcinoma cells, as measured in a clonogenic assay. The results demonstrate that wortmannin, at concentrations ≥10 µM, significantly increased the radiosensitivity of A549 cells (Fig. 4). The concentrations of wortmannin that induce radiosensitization are at least two orders of magnitude higher than those required to abolish PI-3K activity in intact cells, indicating that this drug effect cannot be attributed solely to the inhibition of PI-3K (16). The degree of radiosensitization can be expressed as the ratio of the radiation doses that result in 10% survival (SER_{10}) in the absence versus the presence of drug. The SER_{10} values were 2.2 at 20 µM (P = 0.02), 1.4 at 10 µM (P = 0.05), and 1.0 at 2

![Fig. 2. Covalent modification and inhibition of ATM, DNA-PK, and ATR by wortmannin. ATM (A), DNA-PK (B), and ATR (C) were immunoprecipitated from A549 cell extracts. The immune complexes were treated with wortmannin for 30 min at room temperature, and kinase activities were assayed under linear reaction conditions. The kinase activities for each PIKK were normalized to that measured in the no-drug control. Data points are the means from two experiments; bars, SD. In parallel experiments, anti-ATM (A, insert), -DNA-PK (B, insert), and -ATR (C, insert) immunoprecipitates were incubated with wortmannin (Wm) as described above, and the proteins were separated by SDS-PAGE. Binding of wortmannin to each PIKK was determined by immunoblotting with monoclonal antibody Wm7.1 (a-Wm). Subsequently, the membranes were stripped and reprobed with the indicated PIKK-specific antibodies.](https://cancerres.aacrjournals.org)
**Fig. 3.** Inhibition and covalent modification of PIKKs by wortmannin in intact cells. A549 cells were treated with wortmannin for 1 h at 37°C prior to cell lysis and immunoprecipitation of ATM (A), DNA-PK (B), and ATR (C). Immune complex kinase assays were performed, and kinase activities were determined as described in the Fig. 2 legend. Data points are means from two independent trials; bars, SD. The insets show the level of wortmannin-bound protein, as measured by Wm7.1 immunoreactivity (α·Wm), in parallel immunoprecipitates of each PIKK.

μM wortmannin (P = 0.43). Maximal radiosensitization was observed in cells treated with 20 μM wortmannin (data not shown). Thus, radiosensitization occurs over a wortmannin concentration range that corresponds to the inhibition of ATM and DNA-PK but not ATR kinase activities. Wortmannin treatment induced a notable alteration in the shape of the radiation survival curve for A549 cells. Relative to nondrug-treated cells, the survival curve for cells exposed to 20 μM wortmannin showed an 8-fold increase in the initial slope, α, as described by the linear quadratic equation [ln(S) = -αD - βD^2]. An increase in α is suggestive of the inhibition of DNA repair processes, which results in the conversion of potentially repairable DNA lesions into nonrepairable lesions (17).

The above findings suggest that ATM inhibition might contribute to the radiosensitizing effect of wortmannin in A549 cells. If this is the case, then the wortmannin-treated cells should display the typical cell cycle checkpoint defects observed in ATM-deficient cells. One characteristic of G1-S-phase cells from A-T patients is a prolonged or irreversible arrest in G2 phase after exposure to ionizing radiation. In the present studies, A549 cells were synchronized at the G1-S-phase border with aphidicolin, released briefly from the drug-induced cell cycle block; the resulting S-phase-enriched population was exposed to 5 Gy irradiation. These cells displayed a minor increase in the numbers of G2-M phase cells at 24 h after irradiation, which is indicative of a transient G2 arrest, followed by reentry into the cell cycle. In contrast, cells treated with 20 μM wortmannin prior to irradiation showed a marked accumulation of G2-M-phase cells at 24 h after irradiation (Fig. 5). The protracted, radiation-induced G2-M arrest found in wortmannin-treated cells is highly reminiscent of the abnormal arrest phenotype reported in cells deficient in expression of either DNA-PK or ATM (3, 18).

A hallmark abnormality of A-T cells, but not DNA-PK-deficient cells, is RDS, a reflection of the defective DNA damage-induced S-phase checkpoint in A-T cells (3). We therefore determined whether wortmannin treatment conferred the RDS phenotype in A549 cells. As shown in Fig. 6, A549 cells displayed a significant increase in RDS after treatment with wortmannin, at drug concentrations that correlated with those required for inhibition of ATM kinase activity (Fig. 3a).

