Phosphoaminoglycosides Inhibit SWI2/SNF2 Family DNA-Dependent Molecular Motor Domains†

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ABSTRACT: Members of the SWI2/SNF2 family of proteins participate in an array of nucleic acid metabolic functions, including chromatin remodeling and transcription. The present studies identify a novel strategy to specifically inhibit the functional DNA-dependent adenosinetriphosphatase (ATPase) motor domain common to SWI2/SNF2 family members. We have identified preparations of phosphoaminoglycosides, which are natural products of aminoglycoside-resistant bacteria, as inhibitors of the in vitro activities of three SWI2/SNF2 family members. These compounds inhibit the ATPase activity of the active DNA-dependent ATPase A domain (ADAAD) by competing with respect to DNA and thus have no effect on DNA-independent ATPases or on RNA-dependent ATPases. Within the superfamily of DNA-dependent ATPases, these compounds are most potent toward SWI2/SNF2 family members and less potent toward other DNA-dependent ATPases. We demonstrate that it is feasible to target DNA-dependent ATPases of a particular type without affecting the function of other ATPases. As the SWI2/SNF2 proteins have been proposed to function in all aspects of DNA metabolism, this paper provides an archetype for development of DNA metabolic inhibitors.

DNA-dependent adenosinetriphosphatases (ATPases) play key roles in DNA replication, DNA recombination, DNA repair, chromatin remodeling, and transcription—essentially all DNA metabolic processes. This class of enzymes is therefore an attractive target for inhibition, which should lead to the disruption of DNA metabolism with consequential disruption of the cellular machinery that could ultimately lead to cell death. Hence, inhibitors of DNA-dependent ATPases may have use as chemotherapeutic agents.

The value of metabolic process inhibitors frequently lies in their specificity, and thus, the development of an inhibitor for DNA-dependent ATP hydrolysis should ideally have no effect with respect to DNA-independent ATP hydrolysis. The similarity of the amino acids comprising ATP binding sites for various ATPases (*1*) leads us to hypothesize that development of specificity will depend largely on the ability to differentially affect the function of DNA-dependent and DNA-independent motor domains. The motor domains of some nucleic acid modifying enzymes are known to use the energy of ATP hydrolysis to produce changes in a nucleic acid substrate (i.e., helicases, nucleases, topoisomerases, ligases, and recombinases). However, there are a few nucleic acid-dependent ATPases in which the nucleic acids do not appear to be a substrate for the enzymes, and consequently, the nucleic acid is generally regarded as an effector of the enzymatic activity. Thus, definition of a specific inhibition of function may lie in the ability to understand the interaction of the DNA-dependent ATPase motor domain with its nucleic acid effector.

Initial studies of eukaryotic DNA-dependent ATPase A demonstrated that this enzyme hydrolyzes ATP only in the presence of a DNA effector without apparent modification to the effector (*2*). This protein was originally isolated from calf thymus tissue as a 105-kDa species that undergoes proteolysis to yield two smaller polypeptides of 83 and 68 kDa (*3*). All three polypeptides possessed ATPase activity that was manifested only in the presence of DNA with initial studies indicating that the protein was maximally active in the presence of DNA effectors that have a transition from double-stranded to single-stranded character. The effector specificity is striking and of all the DNA-dependent ATPases characterized is most reminiscent of the gp44/62 and 45 protein complex from T4 bacteriophage (*2, 4*). Our previous studies showed that the monoclonal antibodies raised against DNA-dependent ATPase A also recognize gp44/62, suggesting that ATPase A and the gp44/62 complex share common epitopes (*5*). Gp44/62 protein complex is a DNA-dependent ATPase that hydrolyzes ATP in the presence of primer−template DNA and gp45. The energy released in

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ATP hydrolysis is coupled to the loading of the gp45 sliding clamp onto DNA, which confers processivity onto T4 DNA polymerase (*6*). We hypothesized that DNA-dependent ATPase A, like the gp44/62 protein complex, might be functionally involved in the processes of DNA-dependent protein assembly and/or disassembly.

The presence of common epitopes for gp44/62 and ATPase A, along with the recognition of similar DNA effectors, led to our efforts to establish an understanding of how this particular type of molecular motor functions. We have demonstrated that ATPase A is not significantly similar to gp44/62 by primary amino acid sequence but belongs to the SWI2/SNF2 family of ATPases, whose members have been shown to be involved in most DNA metabolic processes [Muthuswami et al., *J. Biol. Chem*., in press]. All the proteins belonging to this family possess ATPase activity, but the mechanism by which ATP hydrolysis is coupled to their different roles in DNA metabolic function remains to be elucidated. Here we report the use of a bacterial expressed eukaryotic domain, which is required for DNA and ATP binding, for the identification of a family of inhibitors specific for the SWI2/SNF2 family of DNA-dependent ATPases. These inhibitors have been identified as phosphorylated derivatives of aminoglycoside antibiotics naturally produced by some aminoglycoside-resistant bacteria, thereby suggesting a novel and potentially useful role for antibiotic-resistant bacteria in the development of potential chemotherapeutic reagents.

MATERIALS AND METHODS

Chemicals. Unless noted otherwise, chemicals were purchased from either Fisher Scientific or Sigma Chemicals.

Enzymes. F_1 ATPase was a gift from Dr. Robert Nakamoto (University of Virginia). Sarcoplasmic reticulum ATPases were a gift from Dr. Howard Kutchai (University of Virginia). *Escherichia coli* helicase II was a gift from Dr. Steven Matson (University of North Carolina). Bacteriophage T4 gene protein 44/62 and *E. coli* rho protein were a gift from Dr. Peter von Hippel (University of Oregon). *E. coli* rec A was purchased from USB. ADAAD[1] was purified from *E. coli* cells following expression from pRM102, a plasmid based on the pET-24 a(+) vector from Novagen [Muthuswami et al., *J. Biol. Chem*., in press].

ATPase Assays and Protein Estimation. ATPase activity of ADAAD was quantitated by measuring the amount of inorganic phosphate released by the hydrolysis of ATP (*2*). In a typical reaction, ADAAD was incubated with 10 nM stem−loop DNA (5′ GCGCAATTGCGCTCAGCAGTTTTT-TAGCGCAATTGCGC; ON950) at 37 °C for 1 h. For measurement of the inhibition of ADAAD by phosphokanamycin, an assay coupling NADH oxidation to ATP hydrolysis was used (*4*).

The activity of ADAAD at a given ATP concentration was also measured using incubation of [γ-^{32}P]ATP and 400 nM stem−loop DNA (ON950) at 37 °C, followed by thin-layer chromatography (*2*). The amount of phosphate released was quantitated using a phosphor-imager from Molecular Dynamics.

[1] Abbreviations: ADAAD, active DNA-dependent ATPase A domain; APH, aminoglycoside phosphotransferase; PAM, phosphoaminoglycoside.

The ATPase activity of hSWI/SNF complex was assayed in a reaction comprised of 20 mM Tris-HCl, pH 8.8, 5 mM $MgCl_2$, 100 μg/mL bovine serum albumin, 5% glycerol, 0.1% Tween 20, 0.2 mM dithiothreitol, 10 μM denatured calf thymus DNA, and 100 μM [γ-^{32}P]ATP (specific activity 0.6 Ci/mmol). Sufficient hSWI/SNF complex was added to yield approximately 10% hydrolysis of the ATP after 2 h at 37 °C, and the products were analyzed by thin-layer chromatography as above. Protein content was estimated using the Bradford assay (*7*).

Synthesis and Purification of Phosphoaminoglycosides. The overexpression clone for aminoglycoside phosphotransferase (3′)-IIIa (APH (3′)-IIIa) was a gift from Dr. Gerald Wright (McMaster University), and cells were grown according to the published protocol (*8*). After induction with 0.5 mM IPTG, the cells were grown for 4 h at 37 °C. The cells were harvested and resuspended in 10 mL of buffer L (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, and 0.1 mM PMSF). The cells were homogenized using a Dounce homogenizer and lysed by passing twice through a French Press at 1500 psi pressure. The lysate was clarified by centrifugation (12000*g* for 30 min), and the supernatant was diluted 1:5 with buffer A (50 mM Tris-HCl, pH 8.0, and 1 mM EDTA). The resulting solution was loaded onto a DEAE-cellulose column (2 × 13 cm) preequilibrated with buffer A and eluted using a gradient from 0 to 750 mM NaCl in buffer A. Fractions were assayed for APH (3′)-IIIa activity by measuring kanamycin-dependent ATP hydrolysis (*8*). The active APH (3′)-IIIa fractions elute following kanamycin-independent ATP hydrolytic activities and were pooled together for use in phosphoaminoglycoside (PAM) synthesis. This APH (3′)-IIIa preparation is relatively stable at 4 °C and can be used over the course of 10−14 days.

Phosphokanamycin was synthesized by incubating 2 mL of APH (3′)-IIIa (1.5 μmol of kanamycin-dependent ATP hydrolysis min^{-1} mL^{-1}) with 100 mg of kanamycin, HEPES buffer (50 mM HEPES, pH 7.5, and 10 mM $MgCl_2$), and 3 mM ATP in a total volume of 250 mL. The reaction was incubated at 37 °C for 72 h. An additional 2 mL of enzyme was added for each 24 h period, and an additional 1 mM ATP was added prior to the last 24 h to drive the reaction to completion. Bio-Rex 70 (Bio-Rad) (20−30 g) was mixed with the PAM reaction and incubated on a rotator for 2 h at 4 °C. The slurry was poured into a 3 × 14 cm column and extensively washed with water for 12 h. PAMs were eluted with 200 mL of 0.45% ammonium hydroxide, and fractions were collected. These fractions were analyzed for the presence of aminoglycoside by thin-layer chromatography (*8*). The column was subsequently washed with 0.45% ammonium hydroxide for 12 h. The fractions that did not contain aminoglycoside were pooled with this wash and dried (at 37 °C) using a Rotovapor-R. Dried PAM was resuspended in distilled water, and the pH was adjusted to 7.0 using 11.6 M HCl. The purity and concentration of PAM were estimated using thin-layer chromatography as well as mass spectrometry (W. M. Keck Biomedical Mass Spectrometry Laboratory of the University of Virginia).