**Fig. 4.** Wortmannin-induced radiosensitization. A549 cells were exposed to the indicated doses (Gy) of radiation, followed by treatment with drug vehicle only (DMSO, •) or with 2 μM (○), 10 μM (□), or 20 μM (△) wortmannin. The samples were cultured for 14 days prior to fixation and staining. Surviving fraction, the number of viable colonies in the irradiated samples relative to the number measured in unirradiated controls exposed to the same concentration of wortmannin. Data points are means from four independent trials; bars, SE. The plating efficiencies for the unirradiated cells ranged from 65% for DMSO-treated control cells to 57% for 20 μM wortmannin-treated cells.
Fig. 5. Effect of wortmannin on the G2-phase delay of S-phase-irradiated cells. A549 cells were synchronized at the G1/S border by treatment with aphidicolin. The cells were released from the aphidicolin block, and after 3.5 h, the indicated concentrations of wortmannin (Wm) were added to the S-phase-enriched cells. After 30 min, the cells were exposed to 0 or 5 Gy radiation. The drug-containing medium was replaced with fresh culture medium at 30 min after irradiation. Cell cycle distributions shown are from cells harvested 24 h after irradiation. Fixed cells were stained with propidium iodide and analyzed by flow cytometry. Three independent trials were performed. Histograms of red fluorescence intensity (DNA content) from 20,000 ungated events are shown from a representative experiment. The numbers in each panel indicate the percentage of G2-M cells in the test cell population.

DISCUSSION

Members of the PIKK family play critical roles in the regulation of the cell cycle in response to mitogenic signals and unreplicated or damaged DNA. Dysfunction of a subset of PIKKs, including ATM, DNA-PK, and ATR, leads to hypersensitivity to ionizing radiation and other DNA-damaging agents. As such, these kinases represent novel molecular targets for the development of agents that, in principle, could sensitize cancer cells to conventional chemotherapeutic agents or ionizing radiation. Earlier reports that the PI-3K inhibitor, wortmannin, inhibits the protein kinase activities of two PIKK family members, mTOR and DNA-PK, raised the possibility that this drug might be a useful lead compound for proof-of-principle testing of PIKK inhibitors as potential cancer chemotherapeutic agents. Several reports, including the present study, have now documented that wortmannin displays striking radiosensitizing activity in different tumor cell lines (6, 7). In this report, we offer biochemical and pharmacological evidence that DNA-PK and ATM, but not ATR, are relevant targets for wortmannin in A549 lung adenocarcinoma cells.

The objectives of this study required the development of reliable and specific immune complex kinase assays for ATM, DNA-PK, and ATR. The identification of the PHAS-I protein as a suitable substrate for ATM, as well as ATR, adds to the list of PIKK family members with documented phosphotransferase activities toward protein substrates. Although sequence homologies to the catalytic domain of PI-3K provoked speculation that the PIKKs might also be phosphoinositide-directed kinases, none of the PIKKs studied to date have been identified definitively as lipid kinases. The present results, together with previous findings regarding the protein substrates for mTOR and DNA-PK, support the general conclusion that the PIKKs transmit signals via the phosphorylation of specific protein targets.

The consensus sequences for serine-threonine phosphorylation by ATM and ATR have not been determined. The PHAS-I protein, which is phosphorylated by both PIKKs, was shown previously to be a physiologically relevant substrate for mTOR (11). PHAS-I contains both (S/T)P and (S/T)Q sites that serve as target sequences for phosphorylation by mTOR and DNA-PK, respectively (19, 20). Earlier reports suggest that ATM shares with DNA-PK the ability to phosphorylate Abl at Ser (465), which is followed by a Gin residue at the +1 position (14, 21). In contrast, we found that ATR fails to phosphorylate the SQ target sequence-containing peptide substrate for DNA-PK in immune complex kinase assays.6 This difference in substrate specificity may underlie the relative resistance of ATR to modification by wortmannin. The variable potencies with which wortmannin inhibits the kinase activities of members of the PI-3K/PIKK superfamily are determined, at least in part, by the affinity of the drug for the catalytic domains of these kinases. The wortmannin binding results presented in this report suggest that the DNA-PK and ATM kinase domains exhibit higher degrees of conformational similarity to

Fig. 6. Induction of radioresistant DNA synthesis by wortmannin. A549 cells in exponential growth were exposed to the indicated doses of radiation and immediately treated with drug vehicle only (●) or with 3 μM (▲), 30 μM (■), or 100 μM (●) wortmannin. Cells were then pulsed with [3H]thymidine for 40 min, and incorporation of radioactivity into DNA was assayed by liquid scintillation counting. The cpm of radioactivity measured in each sample were normalized to that obtained in the corresponding wortmannin-treated, nonirradiated control. Results are presented as the means of three independent trials. Bars, SE.

6 Unpublished observations.
each other than to ATR. It will be interesting to determine whether the in vivo substrate and/or target site specificities for ATM and ATR are actually nonoverlapping, because these two PIKKs display some functional overlap in cell cycle checkpoint control in intact cells (4).