RESULTS

Biochemical characterization of bovine 105-kDa DNA-dependent ATPase A (*2*) and its partial cleavage products

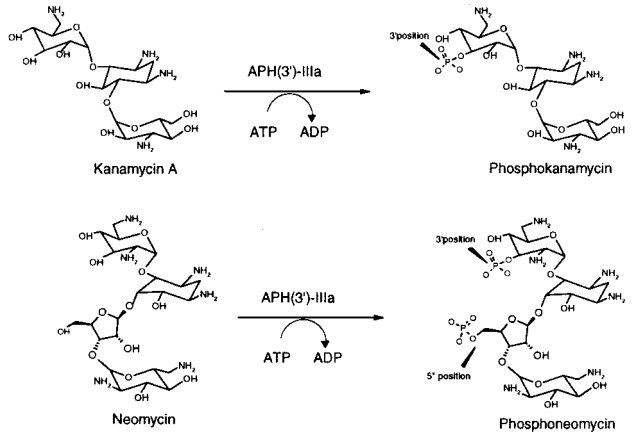

FIGURE 1: Modification of aminoglycosides by phosphorylation reaction. APH(3′)-IIIa transfers a phosphate group onto the 3′ position of kanamycin or the 3′ and 5″ positions of neomycin in an ATP-dependent reaction.

retaining DNA-dependent ATPase activity led to the construction of an overexpression vector for ADAAD [Muthuswami et al., *J. Biol. Chem.*, in press]. The cDNA sequence encoding one of the proteolytic products (82 kDa) was cloned into a pET24d(+) vector (Novagen), which also encoded APH (3′)-IIIa as a kanamycin inactivation selection mechanism (pRM102). While Western blot analysis, using monoclonal antibodies raised against ATPase A (*5*), showed the presence of the ADAAD protein when induced with 0.5 mM IPTG at 37 °C, subsequent purification yielded an inactive protein.

The inactivity of bacterially expressed eukaryotic proteins has frequently been attributed to improper folding (*9*). To produce an active protein, we varied the growth conditions of the bacteria, thereby hoping to optimize the folding process. Neither variations in temperature nor in IPTG concentration appeared to play a significant role in altering the activity of the protein. However, we found that as the concentration of kanamycin decreased below 5 μM in the growth media, the specific activity of ADAAD increased. Maximal ATP hydrolytic activity (16 μmol of ATP hydrolyzed min^{-1} mg^{-1}) peaked in the absence of kanamycin and was comparable to that of the calf thymus 83-kDa ATPase A polypeptide (*3*).

Since the specific activity of ADAAD was dependent upon the concentration of kanamycin present in the growth media, we presumed that kanamycin or a derivative of kanamycin inhibits the ATPase activity of ADAAD. The ATPase activity of purified ADAAD protein in vitro was not inhibited by kanamycin at the concentrations used for bacterial selection ($\sim$50 μM), causing us to hypothesize that a derivative of kanamycin (Figure 1) formed in *E. coli* inhibited the ATPase activity of the eukaryotic ADAAD. Since aminoglycoside resistance vectors are also used in eukaryotic cells, we recognized that proof of this unorthodox postulate would raise significant concern, and we resolved to test the hypothesis.

Identification of the Compound That Inhibits the ATPase Activity of ADAAD. Kanamycin belongs to the aminoglycoside family of antibiotics (*10*). These are naturally occurring compounds containing amino sugars linked by a glycosidic linkage to another amino sugar or cyclic sugar. Kanamycin and other aminoglycosides inhibit protein synthesis by binding to the 16S RNA of the prokaryotic ribosome (*11*). Bacteria can acquire aminoglycoside resistance by a variety of mechanisms, including adenylylation, acetylation, and phosphorylation of aminoglycosides, such that they lose their ability to interact with the ribosomal RNA (*12*). One of the most common modifications is the phosphorylation of aminoglycosides generating PAMs (*12*) (Figure 1). *O*-Phosphotransferases lead to phosphorylation of the aminoglycosides in an ATP-dependent manner and are generally referred to as aminoglycoside phosphotransferases (APH) (*8*). The APH enzymes may lead to phosphorylation at the 4, 6, 3′, and/or 5″ positions on the aminoglycoside rings. The aminoglycoside resistance gene typically used in many vectors encodes for an APH (3′) enzyme.

Comparison of the structures of aminoglycosides, PAMs, and DNA reveals that both DNA and aminoglycosides have a backbone comprised of sugar molecules. Addition of phosphate groups onto the aminoglycosides results in PAMs that may have character similar to the sugar−phosphate backbone of DNA. We therefore hypothesized that PAMs can interact with ADAAD and inhibit its ATPase activity.

We focused our initial studies on two commonly used aminoglycosides: kanamycin and neomycin. These two compounds represent two distinct subfamilies of the aminoglycoside family. The kanamycin subfamily (4,6-aminoglycosides) possesses a central 2-deoxystreptidine ring with aminohexoses attached at positions C-4 and C-6 (Figure 1). Amikacin, geneticin, and gentamicin belong to this subfamily, and all are phosphorylated at the 3′ position. The neomycin subfamily (4,5-aminoglycosides) also contains a central 2-deoxystreptidine ring, but a pentose ring is attached to the C-5 position, and an aminohexose is attached to the C-4 of the central ring. Additional aminosugar moieties may be attached to the pentose ring. Other members of the 4,5-subfamily include butirosin, paromomycin, and lividomycin. With the exception of lividomycin (which lacks a 3′ hydroxyl), all of the 4,5-aminoglycosides can be phosphorylated at the 3′ and/or at the 5″ position (Figure 1).

To examine the inhibitory effects of PAMs, phosphokanamycin and phosphoneomycin were synthesized in vitro using purified APH (3′)-IIIa (*8*). We found that both PAMs were approximately 1000-fold more potent in inhibiting the ATPase activity of ADAAD than the parent aminoglycosides (Figure 2A). These phosphorylated derivatives behaved as competitive inhibitors with respect to DNA (Figure 2B) but did not compete with respect to ATP (Figure 2C). The K_i for inhibition by phosphokanamycin was 580 nM, while the K_i for phosphoneomycin was 11 nM. Although we do not address the issue of the differences in the K_i values here, it should be noted that kanamycin is documented as being phosphorylated at the 3′ position, while the APH (3′) enzyme yields a mixture of 3′ and 5″ phosphorylated species of phosphoneomycin. Therefore, it is possible that the difference in the K_i values for phosphokanamycin and phosphoneomycin are a reflection of the extent of phosphorylation. While the data support the hypothesis that competitive inhibition occurs at the DNA binding site, we note that the apparent competition could also result if the inhibitors prevent a protein conformational change elicited by DNA binding.

Phosphoaminoglycosides Are Specific Inhibitors of the SWI2/SNF2 Family of ATPases. Since this is the first report

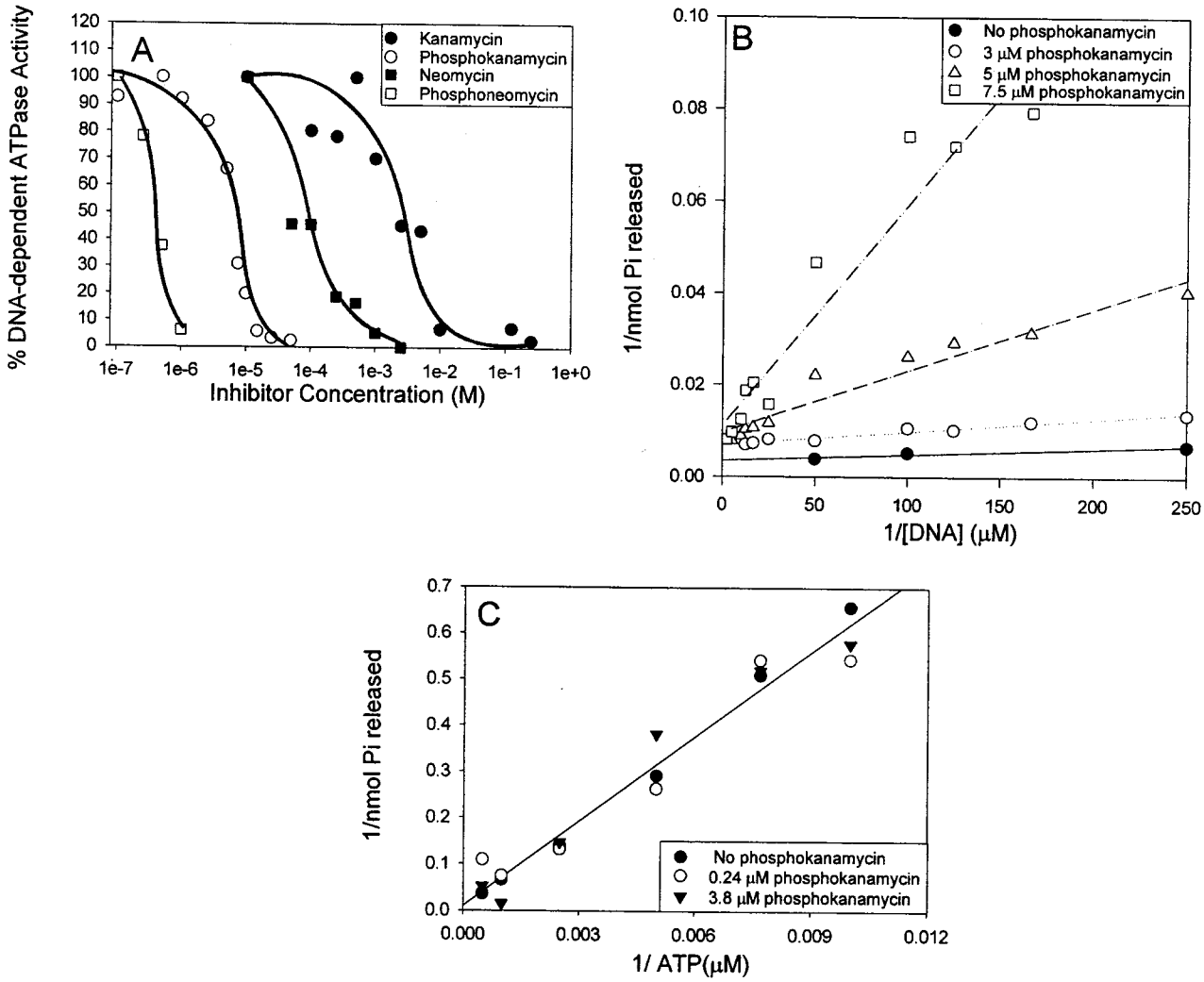

FIGURE 2: (A) Comparison of the inhibitory activities of aminoglycosides and phosphoaminoglycosides. Purified ADAAD was incubated with kanamycin (●), neomycin (■), phosphokanamycin (○), or phosphoneomycin (□) as described in Materials and Methods. Phosphate released was quantitated by colorimetric analysis (data points represent averages of triplicates). (B) Lineweaver−Burk analysis of the inhibitory activity of phosphokanamycin as a function of DNA concentration. Purified ADAAD was incubated in the presence of 2 mM ATP and increasing concentration of stem−loop DNA (ON950) under standard reaction conditions. Activity of ADAAD was measured in the presence of different phosphokanamycin concentrations: 0 (●), 3 (○), 5 (△), and 7.5 μM (□). Data are a single representative set from three repetitions. (C) Lineweaver−Burk analysis of the inhibitory activity of phosphokanamycin as a function of ATP concentration. ADAAD was incubated with 400 nM stem−loop DNA (ON950), [γ-^{32}P]ATP, and the release of phosphate was measured by thin-layer analysis. Activity of ADAAD was measured in the presence of different phosphokanamycin concentrations: 0 (●), 0.24 (○), and 3.8 μM (▼).

demonstrating an inhibitor(s) of ATP hydrolysis that is competitive with respect to DNA, we sought to establish that the inhibitor(s) demonstrated specificity. Recognizing that the PAMs competed with respect to DNA, we hypothesized that these compounds would not inhibit DNA-independent ATPases.