Our results indicate that, of the mammalian PIKKs characterized to date, DNA-PK exhibits the highest level of sensitivity to the inhibitory action of wortmannin. In our studies, the IC50 value for wortmannin was 16 nM with immunoprecipitated DNA-PK as the target. This result differs from the previously reported IC50, which ranges from 250 to 300 nM (7, 10). Although the basis for this discrepancy is unclear at present, a possible explanation lies in the different methodologies used to isolate and assay DNA-PK activity. Native DNA-PK is a heterotrimer comprised of the DNA-PKcs and the regulatory Ku70 and Ku80 subunits. The relatively gentle cell lysis conditions used in our study should have preserved the association between the DNA-PKcs and the Ku proteins. It has been shown previously that dissociation of the Ku subunits after high-salt treatment inhibits the catalytic activity of the DNA-PK (15). Interestingly, Hartley et al. (10) reported that wortmannin inhibited the protein kinase activity of the isolated DNA-PKcs with an IC50 of 250 nM. Perhaps the association of Ku70 and Ku80 with DNA-PKcs alters the conformation of the ATP-binding region of DNA-PKcs, resulting in the generation of a more active kinase with a concomitantly higher level of sensitivity to wortmannin.

The results of this study show that the concentrations of wortmannin required for inhibition of DNA-PK and ATM kinase activities in intact A549 cells are virtually identical to those that induce radiosensitization in these cells. The impact of wortmannin treatment on the shape of the radiation dose-response survival curve is consistent with the notion that the underlying pharmacological mechanism involves inhibition of the DNA damage-responsive PIKKs. Wortmannin treatment caused a marked increase in the initial slope and eliminated the shoulder of the radiation survival curve for A549 cells. These drug-induced alterations are characteristic of those seen following the combination of radiation with DNA damage repair inhibitors (22). Our results lend support to previous findings that the suppressive effect of wortmannin on the repair of DNA double-strand break repair is due, in part, to the inhibition of DNA-PK activity (5).

The present findings implicate an additional PIKK family member as a target for wortmannin at drug concentrations that induce radiosensitization. We observed that treatment of A549 cells with 3–30 µM wortmannin resulted in both the covalent modification and functional inhibition of ATM. The inhibition of ATM kinase activity by wortmannin was accompanied by the appearance of a S-phase checkpoint defect(s), which was manifested as RDS. This phenotypic abnormality is one of the hallmark features of cells from A-T patients but is not observed in DNA-PK-deficient cells from mice with severe combined immunodeficiency (23). The prolongation of the G2-phase arrest found in wortmannin-treated tumor cells is also a feature of the abnormal cell cycle response of ATM-deficient cells to ionizing radiation (24). A recent report showed that wortmannin induces a significantly greater radiosensitization in severe combined immunodeficient cells than in A-T cells (7). Collectively, these findings suggest that inhibition of ATM kinase activity makes an important, perhaps crucial, contribution to the enhancement of radiation-induced cell killing by wortmannin.

The present studies strongly support the hypothesis that specific PIKK inhibitors could represent a novel class of radio- and chemosensitizing agents. Traditional radiosensitizers (e.g., 5-fluorouracil) either enhance the level of initial DNA damage caused by radiation or impede the repair of radiation-induced DNA lesions by inhibiting enzymes involved in DNA metabolism, synthesis, and repair. Unfortunately, the intrinsic cytotoxicity and adverse side effects associated with the administration of such drugs limit their clinical utility as sensitizing agents. In contrast, inhibitors of ATM or DNA-PK should, in principle, exert more selective toxic effects on cells with damaged DNA. Tumor cells bearing genetic defects in other checkpoint proteins, including p53, may be particularly sensitive to drug-induced disruption of PIKK functions in the setting of therapeutically induced DNA damage. In such genetically altered tumor cells, the PIKK inhibitors may promote irreversible G1 arrest or endoreduplication of damaged chromosomes and consequent apoptosis in response to DNA-damaging agents (25).

The relatively broad range of potencies with which wortmannin inhibits different members of the extended PI3K family suggests that chemical modifications of the sterol-like nucleus of wortmannin might yield compounds with more selective inhibitory effects. The assays described in this report are adaptable to a high-throughput screening format and should allow the identification of wortmannin analogues or novel compounds with more selective inhibitory effects on ATM, ATR, or DNA-PK activities. In the meantime, continued studies of the radiosensitizing mechanism of wortmannin in tumor cell lines and animal models will undoubtedly yield additional information regarding the therapeutic potential of PIKK inhibitors in human cancer.

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REFERENCES

INHIBITION OF PIKKs BY WORTMANNIN


Inhibition of Phosphoinositide 3-Kinase Related Kinases by the Radiosensitizing Agent Wortmannin

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