DNA-independent ATPases do not require DNA as an effector for ATP hydrolysis, and these enzymes are involved in a wide range of processes. Examples of DNA-independent ATPases include the following: (1) the F_1 subunit of *E. coli* F_0F_1 ATPase; (2) hexokinase; and (3) sarcoplasmic reticulum ATPases. As shown in Figure 3A, PAMs had no effect on DNA-independent ATPases. Furthermore, PAMs had no effect on the *E. coli* transcription termination factor rho, which hydrolyzes ATP only in the presence of RNA. Thus, these compounds did not inhibit either DNA-independent ATPases or RNA-dependent ATPases.

DNA-dependent ATPases are a heterogeneous class of proteins that use a wide array of DNA effectors either to stimulate the basal ATPase activity of these enzymes or as an essential effector that permits ATP hydrolysis. With our research emphasis on the physical biochemistry of protein−DNA interactions, we have considered classifications based on the preferred single- or double-stranded character of the DNA effector for maximal activation/stimulation of DNA-dependent ATP hydrolysis. We hypothesized that the effects of the PAMs might be directly dependent on the DNA effector utilized for ATP hydrolysis and the protein-binding site specific for that effector. The effects of PAMs on a series of DNA-dependent ATPases were examined at concentrations of PAMs yielding complete inhibition of ADAAD. Topoisomerase II, a double-stranded DNA-dependent ATPase (*13*), was not inhibited in a decatenation assay by the PAMs (data not shown). Both helicase II, which is maximally active in the presence of single-stranded DNA (*14*), and recA protein, which does not show preference for any one type of DNA molecule (*15*), had limited sensitivity to the PAMs (Figure 3B). The bacteriophage T4 gp44/62,

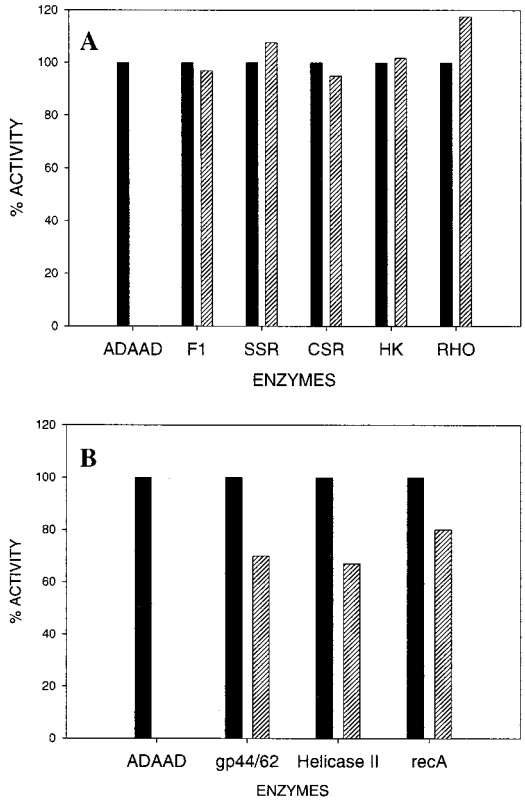

FIGURE 3: (A) Effect of phosphoneomycin on the activity of DNA-independent ATPases. Activity of ADAAD, soluble F_1 of *E. coli* F_1F_0 ATPase, skeletal (SSR) and cardiac (CSR) muscle sarcoplasmic reticulum ATPases, hexokinase (HK), and *E. coli* rho were measured in the absence (solid bars) and presence (hatched bars) of 1 μM phosphoneomycin. Activity of rho was measured in the presence of 36 nM poly r(C). (B) Effect of phosphoneomycin on the activity of DNA-dependent ATPases. Activity of ADAAD, gp44/62, helicase II, and rec A was measured in the absence (solid bars) and in the presence (hatched bars) of 1 μM phosphoneomycin. Each reaction contained DNA and ATP concentrations optimized for the specific enzyme.

which has the most similar DNA effector usage to ADAAD (*16*), represents a final class of DNA-dependent ATPases that recognizes double-stranded to single-stranded transition regions present in a DNA molecule. Gp44/62 also demonstrated limited sensitivity to the PAMs with a K_i of 540 nM, while the same preparation of phosphoneomycin inhibits ADAAD with a K_i of 10 nM when using the same stem−loop DNA effector (ON950) for both assays. Taken together, these results indicate that the PAMs have a high degree of specificity for a limited subset of DNA-dependent ATPases and, in particular, those enzymes that might reasonably be expected to be found near double-stranded to single-stranded transitions in nuclear DNA.

The specificity of the PAMs does not appear to reside entirely in the parent aminoglycoside structures, which are composed of aminosugars linked via glycosidic bonds and whose pK_a is typically estimated to be around 7.5 for the primary amine (*17*). At high concentrations (millimolar), the parent aminoglycosides can inhibit DNA-dependent ATPases (Figure 2A) but do not demonstrate any selectivity among enzymes. We suggest that, under our reaction conditions (pH 7.0), the amino groups will be positively charged and are likely to interact with the negatively charged phosphate groups on the DNA molecule, effectively sequestering the polynucleotide away from the DNA-binding proteins. While the positively charged aminoglycosides may interact directly with DNA, just as they do with rRNA (*11*), it is difficult to envision how the introduction of a negatively charged phosphate group could increase this affinity. Consequently, we suggest that the PAMs inhibit the activity of ADAAD by virtue of interacting specifically with the enzyme and not the DNA.

Evidence for the specificity of the PAMs for the enzyme and not the DNA effector is reflected in the apparent inhibition constants for these compounds. ADAAD and the bacteriophage T4 gp44/62 protein complex both use double-stranded:single-stranded transition DNA molecules to effect ATP hydrolysis, and both are affected by neomycin. The 50-fold difference in K_i leads us to suggest that the PAMs must interact with the protein and not the DNA. While it is conceivable that the PAM could sequester the DNA from one of these enzymes if it had a high dissociation constant for DNA, such an expectation would demand that the ADAAD should have the lower DNA binding affinity. Our estimates of the apparent dissociation constants of these two enzymes demonstrate just the opposite, with ADAAD having an apparent dissociation constant of 1.9 nM for a stem−loop DNA (ON950) [Muthuswami et al., *J. Biol. Chem*., in press] and gp44/62 having an apparent dissociation constant of 23 nM. Thus, we conclude the PAM interaction is specific for ADAAD.

We expanded our observations beyond measurement of ATP hydrolysis by examining the effects of PAMs on two other members of the SWI2/SNF2 family. Mot1 is a transcription repressor from *Saccharomyces cerevisiae*, which binds to a TATA binding protein (TBP)−DNA complex (*18*). A gel shift assay showed that Mot1 dissociated TBP from DNA (Figure 4A, lanes 4−5) in the presence of ATP (*18*). We found that the TBP−DNA complex by itself was not affected by the addition of phosphokanamycin. However, in the absence of ATP, phosphokanamycin disrupted the entire TBP−Mot1−DNA complex (Figure 4A, lanes 1−2). We propose that the PAM disrupts Mot1 conformational changes thereby disrupting the ternary complex. We also suggest that the PAM might disrupt the protein−DNA interaction without affecting the TBP−Mot1 protein−protein interaction, which would account for the loss of the TBP−DNA complex in the presence of both Mot1 and Pkan.

Figure 4B demonstrates the phosphokanamycin-specific disruption of nucleosome remodeling by the human SWI/SNF complex (hSWI/SNF "A" (*19*)). Rotationally phased, end-labeled mononucleosomes generate a 10 bp cleavage ladder upon DNAse I digestion; hSWI/SNF hydrolyzes ATP and alters nucleosome structure such that DNAse I accessibility is increased (compare lane 1 to lane 4) (*20*). Addition of phosphokanamycin inhibited ATP-dependent disruption of nucleosome structure by the SWI/SNF complex, whereas the same concentrations of kanamycin had no effect (compare lanes 5−8 to lanes 9−12). The disruption of the nucleosome remodeling activity appears to be paralleled by disruption of ATP hydrolytic activity as shown in Figure 4C.

Thus, we have demonstrated that PAMs inhibit three structurally related ATPases (ADAAD, Mot1, hSWI/SNF

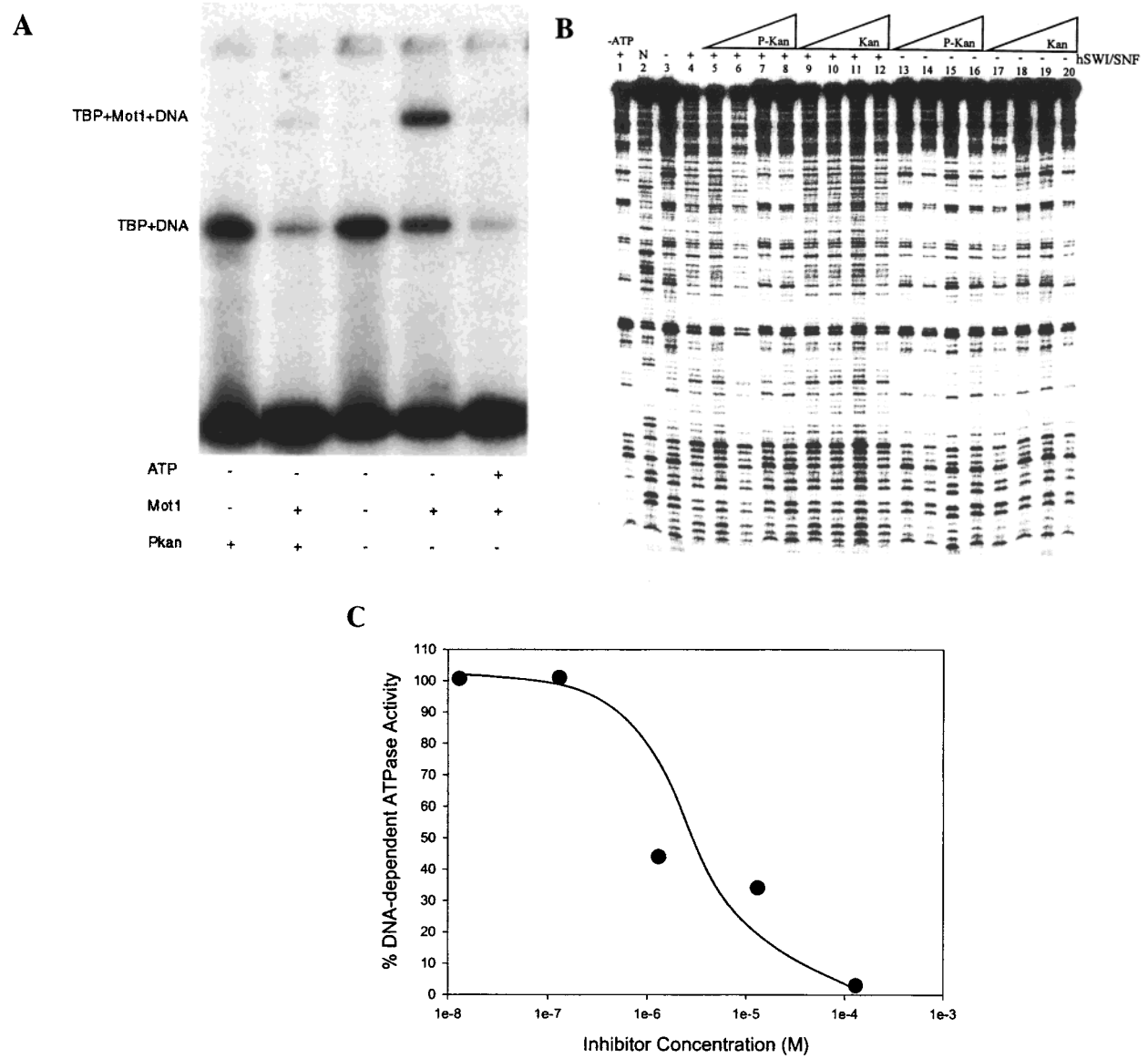

FIGURE 4: (A) Phosphokanamycin disruption of SWI2/SNF2 family activity. Phosphokanamycin (10 μM) was added to a standard gel shift assay Mot1:TBP interaction (*29*). (B) PAM inhibition of ATP-dependent nucleosome disruption assay. Serial 10-fold dilutions of phosphokanamycin (or kanamycin) were added to a standard assay (*19*) yielding a range of concentrations from 146 nM to 146 μM (lanes 5−8 and 13−16). Lanes 1 (no ATP) and 3 (no hSWI/SNF) are control lanes with no nucleosome disruption. Lanes 2 and 4 are controls showing the banding pattern for naked DNA (N) and nucleosome disruption, respectively. (C) Phosphokanamycin disruption of hSWI/SNF DNA-dependent ATP hydrolysis.

complex) from three different eukaryotic organisms (bovine, yeast, and human) as measured by three different assays (ATP hydrolysis, protein−DNA binding, and nucleosome remodeling).

DISCUSSION

The SWI2/SNF2 family of DNA-dependent ATPases are involved in virtually all aspects of DNA metabolism. We have identified inhibitors that specifically inhibit the SWI2/SNF2 family of proteins by competing with respect to the DNA effector that these enzymes need to effect hydrolysis of ATP. These inhibitors, belonging to the aminoglycoside family of antibiotics, are naturally synthesized by the antibiotic-resistant bacteria and were identified initially by their ability to inactivate the "motor" domain of an eukaryotic member of the SWI2/SNF2 family overexpressed in kanamycin-resistant bacteria. Apart from reagents that induce DNA damage and thus inhibit most DNA binding proteins, these data present the first reported identification of inhibitors that specifically inhibit DNA-dependent ATP hydrolysis without having any effect on DNA-independent ATPases.

The family of DNA-dependent ATPases includes a diverse array of enzymes that may require either DNA or a modified form of DNA, such as a nucleosome, to effect ATP hydrolysis that ultimately alters DNA metabolism. As examples, it has been recently demonstrated that the isolated ATPase ISWI subunit of the *Drosophila* CHRAC and NURF nucleosome remodeling complexes is able to carry out ATP hydrolysis, nucleosome remodeling, nucleosome rearrangement, and chromatin assembly reactions (*21*). In a like manner, baculovirus-produced recombinant BRG1 possesses ATPase activity and can alter nucleosome structure in a manner similar to hSWI/SNF complexes purified from cells (*22*). We have narrowed the functional DNA-dependent ATPase activity of a SWI/SNF family member to a polypeptide domain containing the helicase-related motifs, demon-

strated that the DNA and the ATP binding reside within this polypeptide, and overproduced the domain as a functional DNA-dependent ATPase in *E. coli* [Muthuswami et al., *J. Biol. Chem*., in press].

Phosphoaminoglycosides: Inhibitors of the SWI2/SNF2 Family. Overexpression and purification studies demonstrated that production of ADAAD from kanamycin-resistant bacteria resulted in an inactive protein. Further analysis showed that modification of kanamycin results in the generation of a phosphorylated derivative of kanamycin, which is a potent inhibitor of ADAAD. Thus, we have identified an inhibitor for ADAAD that inactivates the enzyme when present in the growth media. In the absence of this compound, we are able to purify ADAAD from the bacterial cells with a specific activity of 16 μmol min^{-1} (mg of protein)$^{-1}$.

We synthesized phosphorylated derivatives of two different aminoglycosides and have found them both to inhibit ADAAD. The inhibition elicited by these compounds is competitive with respect to DNA but not with respect to ATP and is specifically targeted toward the members of the SWI2/SNF2 family. The interaction between the PAMs and the DNA-dependent ATPase appears to be dependent upon both the effector and some sequence/structural element in the protein. Thus, we found that proteins using no nucleic acid effector (hexokinase) or an unrelated effector (rho) were not sensitive to the PAMs, while proteins using similar DNA effectors as ADAAD but with little amino acid sequence similarity also demonstrated limited sensitivity (gp44/62). The response of three different assays demonstrating the sensitivity of three different SWI2/SNF2 family members to the PAMs may be a direct reflection of the ability of the molecular motor domain of these proteins to recognize (or generate) specific DNA structures.

It is rather striking that the PAMs are specific for an enzyme that recognizes neither double-stranded nor single-stranded DNA as effectors of ATP hydrolysis but requires elements of both—a single-stranded:double-stranded DNA junction. Recognition of DNA in a structure-dependent fashion is not unique to DNA-dependent ATPase A but has been demonstrated for a variety of enzymes including SWI/SNF family members (*23*), gp44/62 (*4*, *16*), DNA-dependent protein kinase (*24*), ERCC1-XPF (*25*), and others. SWI/SNF family members either assemble or dissemble multiprotein−DNA complexes in an ATP-dependent fashion (*26*), while the gp44/62 protein complex drives the assembly of multiprotein complexes onto DNA in the presence of ATP (*4*, *16*). Thus, gp44/62 and SWI/SNF family members demonstrate a remarkable physicobiochemical functional similarity despite primary amino acid sequences that are essentially unrelated. Both gp44/62 and ADAAD are more sensitive to the effects of PAMs than other DNA-dependent ATPases, such as topoisomerase II. Furthermore, although DNA-dependent ATPases are sensitive to PAMs, DNA-independent ATPases are resistant to the action of these compounds.

It is noteworthy that the family of DNA-dependent ATPases that is most sensitive to the PAMs is predominately an eukaryotic family with very few prokaryotic members (*27*). This fact may account for the relative insensitivity of bacteria to the PAMs and thus the tolerance to the existence of the aminoglycoside resistance gene products in bacteria. Aminoglycoside antibiotics, like neomycin and geneticin, have been used extensively in selection media to select eukaryotic cells that are resistant to these two compounds (*28*). The observations presented here raise some interesting questions regarding the introduction of aminoglycoside resistance selection systems into eukaryotic cell systems, especially with respect to normalcy of eukaryotic DNA metabolic processes in the transformed cells and the metabolism or transport of the PAMs within the cell. We hope these studies will provide the impetus for a reevaluation of the use of these selection systems in eukaryotic cells.

The discovery of specific inhibitors of the SWI/SNF family of proteins leads us to believe that understanding the interaction between DNA and ATPases could lead to the refinement of our observations and to the development of additional inhibitors that may be specific to only one type of DNA-dependent ATPase. We specifically propose a model wherein a conformational change is induced in the DNA-dependent ATPase by the binding of DNA or some DNA counterpart such as a nucleosome or a Mot1−TBP−DNA complex. In this model, the addition of phosphoaminoglycoside does not result in the displacement of DNA but instead abrogates the conformational change essential for ATP hydrolysis and/or function. Thus, enzymes that recognize similar DNA effectors (e.g., ADAAD and gp44/62) may have significant differences in their interaction with the inhibitor depending on subtleties of the amino acids exposed at the site of PAM binding. This model is also consistent with the observation that a change in the structure of the PAM (e.g., phosphoneomycin versus phosphokanamycin) may have dramatic effect on the function of the protein.

Finally, the discovery of inhibitors to the SWI2/SNF2 family of proteins may provide a valuable tool for the study of assembly and/or disassembly of multiprotein complexes onto DNA. In addition, the SWI2/SNF2 family members have been shown to be involved in all aspects of DNA metabolism; therefore, we hypothesize that any inhibitor of this family should affect DNA metabolic processes driven by DNA-dependent ATP hydrolysis. We are at present investigating the effect of PAMs on cells in both in vitro and in vivo models.

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